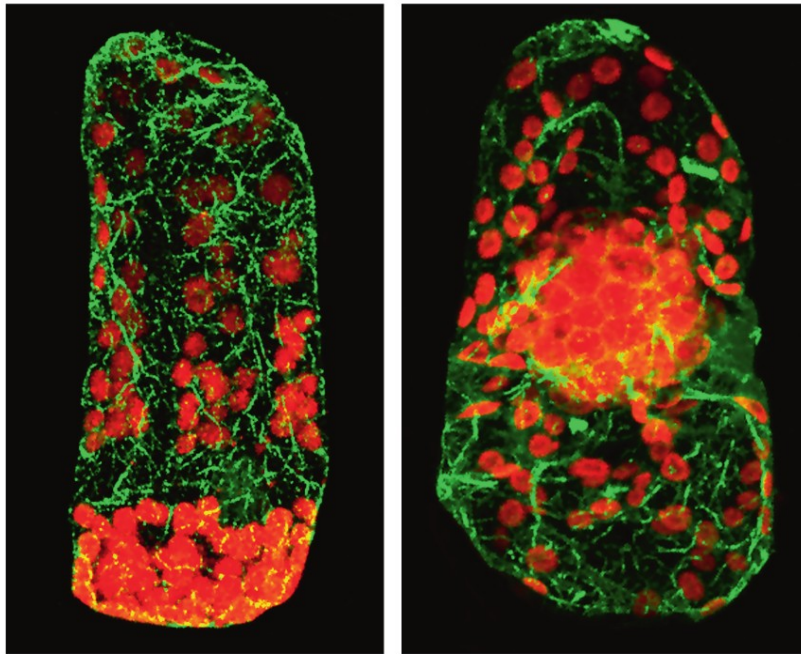


Advances in Photosynthesis and Respiration

Volume 32

C₄ Photosynthesis and Related CO₂ Concentrating Mechanisms



Edited by

Agepati S. Raghavendra

and

Rowan F. Sage



Springer

C_4 Photosynthesis
and
Related CO_2 Concentrating Mechanisms

Advances in Photosynthesis and Respiration

VOLUME 32

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The scope of our series reflects the concept that photosynthesis and respiration are intertwined with respect to both the protein complexes involved and to the entire bioenergetic machinery of all life. *Advances in Photosynthesis and Respiration* is a book series that provides a comprehensive and state-of-the-art account of research in photosynthesis and respiration. Photosynthesis is the process by which higher plants, algae, and certain species of bacteria transform and store solar energy in the form of energy-rich organic molecules. These compounds are in turn used as the energy source for all growth and reproduction in these and almost all other organisms. As such, virtually all life on the planet ultimately depends on photosynthetic energy conversion. Respiration, which occurs in mitochondrial and bacterial membranes, utilizes energy present in organic molecules to fuel a wide range of metabolic reactions critical for cell growth and development. In addition, many photosynthetic organisms engage in energetically wasteful photorespiration that begins in the chloroplast with an oxygenation reaction catalyzed by the same enzyme responsible for capturing carbon dioxide in photosynthesis. This series of books spans topics from physics to agronomy and medicine, from femtosecond processes to season-long production, from the photophysics of reaction centers, through the electrochemistry of intermediate electron transfer, to the physiology of whole organisms, and from X-ray crystallography of proteins to the morphology of organelles and intact organisms. The goal of the series is to offer beginning researchers, advanced undergraduate students, graduate students, and even research specialists, a comprehensive, up-to-date picture of the remarkable advances across the full scope of research on photosynthesis, respiration and related processes.

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C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms

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Cover images: **Single cell C_4 photosynthesis in Chenopodiaceae.** C_4 is developed with the intracellular location of two distinct groups of chloroplasts (indicated by the red fluorescence) held in position by the cytoskeleton (green fluorescence). Borszczowia type (left): One type of chloroplast is more abundant in the proximal end and another type towards the distal end. Bienertia type (right): Dimorphic chloroplasts partition between the peripheral cytoplasm and a central cytoplasmic compartment. These features of single cell C_4 photosynthesis are described in detail by Edwards and Voznesenskaya (Chapter 4). Images were provided by Simon D.X. Chuong, Vincent R. Franceschi and Gerald E. Edwards. Adapted from Chuong et al. (2006), from *Plant Cell* (volume 18, pp 2207–2223).

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From the Series Editor

Advances in Photosynthesis and Respiration **Volume 32: C_4 Photosynthesis and Related CO_2** **Concentrating Mechanisms**

We (Tom Sharkey and I) are delighted to announce the publication, in the Advances in Photosynthesis and Respiration (AIPH) Series, of *C_4 Photosynthesis and Related CO_2 Concentrating Mechanisms*. Two distinguished international authorities in the field of photosynthesis have edited this volume: Agepati S. Raghavendra (University of Hyderabad, Hyderabad, India) and Rowan F. Sage (University of Toronto, Toronto, Canada). Raghavendra, as Raghavendra is called by his friends, has contributed significantly to the topics in this volume and photosynthesis in general, e.g., to the discovery of several C_4 plants, C_3 – C_4 intermediates, regulation of C_4 phosphoenolpyruvate, requirement of mitochondrial respiration for optimizing photosynthesis, and mitochondrial enrichment in bundle sheath cells as the basis of reduced photorespiration in C_3 – C_4 intermediates. Rowan Sage has worked over a remarkably broad range of topics, from biochemistry to ecology of photosynthesis and has been interested in C_4 and its attributes since his Ph.D. research on co-occurrence of C_3 and C_4 weeds. His work has shown that there have been at least 60 independent origins of C_4 photosynthesis, making it the most convergent of evolutionary phenomena known to humanity. His work on C_4 evolution led to his participation in the C_4 rice engineering project; his current research includes the evolution and engineering of C_4 photosynthesis, the impact of temperature and CO_2 variation on the biochemical processes governing C_3 and C_4 photosynthesis, and cold-tolerance in high-yielding C_4 grasses such as *Miscanthus*. This last project is geared toward developing a bioenergy economy based on high-yielding C_4 plants, a very important goal for the benefit of all humanity.

Our Books: 31 Volumes

We list below information on all the 31 volumes that have been published thus far; beginning with volume 31, Thomas D. Sharkey, who had earlier edited volume 9 of this series of book, has joined us as its co-series editor. We are pleased to note that Springer is now producing complete table of content of these books and electronic copies of individual chapters of these books; their web sites include free downloadable front matter as well as indexes. As of July 12, 2010, the only volumes that are not yet complete are: volumes 1, 13, 14, 15 and 17. All the available web sites are listed, within square brackets, at the end of each entry.

- **Volume 31** (2010): ***The Chloroplast: Basics and Applications***, edited by Constantin Rebeiz, Christoph Benning, Hans J. Bohnert, Henry Daniell, J. Kenneth Hooper, Hartmut K. Lichtenthaler, Archie R. Portis, and Baishnab C. Tripathy. Twenty-five chapters, 500 pp, Hardcover, ISBN: 978-90-481-8530-6 available June 2010
- **Volume 30** (2009): ***Lipids in Photosynthesis: Essential and Regulatory Functions***, edited by Hajime Wada and Norio Murata, both from Japan. Twenty chapters, 506 pp, Hardcover, ISBN: 978-90-481-2862-4; e-book, ISBN: 978-90-481-2863-1 [<http://www.springerlink.com/content/978-90-481-2862-4>]
- **Volume 29** (2009): ***Photosynthesis In silico: Understanding Complexity from Molecules***, edited by Agu Laisk, Ladislav Nedbal, and Govindjee, from Estonia, The Czech Republic, and USA. Twenty chapters, 508 pp, Hardcover, ISBN: 978-1-4020-9236-7 [<http://www.springerlink.com/content/978-1-4020-9236-7>]

- *Volume 28* (2009): ***The Purple Phototrophic Bacteria***, edited by C. Neil Hunter, Fevzi Daldal, Marion C. Thurnauer and J. Thomas Beatty, from UK, USA and Canada. Forty-eight chapters, 1014 pp, Hardcover, ISBN: 978-1-4020-8814-8 [<http://www.springerlink.com/content/978-1-4020-8814-8>]
- *Volume 27* (2008): ***Sulfur Metabolism in Phototrophic Organisms***, edited by Christiane Dahl, Rüdiger Hell, David Knaff and Thomas Leustek, from Germany and USA. Twenty-four chapters, 551 pp, Hardcover, ISBN: 978-4020-6862-1 [<http://www.springerlink.com/content/978-1-4020-6862-1>]
- *Volume 26* (2008): ***Biophysical Techniques in Photosynthesis***, Volume II, edited by Thijs Aartsma and Jörg Matysik, both from The Netherlands. Twenty-four chapters, 548 pp, Hardcover, ISBN: 978-1-4020-8249-8 [<http://www.springerlink.com/content/978-1-4020-8249-8>]
- *Volume 25* (2006): ***Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications***, edited by Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger, and Hugo Scheer, from Germany and Australia. Thirty-seven chapters, 603 pp, Hardcover, ISBN: 978-1-40204515-8 [<http://www.springerlink.com/content/978-1-4020-4515-8>]
- *Volume 24* (2006): ***Photosystem I: The Light-Driven Plastocyanin: Ferredoxin Oxidoreductase***, edited by John H. Golbeck, from USA. Forty chapters, 716 pp, Hardcover, ISBN: 978-1-40204255-3 [<http://www.springerlink.com/content/978-1-4020-4255-3>]
- *Volume 23* (2006): ***The Structure and Function of Plastids***, edited by Robert R. Wise and J. Kenneth Hooper, from USA. Twenty-seven chapters, 575 pp, Softcover, ISBN: 978-1-4020-6570-6; Hardcover, ISBN: 978-1-4020-4060-3 [<http://www.springerlink.com/content/978-1-4020-4060-3>]
- *Volume 22* (2005): ***Photosystem II: The Light-Driven Water:Plastoquinone Oxidoreductase***, edited by Thomas J. Wydrzynski and Kimiyuki Satoh, from Australia and Japan. Thirty-four chapters, 786 pp, Hardcover, ISBN: 978-1-4020-4249-2 [<http://www.springerlink.com/content/978-1-4020-4249-2>]
- *Volume 21* (2005): ***Photoprotection, Photoinhibition, Gene Regulation, and Environment***, edited by Barbara Demmig-Adams, William W. Adams III and Autar K. Mattoo, from USA. Twenty-one chapters, 380 pp, Hardcover, ISBN: 978-14020-3564-7 [<http://www.springerlink.com/content/978-1-4020-3564-7>]
- *Volume 20* (2006): ***Discoveries in Photosynthesis***, edited by Govindjee, J. Thomas Beatty, Howard Gest and John F. Allen, from USA, Canada and UK. One hundred and eleven chapters, 1304 pp, Hardcover, ISBN: 978-1-4020-3323-0 [<http://www.springerlink.com/content/978-1-4020-3564-7>] and [<http://www.springerlink.com/content/978-1-4020-3323-0>]
- *Volume 19* (2004): ***Chlorophylla Fluorescence: A Signature of Photosynthesis***, edited by George C. Papageorgiou and Govindjee, from Greece and USA. Thirty-one chapters, 820 pp, Hardcover, ISBN: 978-1-4020-3217-2 [<http://www.springerlink.com/content/978-1-4020-3217-2>]
- *Volume 18* (2005): ***Plant Respiration: From Cell to Ecosystem***, edited by Hans Lambers and Miquel Ribas-Carbo, from Australia and Spain. Thirteen chapters, 250 pp, Hardcover, ISBN: 978-14020-3588-3 [<http://www.springerlink.com/content/978-1-4020-3588-3>]
- *Volume 17* (2004): ***Plant Mitochondria: From Genome to Function***, edited by David Day, A. Harvey Millar and James Whelan, from Australia. Fourteen chapters, 325 pp, Hardcover, ISBN: 978-1-4020-2399-6
- *Volume 16* (2004): ***Respiration in Archaea and Bacteria: Diversity of Prokaryotic Respiratory Systems***, edited by Davide Zannoni, from Italy. Thirteen chapters, 310 pp, Hardcover, ISBN: 978-14020-2002-5 [<http://www.springerlink.com/content/978-1-4020-2002-5>]
- *Volume 15* (2004): ***Respiration in Archaea and Bacteria: Diversity of Prokaryotic Electron Transport Carriers***, edited by Davide Zannoni, from Italy. Thirteen chapters, 350 pp, Hardcover, ISBN: 978-1-4020-2001-8
- *Volume 14* (2004): ***Photosynthesis in Algae***, edited by Anthony W. Larkum, Susan Douglas and John A. Raven, from Australia, Canada and UK. Nineteen chapters, 500 pp, Hardcover, ISBN: 978-0-7923-6333-0
- *Volume 13* (2003): ***Light-Harvesting Antennas in Photosynthesis***, edited by Beverley R. Green and William W. Parson, from Canada and USA. Seventeen chapters, 544 pp, Hardcover, ISBN: 978-07923-6335-4

- *Volume 12* (2003): ***Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism***, edited by Christine H. Foyer and Graham Noctor, from UK and France. Sixteen chapters, 304 pp, Hardcover, ISBN: 978-0-7923-6336-1 [<http://www.springerlink.com/content/978-0-7923-6336-1>]
- *Volume 11* (2001): ***Regulation of Photosynthesis***, edited by Eva-Mari Aro and Bertil Andersson, from Finland and Sweden. Thirty-two chapters, 640 pp, Hardcover, ISBN: 978-0-7923-6332-3 [<http://www.springerlink.com/content/978-0-7923-6332-3>]
- *Volume 10* (2001): ***Photosynthesis: Photobiology and Photobiophysics***, authored by Bacon Ke, from USA. Thirty-six chapters, 792 pp, Softcover, ISBN: 978-0-7923-6791-8; Hardcover: ISBN: 978-0-7923-6334-7 [<http://www.springerlink.com/content/978-0-7923-6334-7>]
- *Volume 9* (2000): ***Photosynthesis: Physiology and Metabolism***, edited by Richard C. Leegood, Thomas D. Sharkey and Susanne von Caemmerer, from UK, USA and Australia. Twenty-four chapters, 644 pp, Hardcover, ISBN: 978-0-7923-6143-5 [<http://www.springerlink.com/content/978-0-7923-6143-5>]
- *Volume 8* (1999): ***The Photochemistry of Carotenoids***, edited by Harry A. Frank, Andrew J. Young, George Britton and Richard J. Cogdell, from UK and USA. Twenty chapters, 420 pp, Hardcover, ISBN: 978-0-7923-5942-5 [<http://www.springerlink.com/content/978-0-7923-5942-5>]
- *Volume 7* (1998): ***The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas***, edited by Jean David Rochaix, Michel Goldschmidt-Clermont and Sabeeha Merchant, from Switzerland and USA. Thirty-six chapters, 760 pp, Hardcover, ISBN: 978-0-7923-5174-0 [<http://www.springerlink.com/content/978-0-7923-5174-0>]
- *Volume 6* (1998): ***Lipids in Photosynthesis: Structure, Function and Genetics***, edited by Paul-André Siegenthaler and Norio Murata, from Switzerland and Japan. Fifteen chapters, 332 pp, Hardcover, ISBN: 978-0-7923-5173-3 [<http://www.springerlink.com/content/978-0-7923-5173-3>]
- *Volume 5* (1997): ***Photosynthesis and the Environment***, edited by Neil R. Baker, from UK. Twenty chapters, 508 pp, Hardcover, ISBN: 978-0-7923-4316-5 [<http://www.springerlink.com/content/978-0-7923-4316-5>]
- *Volume 4* (1996): ***Oxygenic Photosynthesis: The Light Reactions***, edited by Donald R. Ort, and Charles F. Yocum, from USA. Thirty-four chapters, 696 pp, Softcover: ISBN: 978-0-7923-3684-6; Hardcover, ISBN: 978-0-7923-3683-9 [<http://www.springerlink.com/content/978-0-7923-3683-9>]
- *Volume 3* (1996): ***Biophysical Techniques in Photosynthesis***, edited by Jan Ames and Arnold J. Hoff, from The Netherlands. Twenty-four chapters, 426 pp, Hardcover, ISBN: 978-0-7923-3642-6 [<http://www.springerlink.com/content/978-0-7923-3642-6>]
- *Volume 2* (1995): ***Anoxygenic Photosynthetic Bacteria***, edited by Robert E. Blankenship, Michael T. Madigan and Carl E. Bauer, from USA. Sixty-two chapters, 1331 pp, Hardcover, ISBN: 978-0-7923-3682-8 [<http://www.springerlink.com/content/978-0-7923-3681-5>]
- *Volume 1* (1994): ***The Molecular Biology of Cyanobacteria***, edited by Donald R. Bryant, from USA. Twenty-eight chapters, 916 pp, Hardcover, ISBN: 978-0-7923-3222-0

Further information on these books and ordering instructions can be found at <http://www.springer.com/series/5599>. Contents of volumes 1–29 can also be found at <http://www.life.uiuc.edu/govindjee/photosynSeries/ttocs.html>.

Special 25% discounts are available to members of the International Society of Photosynthesis Research, ISPR <http://www.photosynthesisresearch.org/>: See <http://www.springer.com/ispr>

This Book

“*C₄ Photosynthesis and Related CO₂ Concentrating Mechanisms*” is volume 32 of the *Advances in Photosynthesis and Respiration*. The preface of the book on page xix beautifully describes the context of this book; and the contents of this book on page xiii shows the breadth of this book. A unique aspect of this book is tributes to two pioneers: Jagadish Chandra Bose; and Constance E. Hartt just before the topic of the book is introduced. The C₄ pathway, also known as the Hatch and Slack pathway, of photosynthesis was discovered and characterized more than 4 decades ago. The C₄ photosynthesis has had profound impact not only on food production, but on global ecology, and on the

evolutionary development of the modern biosphere, including our own origin and the rise of our civilization. Recent studies have provided new perspectives on the diversity and evolutionary origin of C_4 plants; these plants have independently evolved over 50 times; there are even multiple examples of single-celled C_4 photosynthesis (see the cover of this book). The evolutionary rise of C_4 plants has altered the face of the Earth, and has contributed to the origin of the grassland biota we know today. With new molecular tools, many of the genes controlling C_4 photosynthesis have now been elucidated, allowing us to begin engineering the C_4 pathway into C_3 plants and to domesticate wild C_4 species as new energy crops for our future. This book provides a state-of-the-art overview of basic and applied aspects of C_4 plant biology; its emphasis is on physiology, biochemistry, molecular biology, biogeography and evolution. Further, this book provides a review of developments in the bioengineering of C_4 rice and novel biofuels. We expect that this book will serve as an advanced textbook for graduate students, and a reference for researchers, in several areas of the life sciences, including plant biology, cell biology, biotechnology, agronomy, horticulture, ecology, and evolutionary biology.

Tom Sharkey, who is an expert on the topic of this book, writes “The discovery of C_4 metabolism touched off many investigations about both the commonalities and variation among CO_2 -concentrating mechanisms. The decades from the 1960s to the 1980s saw significant new insights into carbon dioxide acquisition by photosynthesizing organisms. These included advances in understanding the biophysical constraints for CO_2 uptake in C_3 plants, the active uptake of CO_2 and bicarbonate by algae and bacteria, and of course, C_4 metabolism. Since these discoveries, tremendous advances have been made and two world experts, Agepati S. Raghavendra (of India) and Rowan Sage (of Canada), have now edited this volume that makes all of the latest advances available to the interested reader. Clearly, C_4 and related metabolism provides tremendous opportunity to better understand photosynthesis and the possibilities to further adapt it to the needs of people.”

Authors

The current book contains 19 chapters written by 32 international authors from ten different countries (Argentina; Australia; Canada; Germany; India; Ireland; Russia; Turkey; United Kingdom and the United States of America). They are (arranged alphabetically): Carlos S. Andreo (Argentina); Hermann Bauwe (Germany); Andrew A. Benson (USA); James O. Berry (USA); George Bowes (USA); Andrea Bräutigam (Germany); Jim N. Burnell (Australia); Chris J. Chastain (USA); María F. Drincovich (Argentina); Gerald E. Edwards (USA); John R. Evans (Australia); Oula Ghannoum (Australia); Govindjee (USA); Udo Gowik (Germany); Mike B. Jones (Ireland); Ferit Kocacinar (Turkey); Stanislav Kopriva (UK); David S. Kubien (Canada); María V. Lara (Argentina); Andrew Maretzki (USA); Verónica G. Maurino (Germany); Timothy Nelson (USA); Colin P. Osborne (UK); Minesh Patel (USA); Agepati S. Raghavendra (India); Eric H. Roalson (USA); Rowan F. Sage (Canada); Susanne von Caemmerer (Australia); Elena V. Voznesenskaya (Russia); Andreas P. M. Weber (Germany); Peter Westhoff (Germany); and Amy Zielinski (USA).

Future Advances in Photosynthesis and Respiration and Other Related Books

The readers of the current series are encouraged to watch for the publication of the forthcoming books (not necessarily arranged in the order of future appearance):

- Photosynthesis: Perspectives on Plastid Biology, Energy Conversion and Carbon Metabolism (Editors: Julian Eaton-Rye, Baishnab Tripathy, and Thomas D. Sharkey)
- Functional Genomics and Evolution of Photosynthetic Systems (Editors: Robert Burnap and Willem Vermaas)
- The Bioenergetic Processes of Cyanobacteria: From Evolutionary Singularity to Ecological Diversity (Editors: Guenter A. Peschek, Christian Obinger, and Gernot Renger)

- Chloroplast Biogenesis: During Leaf Development and Senescence (Editors: Basanti Biswal, Karin Krupinska, and Udaya Chand Biswal)
- The Structural Basis of Biological Energy Generation (Editor: Martin Hohmann-Marriott)
- Genomics of Chloroplasts and Mitochondria (Editors: Ralph Bock and Volker Knoop)
- Photosynthesis in Bryophytes and Early Land Plants (Editors: David T. Hanson and Steven K. Rice)

In addition to the above contracted books, the following topics are under consideration:

- Artificial Photosynthesis
- ATP Synthase and Proton Translocation
- Biohydrogen Production
- Carotenoids II
- Cyanobacteria
- The Cytochromes
- Ecophysiology
- Evolution of Photosynthesis
- Genomics of Chloroplasts and Mitochondria
- Global Aspects of Photosynthesis
- Green Bacteria and Heliobacteria
- Interactions Between Photosynthesis and Other Metabolic Processes
- Limits of Photosynthesis
- Photosynthesis, Biomass and Bioenergy
- Photosynthesis Under Abiotic Stress
- Plant Canopies and Photosynthesis

If you have any interest in editing/co-editing any of the above listed books, or being an author, please send me an E-mail at gov@illinois.edu, and/or to Tom Sharkey (tsharkey@msu.edu). Suggestions for additional topics are also welcome.

In view of the interdisciplinary character of research in photosynthesis and respiration, it is my earnest hope that this series of books will be used in educating students and researchers not only in plant sciences, molecular and

cell biology, integrative biology, biotechnology, agricultural sciences, microbiology, biochemistry, chemical biology, biological physics, and biophysics, but also in bioengineering, chemistry, and physics.

We take this opportunity to thank and congratulate Agepati S. Raghavendra and Rowan F. Sage for their outstanding editorial work; they have done a fantastic job not only in editing, but also in organizing this book for Springer, and for their highly professional dealing with the typesetting process and their help in preparing this editorial. We thank all the 32 authors of this book (see the list above): without their authoritative chapters, there would be no such volume. We give special thanks to R. Samuel Devanand for directing the typesetting of this book: his expertise has been crucial in bringing this book to completion. We owe Jacco Flipsen, Ineke Ravesloot and André Tournois (of Springer) thanks for their friendly working relation with us that led to the production of this book. Thanks are also due to Jeff Haas (Director of Information Technology, Life Sciences, University of Illinois at Urbana-Champaign, UIUC), Feng Sheng Hu (Head, Department of Plant Biology, UIUC), Tom Sharkey (my co-Series Editor), and my dear wife, Rajni Govindjee for constant support.

August 15, 2010

Govindjee

Founding Series Editor

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The Founding Series Editor



Govindjee

Govindjee was born on October 24, 1932, in Allahabad, India. Since 1999, he has been Professor Emeritus of Biochemistry, Biophysics and Plant Biology at the University of Illinois at Urbana-Champaign (UIUC), Urbana, IL, USA. He obtained his B.Sc. (Chemistry and Biology) and M.Sc. (Botany; Plant Physiology) in 1952 and 1954, from the University of Allahabad. He studied 'Photosynthesis' at the UIUC, under Robert Emerson, and Eugene Rabinowitch, obtaining his Ph.D. in 1960, in Biophysics. He is best known for his research on the excitation energy transfer, light emission, the primary photochemistry and the electron transfer in "*Photosystem II*" (PS II, water-plastoquinone oxido-reductase). His research, with many collaborators, has included the discovery of a short-wavelength form of chlorophyll (Chl) *a* functioning in the Chl *b*-containing system, now called PS II; of the two-light effect in Chl *a* fluorescence; and of the two-light effect (Emerson enhancement) in NADP reduction in chloroplasts. His major achievements include an understanding of the basic relationships between Chl *a* fluorescence and photosynthetic reactions; an unique role of bicarbonate on the electron acceptor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants; the first picosecond measurements on the primary photochemistry of PS II; and the use of fluorescence lifetime imaging microscopy (FLIM) of Chl *a* fluorescence in understanding photoprotection, by plants, against excess light. His current focus is on the "*History of Photosynthesis Research*", in '*Photosynthesis Education*', and in the '*Possible Existence of Extraterrestrial Life*'. He has served on the faculty of the UIUC for ~40 years. Govindjee's honors include: Fellow of the American Association of Advancement of Science (AAAS); Distinguished Lecturer of the School of Life Sciences, UIUC; Fellow and Lifetime Member of the National Academy of Sciences (India); President of the American Society for Photobiology (1980-1981); Fulbright Scholar and Fulbright Senior Lecturer; Honorary President of the 2004 International Photosynthesis Congress (Montréal, Canada); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology, 2006; Recipient of the Communication Award of the International Society of Photosynthesis Research, 2007; and the Liberal Arts and Sciences Lifetime Achievement Award of the UIUC, 2008. Further, Govindjee was honored (1) in 2007, through two special volumes of *Photosynthesis Research*, celebrating his 75th birthday and for his 50-year dedicated research in 'Photosynthesis' (Guest Editor: Julian Eaton-Rye); (2) in 2008, through a special International Symposium on 'Photosynthesis in a Global Perspective', held in November, 2008, at the University of Indore, India. Govindjee is coauthor of 'Photosynthesis' (Wiley, 1969); and editor of many books, published by several publishers including Academic and Kluwer (now Springer). For further information on Govindjee, see his web site at <http://www.life.illinois.edu/govindjee>.

Series Editor



Thomas D. Sharkey

Thomas D. (Tom) Sharkey obtained his Bachelor's degree in Biology in 1974 from Lyman Briggs College, a residential science college at Michigan State University, East Lansing, Michigan. After 2 years as a research technician, Tom entered a Ph.D. program in the federally funded Plant Research Laboratory at Michigan State University under the mentorship of Klaus Raschke and graduated in 1980 after just 3 years and 3 months. Post-doctoral research was carried out with Graham Farquhar at the Australian National University, in Canberra, where he coauthored a landmark review on photosynthesis and stomatal conductance that continues to get over 50 citations per year more than 25 years after its publication. For 5 years he worked at the Desert Research Institute, Reno, Nevada, where Rowan Sage, co-editor of this volume, joined him as a post-doc. After Reno, Tom spent 20 years as professor of botany at the University of Wisconsin in Madison. In 2008, Tom became professor and chair of the Department of Biochemistry and Molecular Biology at Michigan State University. Tom's research interests center on the exchange of gases between plants and the atmosphere. The biochemistry and biophysics underlying carbon dioxide uptake and isoprene emission from plants form the two major research topics in his laboratory. Among his contributions are measurement of the carbon dioxide concentration inside leaves, an exhaustive study of short-term feedback effects in carbon metabolism and a significant contribution to elucidation of the pathway by which leaf starch breaks down at night. In the isoprene research field, Tom is recognized as the leading advocate for thermotolerance of photosynthesis as the explanation for why plants emit isoprene. In addition, his laboratory has cloned many of the genes that underlie isoprene synthesis and published many papers on the biochemical regulation of isoprene synthesis. Tom has edited two books, the first on trace gas emissions from plants in 1991 and then volume 9 of this series on the physiology of carbon metabolism of photosynthesis in 2000. Tom is listed in *Who's Who* and is a "highly cited researcher" according to the Thomson Reuters Institute for Scientific Information, and is grateful to Rowan Sage for contributing to that honor by his early productivity.

Contents

From the Series Editor	v
Contents	xiii
Preface	xix
The Editors	xxiii
Contributors	xxv
Author Index	xxvii

Part I: Tributes & Introduction

1 Sir Jagadish Chandra Bose (1858–1937): A Pioneer in Photosynthesis Research and Discoverer of Unique Carbon Assimilation in <i>Hydrilla</i>	3–11
<i>Agepati S. Raghavendra and Govindjee</i>	
Summary	3
I. Introduction	4
II. Life of Sir J.C. Bose	4
III. Out of Box Concepts and Innovative Instruments for Biological Experiments	5
IV. Classic and Comprehensive Monographs on Physiology of Plants	6
V. Work on Photosynthesis and Focus on <i>Hydrilla</i>	6
VI. Importance of Malate and Operation of C ₄ -like Pathway	7
VII. Contemporary View of his Observations on <i>Hydrilla</i>	7
VIII. Observations on Inhibitors/Stimulants on Photosynthesis in <i>Hydrilla</i>	8
IX. Concluding Remarks: Inspiration for Biology Research in India and a Pioneer of Photosynthesis Research on <i>Hydrilla</i>	9
Acknowledgments	10
References	10
2 Constance Endicott Hartt (1900–1984) and the Path of Carbon in the Sugarcane Leaf	13–16
<i>Andrew A. Benson and Andrew Maretzki</i>	
Summary	13
I. Biography of Constance Hartt: Early Period and Her Move into Hawaii	14
II. Work at Hawaiian Sugar Planters' Association: Focus on Biosynthesis and Transport of Sugar in Sugarcane	14
III. Discovery of the Role of Malate in Carbon Assimilation and Sucrose Biosynthesis	14
IV. Concluding Remarks	15
Acknowledgments	16
References	16

3	Introduction	17–25
	<i>Agepati S. Raghavendra and Rowan F. Sage</i>	
	Summary	17
	I. Introduction	18
	II. New Physiological and Developmental Perspectives	20
	III. Molecular Basis of the C ₄ Pathway	22
	IV. Systematics, Diversity and Evolution	23
	V. New Uses of C ₄ Photosynthesis	24
	VI. Conclusions	24
	Acknowledgments	24
	References	24
	Part II: New Physiological and Developmental Perspectives	
4	C₄ Photosynthesis: Kranz Forms and Single-Cell C₄ in Terrestrial Plants	29–61
	<i>Gerald E. Edwards and Elena V. Voznesenskaya</i>	
	Summary	30
	I. Introduction	30
	II. Structural and Biochemical Diversity in Kranz Type Anatomy	31
	III. Single-Cell C ₄ Photosynthesis in Terrestrial Plants	48
	IV. Future Perspectives	55
	Acknowledgments	56
	References	56
5	Single-Cell C₄ Photosynthesis in Aquatic Plants	63–80
	<i>George Bowes</i>	
	Summary	63
	I. Introduction	64
	II. Unraveling the Single-Cell C ₄ System	64
	III. HCO ₃ ⁻ -Use Mimics C ₄ Photosynthetic Gas Exchange Characteristics	76
	IV. Concluding Thoughts	76
	Acknowledgments	77
	References	77
6	Photorespiration: The Bridge to C₄ Photosynthesis	81–108
	<i>Hermann Bauwe</i>	
	Summary	81
	I. Introduction	82
	II. Biochemistry and Genetics of the C ₂ Cycle	84
	III. Related Reactions and Interactions with Other Metabolic Pathways	93
	IV. Measurement of Photorespiration	95
	V. The Role of Photorespiration for the Evolution of C ₄ Photosynthesis	97
	VI. Future Prospects	101
	Acknowledgments	103
	References	103

7	Nitrogen and Sulfur Metabolism in C₄ Plants	109–128
	<i>Stanislav Kopriva</i>	
	Summary	109
	I. Introduction	110
	II. Nitrogen Assimilation	110
	III. Sulfate Assimilation	114
	IV. Glutathione Synthesis and Reduction	117
	V. Physiological Significance of the Distribution of Nitrate and Sulfate Assimilation	120
	VI. Conclusions	122
	Acknowledgments	122
	References	123
8	Nitrogen and Water Use Efficiency of C₄ Plants	129–146
	<i>Oula Ghannoum, John R. Evans, and Susanne von Caemmerer</i>	
	Summary	129
	I. Introduction	130
	II. Nitrogen Use Efficiency	131
	III. Water Use Efficiency	138
	IV. Conclusions	143
	References	143
9	Development of Leaves in C₄ Plants: Anatomical Features That Support C₄ Metabolism	147–159
	<i>Timothy Nelson</i>	
	Summary	147
	I. Introduction	148
	II. Overview of C ₄ Biology and Leaf Anatomy	148
	III. Quantitative Variation in Leaf Traits	149
	IV. Complex Traits and Systems Analysis	154
	Acknowledgments	155
	References	155
10	C₄ Photosynthesis and Temperature	161–195
	<i>Rowan F. Sage, Ferit Kocacinar, and David S. Kubien</i>	
	Summary	162
	I. Introduction	162
	II. The Temperature Responses of C ₄ Photosynthesis and Growth	163
	III. The Biogeography of C ₄ Photosynthesis	168
	IV. The Temperature Response of C ₄ Photosynthesis: Biochemical Controls	175
	V. Fluorescence at Low Temperature	184
	VI. Stomatal Limitations	185
	VII. Thermal Acclimation of C ₄ Photosynthesis	185
	VIII. Conclusion: Are C ₄ Plants Inherently More Sensitive by Low Temperature Than C ₃ Plants?	187
	Acknowledgments	187
	References	188

Part III: Molecular Basis of C₄ Pathway

11	Transport Processes: Connecting the Reactions of C₄ Photosynthesis	199–219
	<i>Andrea Bräutigam and Andreas P. M. Weber</i>	
	Summary	199
	I. Introduction	200
	II. Intercellular Fluxes	203
	III. Transport Processes in the NADP-Malic Enzyme Type	203
	IV. Transport Processes in the NAD-Malic Enzyme Type	209
	V. Transport Processes in the PEP Carboxykinase (PEP-CK) Type	211
	VI. Transport Processes in Single Cell C ₄ Metabolism	212
	VII. Future Prospects	213
	Acknowledgments	214
	References	215
12	C₄ Gene Expression in Mesophyll and Bundle Sheath Cells	221–256
	<i>James O. Berry, Minesh Patel, and Amy Zielinski</i>	
	Summary	221
	I. Introduction and Overview	222
	II. C ₄ Gene Expression in Bundle Sheath Cells	225
	III. C ₄ Gene Expression in Mesophyll Cells	235
	IV. C ₄ Gene Expression in Organelles	240
	V. Factors Affecting C ₄ Gene Expression in BS and MP Cells	240
	VI. Levels of C ₄ Gene Regulation	243
	VII. Conclusions, Future Directions, and Molecular Engineering of C ₄ Capability	248
	Acknowledgments	249
	References	250
13	C₄-Phosphoenolpyruvate Carboxylase	257–275
	<i>Udo Gowik and Peter Westhoff</i>	
	Summary	257
	I. Phosphoenolpyruvate Carboxylase: An Overview	258
	II. Evolutionary Origin of C ₄ PEPCs	263
	III. Molecular Evolution of C ₄ PEPCs	266
	IV. Outlook	272
	References	272
14	C₄ Decarboxylases: Different Solutions for the Same Biochemical Problem, the Provision of CO₂ to Rubisco in the Bundle Sheath Cells	277–300
	<i>María F. Drincovich, María V. Lara, Carlos S. Andreo, and Verónica G. Maurino</i>	
	Summary	277
	I. Introduction	278
	II. NADP-Malic Enzyme, the Most Studied C ₄ Decarboxylase	280

III.	Plant Mitochondrial NAD-ME, a Hetero-Oligomeric Malic Enzyme	286
IV.	Plant PEPCK: the Cytosolic Gluconeogenic Enzyme Involved in C ₄ Photosynthesis	290
V.	Future Perspectives	295
	Acknowledgments	295
	References	295
15	Structure, Function, and Post-translational Regulation of C₄ Pyruvate Orthophosphate Dikinase	301–315
	<i>Chris J. Chastain</i>	
	Summary	301
I.	Introduction	302
II.	Post-translational Regulation of C ₄ PPDK	305
III.	Functional and Bioinformatic Analysis of Cloned Maize C ₄ and <i>Arabidopsis</i> C ₄ -Like PPDK-Regulatory Protein	310
IV.	Future Directions	313
	Acknowledgments	313
	References	313
Part IV: Diversity and Evolution		
16	C₄ Photosynthesis Origins in the Monocots: A Review and Reanalysis	319–338
	<i>Eric H. Roalson</i>	
	Summary	319
I.	Introduction	320
II.	Alismatales	320
II.	Cyperaceae	323
IV.	Poaceae	326
V.	Conclusions	332
	References	335
17	The Geologic History of C₄ Plants	339–357
	<i>Colin P. Osborne</i>	
	Summary	339
I.	Introduction	340
II.	Geologic Evidence	340
III.	Origin of C ₄ Photosynthesis	345
IV.	Expansion of C ₄ Grasslands	347
V.	Conclusions	353
	Acknowledgments	354
	References	354

Part V: C₄ Engineering and Bioenergy

18	Hurdles to Engineering Greater Photosynthetic Rates in Crop Plants: C₄ Rice	361–378
	<i>James N. Burnell</i>	
	Summary	361
	I. Introduction	362
	II. Why Try to Engineer a C ₄ Crop Plant?	362
	III. How Can Crop Productivity Be Increased by C ₄ Photosynthesis?	363
	IV. The Requirements for C ₄ Photosynthesis	363
	V. Which Plant Should We Transform?	366
	VI. Which Mechanism of C ₄ Photosynthesis Should Be Used and Why?	367
	VII. Early Attempts at Transferring C ₄ -Traits into C ₃ Plants	369
	VIII. Alternate Approaches to Improving Photosynthetic Rates	372
	IX. Hurdles to Engineering C ₄ Crops	373
	X. Assessment of C ₄ -ness	374
	XI. Conclusions	374
	Acknowledgment	375
	References	375
19	C₄ Species as Energy Crops	379–397
	<i>Michael B. Jones</i>	
	Summary	379
	I. Introduction	380
	II. What Are the Qualities of an ‘Ideal’ Energy Crop?	381
	III. C ₄ Species as Energy Crops in Cool-Temperate Climates	383
	IV. Examples of C ₄ Species as Biofuel Feedstock	385
	V. Prospects for Energy Crop Improvement	388
	VI. The Environmental Debate and Bioenergy Crops	389
	VII. Economic and Energetic Costs and Benefits	391
	VIII. Conclusions and Perspectives	392
	References	392
	Index	399–410

Preface

C_4 photosynthesis is carbon concentrating system that uses a metabolic cycle centered around phosphoenolpyruvate (PEP) carboxylation to concentrate CO_2 into an internal compartment where Rubisco (Ribulose bis-phosphate carboxylase oxygenase) has been localized. In doing so, it greatly reduces photorespiratory inhibition of photosynthesis and increases the carboxylation capacity of Rubisco over what would be possible in C_3 plants under similar conditions. Approximately 7,500 plant species in 19 families of vascular plants use the C_4 photosynthetic pathway as an alternative to the C_3 pathway. Even though C_4 plants make up to only about 3% of angiosperm species, they account for one-fourth of global terrestrial productivity, and are the most productive and resource-use efficient plants exploited by humanity.

With the discovery of the C_4 pathway in the 1960s by Marshall (Hal) D. Hatch, C. Roger Slack and colleagues, humans quickly recognized its superior performance relative to C_3 photosynthesis. This recognition led to a surge in research of all things C_4 , and by the mid-1970s, the general patterns of ecology, physiology, systematics and biochemistry of the C_4 pathway had been described. This rapid expansion of knowledge of C_4 photosynthesis following the first publication of the C_4 pathway in 1967 stands out as one of the most exciting eras in the plant sciences.

As the C_4 photosynthesis was characterized, plant biologists were able to explain in mechanistic terms many patterns long recognized by humanity. The classic example is the function of Kranz anatomy, which was first described in the 1880s by the Austrian/German botanist Gottlieb Haberlandt, but had no known purpose. The C_4 discovery demonstrated that the enlarged bundle sheath of Kranz anatomy is the internal compartment where CO_2 is concentrated around Rubisco by the C_4 metabolic cycle. The geographical separation of warm-season “sour” grasses from cool season “sweet” grasses that was long noticed by pastoralists became clear – sour grasses are C_4

species, while the sweet grasses were C_3 species. Weed biologists quickly realized that there was a physiological explanation for the severity of the world’s worst weeds; it turned out that most of the severe weeds utilized the C_4 pathway and thus were highly productive in the presence of C_3 crops. Furthermore, with the discovery of the photorespiratory pathway in the late 1960s, plant biologists were able to explain the biogeographical segregation between C_3 and C_4 grasses and sedges, and thus the reason for the long-noted pattern that Kranz species occur in warm climates became known. In short order, the discovery of C_4 photosynthesis revolutionized our understanding of the biological world and our place in it, and in doing so, provided society a means to better manipulate the natural world to meet the food, fiber and fuel needs of human society.

The four decades since the discovery of the C_4 pathway have produced a widening arc of discovery that has spread well beyond the plant sciences to influence a wide range of biological disciplines, as well as fields outside of biology such as geology and anthropology. With the advent of modern phylogenetics, it has become possible to precisely identify the lineages where C_4 photosynthesis independently arose. This understanding laid the foundation for the realization that C_4 photosynthesis is one of the most convergent of evolutionary phenomenon, having independently evolved at least 60 times. Molecular phylogenetics, along with advances in the use of isotopic tracers, provide strong evidence for the first origin of the C_4 pathway some 25 million years ago, at a time when the climate of the earth was becoming cooler and drier, and atmospheric CO_2 levels were falling to values lower than currently observed. The rise of functional and comparative genomics have provided physiologists with important new tools for identifying genes, enzymes and regulatory systems that are essential for C_4 function. In the past decade, these tools have allowed for

the identification of the evolutionary changes within the genome during the evolution of the C_4 pathway. With these discoveries, scientists now have some of the key elements needed to engineer C_4 photosynthesis into C_3 plants, potentially bringing the greater productivity of the C_4 pathway to a wide range of plants used in agriculture and forestry. Because of the magnitude and complexity of the task, C_4 engineering will require unprecedented coordination between specialists in basic research disciplines (plant physiology, genetics and genomics, molecular and systems biology, and bioinformatics) and related applied fields such as crop breeding, agronomy, and weed science. Many scientists, both old and new, will need to become familiar with a wide range of topics concerning C_4 photosynthesis. As such, a new text is needed that provides up-to-date summaries of the latest developments in C_4 plant biology. To this end, we and the authors of the chapters in this volume of the *Advances in Photosynthesis and Respiration* series provide in-depth summaries of the state of our understanding of the structure, function, evolution and potential for novel applications of C_4 plants.

Since the discovery of the C_4 pathway in the 1960s, there have been three major treatises on C_4 photosynthesis. The first was *Photosynthesis and Respiration* (1971) edited by M.D. Hatch, C.B. Osmond and R.O. Slatyer (Wiley-Interscience Publishers). This book arose out of a highly influential conference held in December 1970 at the Australian National University in Canberra where many of the disparate elements of the C_4 story first came together. As summarized by the editors, this meeting “permitted a consensus of opinion on matters of interest or controversy regarding the new and rapidly advancing areas” of C_4 photosynthesis and photorespiration. One seminal feature of this meeting and the resulting book was the first realization of the significance of photorespiration for the existence and success of the C_4 pathway. To this day, the importance of this meeting is heralded by old-timers and youngsters alike, as demonstrated at the 2007 C_4 -CAM International Congress held in Cambridge, England where the attendees honored the early pioneers of C_4 research by singing “*The C-Two Three Through Four Pathway*” first sung by the participants of the 1970 conference.

The second notable treatise was C_3 - C_4 : *Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis* by Gerry Edwards and David Walker (Blackwell Scientific, 1983). This book was notable in that it provided the first in depth, textbook style-summary of the C_3 , C_4 and CAM pathways as understood at that time. For the second generation of C_4 plant biologists who came of age in the late-1970s and 1980s, this book was the C_4 bible, the text to memorize, and later, when they were academics, the book to assign to their students. For nearly 20 years, one could not be a C_4 biologist without having intimate familiarity of C_3 - C_4 , for its breadth of scope addressed everything from the detailed biochemistry to ecological performance of C_3 , C_4 and CAM species. Even today, nearly 30 years later, C_3 - C_4 remains one of the most straight-forward and understandable introduction to C_4 plant biology for students as they move beyond the simple treatments in plant physiology textbooks.

The third and most recent comprehensive overview on C_4 photosynthesis was prepared a decade ago by one of us (R.F.S.) and Russ Monson (*C_4 Plant Biology*, Academic, San Diego, CA, USA, 1999). This book was noted for its breadth, and the depth with which its authors reviewed the biochemical, physiological, evolutionary, ecological, agronomic and anthropological aspects of C_4 plant biology. Notable contributions from this volume included a series of cogent arguments for why the C_4 pathway existed, when it had evolved and how it had influenced the rise of humanity. The first comprehensive phylogenetic pattern of the world's C_4 flora was presented, along with the first detailed theoretical model of C_4 photosynthesis. The distribution of the C_4 flora around the world, and underlying ecological and physiological drivers for the distribution were reviewed, and for the first time, a complete compilation of the many types of Kranz anatomy was presented. As C_3 - C_4 have been to the C_4 plant scientists coming of age in the 1980s and 1990s, *C_4 Plant Biology* became the main text for the most recent generation of plant biologists, many of whom are represented in this volume either as authors, or colleagues whose research is summarized in the many chapters.

Since *C_4 Plant Biology*, there has been rapid progress in our understanding of the C_4 pathway,

with new emerging concepts, particularly in relation to evolution, novel single-cell C_4 plants, molecular biology of gene expression, genetic engineering of C_4 traits and novel ways to exploit C_4 plants for food and fuel. The high-throughput techniques of molecular biology are responsible for many of the new insights, but the widening realization that C_4 plants had a great impact on the evolution of the biosphere in recent geological time has brought new approaches and perspectives to the study of C_4 plants. Thus, zoologists, geologists and anthropologists have provided important contributions to our understanding of C_4 plant biology, and knowledge of C_4 photosynthesis is considered important for specialists in each of these disciplines. The need to summarize these recent developments in C_4 research for a broad audience that extends beyond the traditional core of plant physiologists has been a major impetus in the development of this book.

This current book on C_4 plants and algae is broadly divided into four parts. Part I starts with two tributes: one to Jagadish Chandra Bose (Chapter 1) and a second to Constance Hartt (Chapter 2), two of the early discoverers of C_4 -like characteristics in plants. This is followed by an introduction to the book (Chapter 3). Part II addresses new physiological and developmental perspectives of the C_4 pathway. This part has the largest number of chapters (seven in total), reflecting the expansion in our knowledge of this traditional core area of C_4 research. Topics covered in this part include: single-cell C_4 systems in terrestrial and aquatic plants (Chapters 4 and 5); photorespiration (Chapter 6); nitrogen/sulphur metabolism (Chapter 7); nitrogen and water use efficiency (Chapter 8); the development of leaves and the specialized anatomy required for C_4 photosynthesis (Chapter 9); and finally, a review of the temperature responses of C_4 photosynthesis (Chapter 10).

Part III, with five chapters (11–15), provides descriptions of the molecular basis of the C_4 pathway. The intercellular and intracellular transport processes unique for C_4 leaves are described in Chapter 11, while the different patterns of gene expression in mesophyll and bundle sheath cells are outlined in Chapter 12. The molecular and biochemical properties of the key enzymes of C_4 pathway, namely PEP

carboxylase, pyruvate orthophosphate dikinase and C_4 acid decarboxylases, are presented in Chapters 13–15. Part IV contains reviews of the multiple origins of the C_4 pathway in the monocots (Chapter 16) and the geologic history of C_4 plants (Chapter 17). In Part V, Chapter 18 focuses on novel applications of C_4 photosynthesis and how our current knowledge can be exploited for engineering of C_4 rice. The very last chapter (Chapter 19) addresses the use of C_4 species as energy crops.

We are confident that the present volume will follow in the footsteps of the earlier treatises and serve as an important milestone in the literature on C_4 pathway. The information provided here should stimulate further research and pave the way for interdisciplinary interactions, and may be key in inspiring a new generation of researchers to build on the successes of their fore-bearers. The book would be a useful tool in diversifying the research on C_4 photosynthesis and in exploiting C_4 plants for the benefit and advancement of all humanity.

We dedicate this volume to the memory of the many scientists whose early efforts created the knowledge base that made the C_4 discovery possible. While the scientific endeavor is punctuated by significant discoveries that are often attributed to one or a few individuals, it is the efforts of those who have gone before, many of whom are never recognized for their contributions that made the great discoveries such as C_4 photosynthesis possible. In this volume, we have specifically recognized Jagadish Chandra Bose and Constance Hartt, but to this list we would like to add Gottlieb Haberlandt, who first published the term Kranz anatomy (*Kranz-typus*) and recognized that there could be a functional specialization of the mesophyll and bundle sheath cell types. While many know of Haberlandt and Kranz anatomy, few in the C_4 community know his first name, the circumstances of his life, and that he is also considered the father of plant tissue culture. A fascinating aspect of the C_4 story is how independent lines of inquiry suddenly converged in 1966–1968 to produce the understanding that holds today. Names worth recalling from these different lines of inquiry include Heinrich Moser (Austria), Roger Black (Australia) and Tana Bisalputra (Australia and Canada) whose anatomical work between 1934 and 1960 drew

attention to Kranz anatomy in the dicots. Bisalputra may have played a key role in linking the early use of the term “Kranz” with the newly described C_4 physiology, for he brought his knowledge of the early anatomical literature to the lab of Bruce Tregunna in Vancouver, Canada, and published with Tregunna and John Downton the paper that first applied the term “Kranz” anatomy to C_4 photosynthesis (*Canadian Journal of Botany* **47**: 915, 1969). From the cell biology perspective, the possible significance of the distinct cell structure of maize was discussed in some depth in 1944 by M.M. Rhoades and A. Carvalho in a light microscopy analysis. Following the introduction of the electron microscope, A.J. Hodge, J.D. McLean and F.V. Mercer described the ultrastructure of maize chloroplasts in 1955, and W.M. Laetsch and co-workers followed with studies on sugarcane and C_4 dicots in the 1960s. On the gas exchange front, an important node was the lab of Roger Musgrave and students (D.N. Baker, D.N. Moss and J.D. Hesketh) and later, Hesketh’s group which included Mabrouk El-Sharkaway and H. Muramoto in Arizona. These workers, along with Y. Murata and J. Iyami in Japan produced an extensive body of photosynthesis data in the early-to-mid 1960s that drew attention to the distinctive characteristics of what would soon be known as C_4 photosynthesis. On the biochemical front, two important contributions preceded the work of Hatch and Slack. One was from the research team of Hugo Kortschak, Constance Hartt and George Burr at the Hawaiian Sugar Planter’s Association, and the other was the team of Yuri Karpilov in the former Soviet Union. These groups independently demonstrated C_4 acid flux in maize and sugarcane in the 1950s. Unfortunately, Karpilov’s work did not come to the attention of western scientists until the late 1960s, after the C_4 pathway had been described. The Hawaiian results proved instrumental in stimulating Hal Hatch and Roger Slack to begin their experiments on sugarcane in the early-to-mid-1960s, which quickly led to the elucidation of the C_4 pathway (see Hatch in *Photosynthesis Research* **73**: 251–256, 2002). Also of note, Barry Osmond produced a significant paper in 1967 showing that dicots also exhibited the C_4 -type of metabolism. This work, along with the studies by Hatch and Slack, allowed John

Downton and Bruce Tregunna to produce a series of papers in 1968–1970 that pulled the C_4 story together by linking C_4 biochemistry, C_4 anatomy, and the biogeography of C_4 plants.

It is the efforts of these and the many other researchers who made the telling of the C_4 story possible. Their history deserves a dedicated volume, for the discovery of the C_4 story is a compelling example of how disparate and perhaps mundane observations converge in an instant in time with a profound realization that impacts the human condition. With much of this early research now available on-line, we urge the new generation of C_4 plant biologists to examine the contributions of the early pioneers of the C_4 story, both to see how prescient their work was in retrospect, but also to appreciate the context in which they studied. Unlike us, they had no idea of the big discovery that lay just around the corner.

We thank all the authors who made this book possible with their excellent contributions. We owe special thanks to the reviewers who read the drafts and helped to improve the chapters. In particular, we thank Govindjee for his significant assistance, from the beginning of this project until final publication of the manuscripts, and as the founding series editor, author, and critical advisor on formatting/editorial issues. We also welcome Thomas D. Sharkey who has joined this series, from volume 31, as a co-series editor. We appreciate the help and services of Jacco Flipsen, Noeline Gibson (who has now retired), Ineke Ravesloot at the Springer office in Dordrecht, the Netherlands and R. Samuel Devanand, SPi Technologies, India.

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Agepati Srinivasa Raghavendra was born on 17 November 1950 in India. He is now a Professor and J.C. Bose National Fellow at the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, India. He earned a B.Sc. (1969), an M.Sc. (1971) and a Ph.D. (1975), all from Sri Venkateswara (S.V.) University, Tirupati. Availing the Humboldt Foundation Fellowship, he worked with leading plant physiologists/biochemists in Germany, including Ulrich Heber, Hans Walter Heldt, Peter Westhoff and Renate Scheibe. He also collaborated with scientists from Japan, France, Germany and U.K. for extended periods. He started his career as scientist at Central Plantation Crops Research Institute (Indian Council of Agricultural Research, ICAR), Vittal in 1974; worked as Assistant Professor, Botany Department, S.V. University (1976–1982); Deputy Director and Head, Plant Physiology Division, Rubber Research Institute, Kottayam (1982–1985); and Associate Professor (1985), Professor (1996–current), Department of Plant Sciences, and Dean, School of Life Sciences (2004–2010), all at University of Hyderabad. Ragha, as he is called by his friends, contributed significantly towards the discovery of several C_4 plants, C_3 – C_4 intermediates; regulation of C_4 -phosphoenolpyruvate carboxylase, essentiality of mitochondrial respiration for optimizing photosynthesis, mitochondrial enrichment in bundle sheath cells as the basis of reduced photorespiration in C_3 – C_4 intermediates and mechanisms of stomatal closure. He has published more than 190 research papers, and authored a number of reviews and book chapters, besides a highly referred book (*Photosynthesis: A Comprehensive Treatise*, Cambridge University Press, 1998 and 2000). He established an active research group to study photosynthetic carbon assimilation initially at the S.V. University and later at the University of Hyderabad. His current research interests include biochemistry of C_4 photosynthesis, chloroplast–mitochondria interactions and signal transduction in stomatal guard cells. Ragha is on the editorial board of the journal *Photosynthesis Research* and was on the advisory editorial board of the *Advances in Photosynthesis and Respiration*, both published by Springer, Germany. Currently, he is editor-in-chief of *Journal of Plant Biology*. In recognition of his research contributions, Ragha was elected Fellow of all the three Indian Science Academies (Indian National Science Academy, Indian Academy of Science, and the National Academy of Sciences), besides the National Academy of Agricultural Sciences and the prestigious Third World Academy of Sciences, Trieste, Italy.



Rowan F. Sage

Rowan Frederick Sage was born on September 2, 1958 in Reno, Nevada USA, and now lives in Toronto, Canada where he is a Professor of Botany in the Department of Ecology and Evolutionary Biology, University of Toronto, St. George Campus, Toronto, Ontario, Canada. He received a B.Sc. degree in 1980 from Colorado College, in Colorado, USA and his Ph.D. in 1986 from the University of California, Davis under the supervision of Professor Robert W. Pearcy. His Ph.D. dissertation addressed the nitrogen use efficiency of C_4 photosynthesis in the ecologically similar weeds *Chenopodium album* (C_3) and *Amaranthus retroflexus* (C_4). From Davis, he returned to Reno for a post-doctoral appointment in the labs of Thomas D. Sharkey and Jeffrey Seemann at the Desert Research Institute, where he studied the biochemical limitations on C_3 photosynthesis in response to temperature and CO_2 . After 2 years in Reno (1986–1987), he accepted his first faculty appointment at the University of Georgia, where he remained for 5 years (1988–1993). In 1993, he joined the faculty at the University of Toronto, where he reactivated his C_4 research. At the University of Toronto, he served as associate chair (1996–2003) and chair (2004–2006) of the Botany department. Initially, the C_4 research during his Toronto years addressed whether Rubisco limits C_4 photosynthesis at cooler temperatures, rather than pyruvate–phosphate dikinase, which at the time was the prevailing hypothesis. Following the publication of *C_4 Plant Biology* in 1999, which he edited with Russ Monson, Rowan embarked on a 10-year program to study the evolution of C_4 photosynthesis in the dicots. A highlight of this work was the compilation of every known C_4 evolutionary lineage, which at the latest count shows at least 60 independent origins of C_4 photosynthesis, making it one of the most convergent of evolutionary phenomena known to humanity. Rowan’s work on C_4 evolution led to his participation in the C_4 Rice Engineering project, which was initiated by John Sheehy at the International Rice Research Institute in 2006. His current research includes the evolution and engineering of C_4 photosynthesis, the impact of temperature and CO_2 variation on the biochemical processes governing C_3 and C_4 photosynthesis, and cold-tolerance in high-yielding C_4 grasses such as *Miscanthus*. This last project is geared toward developing a bioenergy economy in Canada based on high-yielding C_4 plants. In addition to his research and teaching (of physiological ecology and global change ecology), he is a handling editor for *Global Change Biology* and *Oecologia*, an associate editor for the *Journal of Integrative Plant Sciences*, and serves on the editorial board of *Plant, Cell and Environment*, *Plant and Cell Physiology*, and *Photosynthesis Research*.

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Author Index

- Andreo, C.S., 277–300
- Bauwe, H., 81–108
Benson, A.A., 13–16
Berry, J.O., 221–256
Bowes, 63–80
Bräutigam, A., 199–219
Burnell, J.N., 361–378
- Chastain, C.J., 301–315
- Drincovich, M.F., 277–300
- Edwards, G.E., 29–61
Evans, J.R., 129–146
- Ghannoum, O., 129–146
Govindjee, 3–11
Gowik, U., 257–275
- Jones, M.B., 379–397
- Kocacinar, F., 161–195
Kopriva, S., 109–128
Kubien, D.S., 161–195
- Lara, M.V., 277–300
- Maretzki, A., 13–16
Maurino, V.G., 277–300
- Nelson, T., 147–159
- Osborne, C.P., 339–357
- Patel, M., 221–256
- Raghavendra, A.S., 3–11, 17–25
Roalson, E.H., 319–338
- Sage, R.F., 17–25, 161–195
- von Caemmerer, S., 129–146
Voznesenskaya, E.V., 29–61
- Weber, A.P.M., 199–219
Westhoff, P., 257–275
- Zielinski, A., 221–256

Part I

Tributes & Introduction

Chapter 1

Sir Jagadish Chandra Bose (1858–1937): A Pioneer in Photosynthesis Research and Discoverer of Unique Carbon Assimilation in *Hydrilla*

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Summary	3
I. Introduction.....	4
II. Life of Sir J.C. Bose	4
III. Out of Box Concepts and Innovative Instruments for Biological Experiments.....	5
IV. Classic and Comprehensive Monographs on Physiology of Plants	6
V. Work on Photosynthesis and Focus on <i>Hydrilla</i>	6
VI. Importance of Malate and Operation of C ₄ -like Pathway.....	7
VII. Contemporary View of his Observations on <i>Hydrilla</i>	7
VIII. Observations on Inhibitors/Stimulants on Photosynthesis in <i>Hydrilla</i>	8
IX. Concluding Remarks: Inspiration for Biology Research in India and a Pioneer of Photosynthesis Research on <i>Hydrilla</i>	9
Acknowledgments	10
References	10

Summary

Sir Jagadish Chandra Bose (1858–1937) is acknowledged as the greatest interdisciplinary scientist in India; he was a pioneer of not only Physics, but of Plant Biology. Essentially, he was the father of Biophysics, long before it became a field. He was almost 60 years ahead of his time in his ideas, research and analysis. Bose had several out-of-box concepts and designed his own innovative instruments to facilitate his research. He made several discoveries during his studies on physiology and biophysics of plants, particularly the electrical nature of conduction of various stimuli. His interest shifted during early 1920s from physics towards the physiology of plant movements and then photosynthesis. He fabricated and used a unique photosynthesis recorder to study extensively the carbon assimilation pattern, actually measured through oxygen evolution, in an aquatic plant, *Hydrilla verticillata*. Bose made a phenomenal discovery that a unique type of carbon fixation pathway operated in *Hydrilla*. The plants of *Hydrilla* during summer time were more efficient in utilizing CO₂ and light. The summer-type plants used malate as

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a source of CO₂ and appeared to be different from Crasulacean Acid Metabolism (CAM) plants. These findings of Bose appeared anomalous at his time but are now known to illustrate an instance of non-Kranz single cell type C₄-mechanism. In view of his major research contributions, we consider J.C. Bose as a pioneer of photosynthesis research not only in India but also in the world.

I. Introduction

Two prominent names come up when we consider the scientific research in contemporary India: Sir Jagadish Chandra Bose (also known as Jagdish Chander Basu; November 30, 1858 to November 23, 1937), popularly known as J.C. Bose and Sir Chandrasekhara Venkata Raman (November 7, 1888 to November 21, 1970), who was a 1930 Nobel laureate in Physics, for the discovery of the Raman Effect. In biology, the contributions of Sir J.C. Bose (Fig. 1) are awesome and outstanding. Bose was an outstanding physicist as well as a biologist, a pioneer of Biophysics. With his initial interest in electromagnetism and exploitation of electromagnetic waves, he invented several devices for radio-communication with short waves. Later, his attention turned towards the movements and electrical responses in biological systems, mainly of plants. He was a rare genius who was highly versatile and contributed to diverse fields of not only science (physics/biology/botany/biophysics/ archaeology) but also the Bengali literature; he also wrote science fiction in Bengali. See Geddes (1920), Ray and Bhattacharya (1963) and Salwi (2002) for his biography.

II. Life of Sir J.C. Bose

Sir Jagadish Chandra Bose was born on 30 November 1858, at Mymensingh, now in Bangladesh. He had a graduate degree in science from St. Xavier's College, Calcutta (now Kolkata) and obtained an honors degree, as well as a National Science Tripos in Physics, from Cambridge University, UK, in 1884. Soon after his return from Cambridge in 1885, he was appointed a Professor of Physics in Presidency College, Calcutta. Here, he initiated his experiments in various areas in physics and botany. He received the D.Sc. degree of London

University in 1896 for his work on the determination of wavelength of electric radiation by diffraction grating (Ray and Bhattacharya, 1963).

Bose retired from the Presidency College in 1915 and joined in the same year as an Emeritus Professor in the newly founded Department of Physics in the University College of Science, Calcutta. He utilized his scholarly background in



Fig. 1. (a) Portrait of J.C. Bose. (b) J.C. Bose at the Royal Institution, London, with his radio equipment. The date is 1897, prior to his plant research. (c) The Museum located in Bose institute, displaying the work and several innovative instruments developed by J.C. Bose. (d) Bust of Sir J.C. Bose, in the Museum; on the right of the bust, a potted plant of *Mimosa pudica* can be seen. (e) Plaque of J.C. Bose, in the museum, Bose Institute, Kolkata. (f) *Samadhi* (holy grave) of Sir J.C. Bose, in the courtyard of the main campus of the Bose Institute (Courtesy: Bose Institute).

Physics to initiate path-breaking work in Plant Biology, specifically Plant Physiology. He could unequivocally demonstrate scientifically that plants had life, something everyone knew, and responded to stimuli, as in the case of animals (Sen, 1997). Bose was conferred the Knighthood in 1916 and was elected a Fellow of the Royal Society, England, in 1920. He is respected throughout India as ‘Acharya’, meaning the most revered teacher. He established the Bose Institute (then called Basu Bigyan Mandir) in 1917. The Bose Institute (Fig. 2) was then devoted mainly to the study of plants. The Institute’s research interests expanded gradually into several other related subjects. At present, Bose Institute is one of the pioneering research institutions in India (for further details of the Bose Institute, visit their website: <http://www.boseinst.ernet.in>). J.C. Bose passed away on 23 November 1937 at Giridih in Bihar, India.

The extensive studies of J.C. Bose on the photosynthetic characteristics of *Hydrilla*, and his leading contributions to photosynthesis research in India are highlighted in several articles (see S. Bose, 1982; S. Bose and Rao, 1988; Raghavendra et al., 2003; Mukherjee and Sen, 2007).

His basic approach was to study electromagnetic waves, their properties and their practical applications in both living and nonliving objects. This approach of applying physical principles to biological system developed into the exciting field of biophysics. Despite his inventing the radio,

contemporarily with Marconi of Italy, Bose did not get proper recognition, as he did not patent the device. One of the innovative concepts of Bose was that plants and metals have ‘life’ on the basis of their electrical responses. We, of course, know that life, as we understand it today, is not in ‘metals’: it was only a way of expressing himself at that time because he was trying to bridge physics and biology! He proved that plants as well as animals use electric signals to carry and convey information.

III. Out of Box Concepts and Innovative Instruments for Biological Experiments

Several of the concepts/explanations proposed by Sir J.C. Bose were all of out-of-box approach at those times. His comprehensive experiments in photosynthesis, physiology, physics, his monumental monographs and his innovative work on plant physiology, made him a pioneer and an icon of biological research in India. His contributions to the communication systems in biology as well as physics are amazing. He devoted strong attention to studies on the biology of movements, feelings and nervous system. The word ‘feelings’ was used for plants, but clearly this is a matter of semantics; plants react both chemically and physically to touch, but to use the word ‘feeling’ or ‘sensation’ as we know it is quite different. The simple experiments



Fig. 2. Bose Institute, Kolkata. On the left (a) is the Main Campus, started in 1917 and located on Acharya Prafulla Chandra Road, near Raja Bazaar in Kolkata. On the right (b) is New Building, in the “Acharya J.C. Bose Centenary Campus” at Kankurgachi, Kolkata. This campus was built to commemorate the birth centenary of Sir J.C. Bose (Courtesy: Bose Institute, Kolkata, 2008).

of Bose revealed a high degree of similarity in the responses of plant and animal tissues to external stimuli. This principle was amply demonstrated later by biophysicists, using highly sophisticated instruments (Shepherd, 1999, 2005).

The areas of Bose's research included electrophysiology, physiology of ascent of sap, movement in plants, mechanisms of plant response to varieties of stimuli, and physiology of photosynthesis. Three notable features/objectives of his research were: (a) to measure the responses *quantitatively*; (b) to design and to build the physical instruments required for the purpose; and (c) to interpret the results quantitatively in terms of the physicochemical principles known at that time to him. During his research career, Bose designed and utilized several innovative instruments, which looked simple, but were very sensitive and capable of measuring minute changes (Table 1). Only some of these instruments were patented. One of these most fascinating instruments was the Photosynthesis Recorder that can detect the formation of carbohydrate as a millionth of a gram per minute, and record the rate of photosynthetic activity (Fig. 3).

IV. Classic and Comprehensive Monographs on Physiology of Plants

Sir J.C. Bose was never in a hurry to publish small scientific articles. He studied the phenomena in detail and then published his observations com-

Table 1. A partial list of the novel and innovative instruments fabricated by Sir J.C. Bose.

Instrument	Purpose/parameter of measurement
Oscillating recorder	Ascent of sap
Photosynthetic recorder	Rate of carbon assimilation by plants
Crescograph	Growth of a plant
Magnetic Crescograph	Movements beyond the magnifying capacity of light microscope
Transpirograph	Quantity of water transpired by a single stoma of the leaf
Magnetic radiometer	Measure energy of every ray in the solar spectrum
Resonant recorder	Determination of the latent period of the plant within millisecond
Conductivity balance	Determine the effect of various drugs on electrical impulse

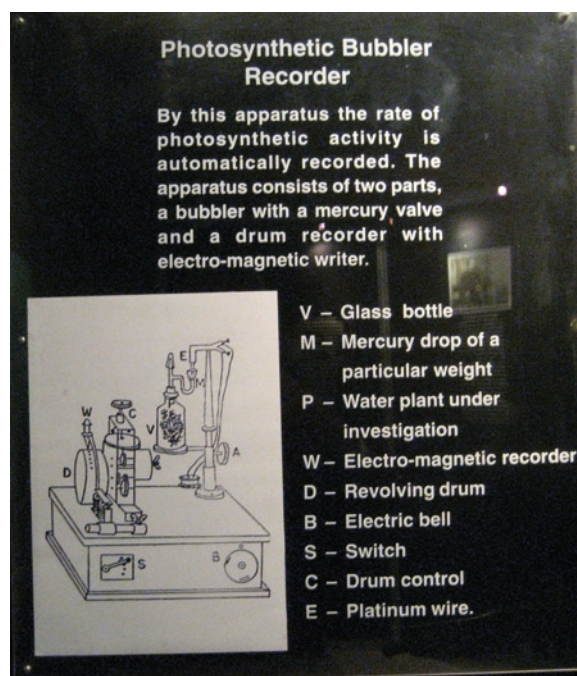


Fig. 3. The *Photosynthesis Recorder* fabricated by J.C. Bose and used extensively for his experiments on photosynthesis in *Hydrilla* (Bose, 1924). This photograph is that of an exhibit in the J.C. Bose Museum in Kolkata, taken by one of us (Govindjee) in January, 2008.

prehensively in the form of books – monographs. This apparently had the disadvantage of his research being not in a format for scrutiny by the peers since often experimental details were not available. One of his books was on “*Responses in the Living and Non-living*”, published by Longman in 1902. This monograph made him a celebrity in the world of science. His other important publications again were mostly monographs, including the one on “*Physiology of Photosynthesis*”, published in 1924 (Table 2). His observations have been published in several volumes by Longmans, Green and Co. Ltd., England during 1902–1928.

V. Work on Photosynthesis and Focus on *Hydrilla*

During his research life, Bose carried out important and thought-provoking experiments on photosynthesis, particularly on its physiological aspects. In the simplest terms, photosynthesis in plants may be described as the process by which

Table 2. Books written by J.C. Bose on physiology and physics of plant cells, including photosynthesis.

Bose JC (1902) Response in the living and nonliving. Longmans, Green & Co., London
Bose JC (1906) Plant response as a means of physiological investigation. Longmans, Green & Co., London
Bose JC (1907) Comparative electrophysiology. Longmans, Green & Co., London
Bose JC (1913) Researches on the irritability of plants. Longmans, Green & Co. London
Bose JC (1923) The physiology of the ascent of sap. Longmans, Green & Co., London
Bose JC (1924) The physiology of photosynthesis. Longmans, Green & Co., London
Bose JC (1926) The nervous mechanism of plants. Longmans, Green & Co., London
Bose JC (1927) The plant autographs and their revelations. The Macmillan Company, New York
Bose JC (1928a) The motor mechanism of plants. Longmans, Green & Co., London
Bose JC (1928b) Growth and tropic movements of plants. Longmans, Green & Co. London
Bose JC (1985) Life movements in plants. Reprinted and distributed by D.K. Publishers' Distributors, Kolkata

CO₂ and H₂O are taken up, forming carbohydrate and releasing oxygen, using Light. Bose presented the results of his comprehensive studies on photosynthesis, in the form of a book, '*The Physiology of Photosynthesis*' (Bose, 1924). The comparative results and discussion of Bose's investigation with *Hydrilla* in summer and winter seasons are available in the articles of S. Bose (1982) and S. Bose and Rao (1988). During J.C. Bose's time, biochemical interpretations were not available. Subsequent work provided detailed explanations of unique photosynthetic characteristics of *Hydrilla*, which could be ascribed to a variant of carbon assimilation or CO₂ concentrating mechanism called C₄-pathway (Leegood et al., 2000).

J.C. Bose had selected the aquatic plant *Hydrilla* and used it extensively for his studies. He ascribed the following reasons for selecting the plant (Bose, 1924): (a) The plant can be maintained under normal conditions in a vessel of water; (b) The leaves have no stomata and there is no transpiration, making the system very simple; and (c) The oxygen released into intercellular spaces can easily escape out into the medium. Bose (1924) investigated the relation between CO₂ supply and photosynthesis and defined the coefficient for CO₂ concentration as a measure of the efficiency of CO₂ utilization. The average value of CO₂

coefficient of *Hydrilla* in winter was about 40, which nearly doubled in summer (Table 3). This was a clear demonstration of the marked increase in the photosynthetic efficiency of carbon assimilation in *Hydrilla* during summer time.

VI. Importance of Malate and Operation of C₄-like Pathway

In the early 1920s, Sir J.C. Bose showed that in the aquatic plant, *Hydrilla*, the photosynthetic characteristics in summer were quite different from those in winter. Some of his major observations are summarized in Table 3. Bose (1924) further observed that 'while the juice of the plant was practically neutral in winter and spring, it was very strongly acid in summer'. Furthermore, 'the acidity of the plants was found to be due to the presence of malic and oxalic acids, the latter in small quantities'.

Bose (1924) observed that photosynthesis in *Hydrilla* was unique, because of the following features: (a) Acids, mainly malate, accumulated; (b) Malic acid was a source/substitute for CO₂; and (c) Photosynthesis could occur without external addition of CO₂. *Hydrilla* plants, at high temperatures of summer, became acidic. Photosynthesis, measured by the evolution of oxygen, apparently, occurred also in the complete absence of externally added CO₂. Bose studied the assimilation of organic acids by substituting malic acid for CO₂ and found that the photosynthesis curves of *Hydrilla* under increasing concentration of CO₂ or of malic acid solutions were quite similar (Bose, 1924; see S. Bose and Rao, 1988). Thus, he demonstrated that during photosynthesis, *Hydrilla* assimilated malate instead of CO₂ and that uptake of CO₂ by these plants is less than normal. It is quite astonishing that as early as 1924, Bose had visualized the idea of the operation, in *Hydrilla*, of a quite different photosynthetic pathway, which utilizes malate.

VII. Contemporary View of his Observations on Hydrilla

The primary route of carbon assimilation through Calvin-Benson-Bassham cycle or C₃-pathway was established by the research group of Melvin

Table 3. Photosynthetic characteristics of winter and summer *Hydrilla*.

Characteristic	Form of hydrilla	
	Winter	Summer
Optimum temperature of photosynthesis (°C)	28	33
Light-saturated rate of photosynthesis (arbitrary units, cm m ⁻¹ h ⁻¹) ^a	147	362
Light compensation point (in lux)	205	25
Relative quantum yield (initial slope of photosynthesis <i>versus</i> light intensity curve)	12	25
Efficiency of CO ₂ utilization (initial slope of photosynthesis <i>versus</i> CO ₂ concentration curve)	40	71
CO ₂ compensation point (mg CO ₂ water (100 mL) ⁻¹)	1.2	0

Data adapted from Bose (1924); average values are shown

^aDisplacement of air column by O₂

Calvin and Andy Benson and their coworkers (Bassham and Calvin, 1957; Benson, 2002; Bassham, 2003). The variant of carbon assimilation through C-4 acids was identified and characterized more than a decade later (see e.g., Hatch, 2002). The third type of carbon assimilation is Crassulacean Acid metabolism (CAM) that also uses malate and other acids for concentrating CO₂ inside the cells during darkness (or night); they use these acids up during subsequent day time (Black and Osmond, 2003).

Bose was aware that his observation with summer *Hydrilla* was different from the phenomenon of acid accumulation by many succulent or CAM plants. He said: ‘The organic acids stored during the night (in succulent plants) provide *indirect* material for photosynthesis during the day in the form of CO₂. The *Hydrilla* plant appeared to be most suitable for further investigation on the subject that the organic acid served *directly* for photosynthesis’ (Bose, 1924). Although he proposed that malic acid was used *directly* as a substitute of CO₂ by summer plants, Bose’s observations and the available biochemistry were not detailed enough to suggest any C₄-mechanism in *Hydrilla*. Although the knowledge of biochemistry of photosynthesis was almost nonexistent in the 1920s, his observations and inference, nevertheless, clearly indicated a mechanism different from CAM and which is now known as the C₄-pathway (Bowes et al., 2002).

The physiology, biochemistry and molecular biology of photosynthetic carbon assimilation in aquatic plants, including *Hydrilla verticillata*,

were studied in detail after more than 50 years, by the research group of George Bowes. Their results offered a candid explanation of several of the observations made by J.C. Bose (Table 4). The carbon assimilation pathway in *Hydrilla* turned out to be quite unique and is now being termed as an example of non-Kranz single cell C₄-pathway operating in aquatic angiosperms (see Chapter 5, by Bowes, this volume).

VIII. Observations on Inhibitors/Stimulants on Photosynthesis in *Hydrilla*

J.C. Bose examined the effects of several compounds which either stimulated or inhibited the rate of photosynthesis depending on the nature and concentration of the compounds (Bose, 1923). His observations on the stimulatory effects by almost infinitesimal quantities of different chemical agents were triggered by a casual observation that the rate of photosynthesis of certain water plants increased sharply during a thunderstorm. Bose attributed this phenomenon to the oxides of nitrogen produced by electric discharges in the atmosphere; this conclusion induced him to investigate the effects on photosynthesis of various stimulants. He found that the photosynthesis of *Hydrilla verticillata* was tripled by *nitric acid* and doubled by *thyroid gland extract*. *Iodine* and *formaldehyde* increased the photosynthetic rate 60% and 80%, respectively.

Table 4. The simple observations by J.C. Bose and the independent biochemical characterization of photosynthesis in *Hydrilla*, made by the group of George Bowes.

Observation by J.C. Bose ^a	Biochemical basis	Reference ^b
Low light compensation in summer	Low light compensation point compared to other hydrophytes, such as <i>Myriophyllum</i> or <i>Ceratophyllum</i>	Van et al., 1976
Summer/winter type	Result of daylength and the temperature. Summer type at 27°C/14-h photoperiod and winter type at 11°C/9-h photoperiod	Holaday and Bowes, 1980
Malate is a major product of photosynthesis	Over 50% of carbon assimilated into malate, as shown by the incorporation of ¹⁴ CO ₂	Salvucci and Bowes, 1983
CO ₂ compensation point	Measured precisely; CO ₂ compensation points of >50 mL L ⁻¹ in summer type and 1–25 mL L ⁻¹ in winter type	Magnin et al., 1997
Photosynthetic rate in summer type plants is 2.5 times greater than that of winter type	Activity of PEP (phospho-enol pyruvate)carboxylase, the key enzyme for carbon fixation, is enhanced nearly 10 times in summer forms (C ₄ -type), compared to winter form (C ₃ -type)	Rao et al., 2006
Malate is a source of CO ₂ for photosynthesis	Efficient utilization of malate leading to a reduction in photorespiration; Malate decarboxylated by NADP malic enzyme	Estavillo et al., 2007

^aFrom Bose (1924)

^bArranged in chronological order

IX. Concluding Remarks: Inspiration for Biology Research in India and a Pioneer of Photosynthesis Research on *Hydrilla*

The observations of Sir Jagadish Chandra Bose on “feelings” and movements in plants can be treated as the earliest studies on the “intelligence” of plants, which is being termed by some as ‘plant neurobiology’ (Brenner et al., 2006). As mentioned earlier, the use of the words “feelings” and “intelligence” for plants is a matter of semantics, and we need to caution the readers against their misinterpretation. However, the experiments of J.C. Bose to measure minute electrical signals in plants have been recognized and have paved the way for the biophysics of plant cells (Shepherd, 1999, 2005). The anomalies recorded by Bose in the patterns of plant growth are now confirmed to be due to their oscillatory behavior, found by much sophisticated computer based image analysis system (Jaffe et al., 1985). The biological significance of seasonal and diurnal adaptation became the subject matter of modern research in chronobiology (Chandrasekharan, 1998). Bose’s, 1924 work on photosynthesis with *Hydrilla* is a landmark in photosynthetic research. Sir J.C. Bose is therefore rightly considered as an

early pioneer in research in the field of photosynthesis, particularly carbon assimilation. It seems that Eugene Rabinowitch did not discuss Bose’s work, perhaps because it was not published in regular journals, yet Rabinowitch (1951, p. 1079) did mention his 1924 book.

Bose’s thoughts and vision have illuminated the path of research since 1920s and they became a source of inspiration to several of his students, who all became great scientists in either physics or biology. Among these stalwarts are: Meghnad Saha, J.C. Ghosh, S. Dutta, Satyendra Nath Bose, D.M. Bose, N.R. Sen, J.N. Mukherjee and N.C. Nag, to name a few. Among his students, Satyendra Nath Bose (January 1, 1891 to February 4, 1974) was the most famous as he is known the world-over for the Bose-Einstein’s statistics, and for the particle ‘Boson’ named after him. The (J.C.) Bose Institute in Kolkata is keeping up his motto and is training several young Indian scientists and offering state-of-the-art facilities in physics and biology. The Bose Institute organized an year-long celebrations of the 150th birth anniversary of its founder during 2008 (Fig. 4). It is no wonder that Sir J.C. Bose is treated as the first Modern Scientist and a pioneer in India (Salwi, 2002; Yadugiri, 2010).



Fig. 4. One of us (Govindjee) honoring Sir J.C. Bose by lighting a lamp on November 24, 2008, in front of his statue, located at the entrance of Acharya Jagadish Chandra Bose's Museum, on the Main campus of Bose Institute, Kolkata. This photograph was taken on the occasion of the Inaugural function of an International Symposium, commemorating the 150th birth year of Sir J.C. Bose. Prof. Arun Lahiri Majumder and three Ph. D. students, of the Bose Institute, are also in the picture (Courtesy: Arun Lahiri Majumder and Sampa Das, 2008).

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Chapter 2

Constance Endicott Hartt (1900–1984) and the Path of Carbon in the Sugarcane Leaf

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Summary	13
I. Biography of Constance Hartt: Early Period and Her Move into Hawaii	14
II. Work at Hawaiiin Sugar Planters' Association: Focus on Biosynthesis and Transport of Sugar in Sugarcane	14
III. Discovery of the Role of Malate in Carbon Assimilation and Sucrose Biosynthesis	14
IV. Concluding Remarks	15
Acknowledgments	16
References	16

Summary

A short biography of Constance Endicott Hartt is presented here, followed by her research on the biochemical mechanism of sucrose synthesis in the sugarcane leaf. The excellence of her approach led to delineation, in the sugarcane leaf, of much of the path of carbon in photosynthesis and the unique involvement of malic acid in the production of sucrose, two decades before the classic publication by H.P. Kortschak, C. Hartt and G.O. Burr in 1965 (Plant Physiol. 40: 209–213). The impressive contributions of Constance Hartt to plant biological science had been overlooked because of her isolation from the mainstream plant biochemistry and by the passage of time. Not until the 7th International Botanical Congress in Stockholm, 1950, and her participation in the 1958 Plant Physiology annual meeting at Bloomington, Indiana, USA, did she have an opportunity to interact with scientists in the field of sucrose biosynthesis and, even then, not extensively. Her papers, since 1935, published in The Hawaiian Planters' Record, are no longer widely available. In this Tribute to Constance Hartt, we hope to define her role in developing concepts of the unique process by which the sugarcane leaf produces sugar, besides her contributions to the landmark discovery of unique (now known as C_4) pathway of carbon fixation in sugarcane.

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I. Biography of Constance Hartt: Early Period and Her Move into Hawaii

Constance Endicott Hartt was born on November 2, 1900 in Passaic, New Jersey. She was educated at Mount Holyoke College and at the University of Chicago with a Ph.D. dissertation, 'Potassium Deficiency in Sugar Cane', December, 1928, under Professor Charles Albert Shull (1879–1962), founder of the American Society of Plant Physiologists and its journal, *Plant Physiology*. She had been an Instructor in Hygiene at North Carolina College for Women, 1922–1923. She then taught Biology at St. Lawrence University for 6 years and was an Assistant Professor of Botany and co-chairman of the Department at Connecticut College for Women during 1931–1932. In 1930, she attended the International Botanical Congress at Cambridge, England, and met there Harold L. Lyon (1879–1957), Botanist at The Hawaiian Sugar Planters' Association (HSPA) in Honolulu, Hawaii. Dr Lyon invited her to spend a postdoctoral year in Hawaii on a Sarah Berliner Research Fellowship (from American Association of University Women) followed by another year with a fellowship sponsored by the HSPA. From then on, Hawaii was her home and the HSPA her place of employment as an Associate Research Plant Physiologist for 23 years, from 1936 to 1959. In 1959, she was promoted to 'Senior Plant Physiologist' until her retirement in 1963.

Harold Lyon was a moving force for botanical and plant pathology research in Hawaii and major Pacific islands for more than 50 years since his arrival in 1907. Lyon had a profound influence on the research performance of Constance Hartt. She valiantly pursued her search for the Green Secret of sucrose biosynthesis in the sugarcane leaf, besides playing an important role in the botanical and plant cultural groups of Honolulu for appreciation and protection of plants in Hawaii.

II. Work at Hawaiian Sugar Planters' Association: Focus on Biosynthesis and Transport of Sugar in Sugarcane

Once at the Experiment Station of the HSPA, Constance continued her research work on potassium nutrition (Hartt, 1929). Later, she

extended her studies to search for formation, transport and storage of sucrose in the sugarcane leaf and stalk and the patterns of mineral absorption, with particular emphasis on potassium (Hartt, 1929, 1933, 1934b, c). Her publications were professionally impressive, quite detailed and had the punctiliousness of the distinguished contemporary European publications. She studied also the effects of water on cane ripening (Hartt, 1934a).

In the subsequent years, including the turbulent years of the World War II (1941–1943), Hartt studied in detail the synthesis and transport of sucrose in excised cane blades. Enzymology was not Constance's primary strength but she started to study the enzymes involved in the biosynthesis of sucrose in leaves of sugarcane (Hartt, 1943, 1944, 1947). The results obtained by Hartt led to the conclusion that the formation of sucrose fits into the general scheme of carbohydrate metabolism. She observed that the inhibition of formation of fructose diphosphate (also known as fructose bisphosphate) restricted the formation of sucrose, whereas inhibiting the breakdown of fructose diphosphate increased sucrose synthesis. Hartt concluded that fructose diphosphate is a stepping stone in the formation of sucrose by the sugarcane plant (Hartt, 1943).

III. Discovery of the Role of Malate in Carbon Assimilation and Sucrose Biosynthesis

Based on the studies using specific enzyme inhibitors, Hartt recognized the role of triose-phosphate and fructose diphosphate as intermediates and pointed out the involvement of malate dehydrogenase and malate being involved in sucrose production (Hartt, 1943, 1944). The effect of methane arsenate inhibition of malic enzyme was later considered in a report of inhibition of sucrose synthesis in the C_4 plant, Johnson grass (Knowles and Benson, 1983). Hartt concluded that sucrose synthesis involves respiration and phosphorylation, hexokinase and ATP. She predicted that fructose phosphate and fructose diphosphate are involved in the production of sucrose phosphate with its phosphate being recycled to the pool of ATP.

During the later part of her stay in HSPA, George O. Burr (1896–1987), a distinguished University of Minnesota scientist, was hired to supervise the research work in physiology and biochemistry of the HSPA. In 1946, Burr brought his research experience and international contacts with nuclear scientists to bear on the problems of photosynthesis in the sugarcane leaf. With a combination of his early experience with the isotope, ^{13}C , and realization of the power of two-dimensional paper chromatography, Burr encouraged Constance Hartt to try repeating the work being done at the University of California at Berkeley with sugarcane. She was enthusiastic, offering the full facilities of her laboratory and her capable assistants. Shortly after the end of the World War II, and with Burr's arrival in the Sugar Planters' laboratory and, no doubt with Burr's encouragement, Constance Hartt and Burr started to work with radioactive carbon dioxide fixation and translocation in sugarcane leaves (Hartt and Burr, 1951; Burr et al., 1957; Hartt et al., 1963a, b, 1964).

Hartt also concluded that sucrose synthesis involves respiration and phosphorylation, hexokinase and ATP. She predicted that fructose phosphate and fructose diphosphate were involved in the production of sucrose phosphate with its phosphate being recycled to the pool of ATP (Hartt, 1973). Hartt et al. (1963a) had earlier observed the production of C-14 malic acid in 6 s and its disappearance during 12 min in C-12 carbon dioxide. This is consistent with malate being a primary product of CO_2 fixation. Though Hartt previously had suggested involvement of malate in sucrose synthesis (Hartt, 1944), this publication had failed to recognize the exact relationship. It seems that Constance Hartt determinedly avoided discussion of short time products of photosynthesis in $^{14}\text{CO}_2$. She seemed overly self-conscious of her very early conclusions regarding production of malic acid and its involvement in sucrose synthesis. Hartt continued her extensive studies on the mechanism and the regulation of translocation in sugarcane leaves using radioactive carbon C-14. A series of her publications on the effects of light, temperature, watering, and mineral nutrition, particularly with reference to potassium, phosphorus and nitrogen were published mostly in *Plant Physiology* (Hartt, 1965a, b, 1966, 1967, 1969, 1970, 1971, 1972, 1973; Hartt and Kortschak, 1964).

For completeness and further history on the topic of C_4 photosynthesis, we refer the readers to Kortschak et al. (1957, 1965); Burr (1962); Hartt et al. (1963); Benson (2002) and Hatch (2002).

IV. Concluding Remarks

Constance Hartt was an avid botanist and elected President of the Hawaiian Botanical Society. Figure 1 shows a 1957 photograph of Hartt. As an enthusiastic gardener, she became “the most professional of amateur rose growers”. The 1948 American Rose Annual published an article by Hartt entitled, ‘Roses – A New Hawaiian Hobby.’ She was probably the first successful rose enthusiast in Hawaii. On Friday, December 21, 1984 at the age of 84 this great lady ascended to her ‘Garden in the Sky’. With the simplest of methods and decades before the rest of science,



Fig. 1. Constance Hartt in Honolulu, 1957 (Photo taken by AAB, one of the authors)

Constance Hartt left plant physiologists proud of her immense contributions to our understanding of physiology and metabolism of sucrose production in the sugarcane leaf.

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Chapter 3

Introduction

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Summary	17
I. Introduction.....	18
II. New Physiological and Developmental Perspectives	20
III. Molecular Basis of the C_4 Pathway.....	22
IV. Systematics, Diversity and Evolution.....	23
V. New Uses of C_4 Photosynthesis.....	24
VI. Conclusions.....	24
Acknowledgments	24
References	24

Summary

This chapter introduces the topics covered in the present volume of the *Advances in Photosynthesis* series addressing C_4 photosynthesis. Tremendous progress has been made in our understanding of C_4 photosynthesis since the discovery of the pathway in the mid-1960s. C_4 photosynthesis appears to have evolved as a response to a reduction in CO_2 during the late Oligocene Epoch some 25–30 million years ago, but C_4 species did not begin to dominate the grassland biome until the late-Miocene epoch between 6 and 10 million years ago. Evolutionarily, the C_4 pathway is highly convergent, having evolved independently over 50 times, with the grass family having the most C_4 species and distinct C_4 lineages of all plant families. While serving a common function of concentrating CO_2 around Rubisco, the many independent lineages of C_4 photosynthesis often achieve CO_2 concentration via distinct anatomical and biochemical features. Only the phosphoenolpyruvate (PEP) carboxylation step is common to all forms of C_4 photosynthesis. Thus, C_4 photosynthesis is perhaps better characterized as a syndrome of distinct traits that share a common function, rather than a single metabolic pathway. Molecular studies now show that the C_4 pathway is derived from modifications to pre-existing enzymes and regulatory networks within C_3 ancestors, rather than the evolution of completely new genes and traits. With this information, humanity is now poised to manipulate C_4 photosynthesis to better address shortages of food and fuel predicted for the coming century. Among the leading innovations proposed for improving food and energy

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supplies are the engineering of C_4 photosynthesis into C_3 crops such as rice, and the domestication of highly productive C_4 grasses to support a cellulosic-based bioethanol industry.

I. Introduction

In terrestrial plants, the C_3 photosynthetic pathway is the predominant form of carbon fixation, occurring in over 90% of land plant species. About 7,500 plant species in 19 families of vascular plants use the C_4 photosynthetic pathway as an alternative to the C_3 pathway (Sage et al., 1999a; Sage 2004). Although small in number compared to C_3 species, the C_4 species of the planet account for about a fourth of the earth's primary productivity, and can dominate grassland and arid shrub communities in warm temperate to tropical climates (Sage et al., 1999b; Gillion and Yakir, 2001). In many ways, C_4 photosynthesis is an astounding development in the history of life on earth. It independently evolved over 50 times (Muhaidat et al., 2007), making it one of the most convergent of evolutionary phenomena. C_4 grasses and sedges contributed to the expansion of the grassland and savanna biomes, leading in turn to an evolutionary diversification of mammalian herbivore guilds, possibly including our own genus *Homo* (Sage, 2004; Osborne, Chapter 17, this volume). By suppressing photorespiration, C_4 plants exhibit greater resource use efficiency and potential productivity than is possible with C_3 photosynthesis. Humans have exploited these characteristics in the domestication of C_4 species with great success. Ancient civilizations in the Western Hemisphere and Africa were supported by the highly productive C_4 crops such as maize, sorghum and tef (van der Merwe and Tschauner, 1999). Today, the affluent lifestyle of developed countries depends upon inexpensive maize grain for the production of meat and dairy

products, while maize, sorghum and sugarcane provide cheap sugar in beverages and processed foods (Brown, 1999).

As we look to the future, humanity might better exploit the productive power of C_4 photosynthesis to address some of the critical problems of the twenty-first century, notably, the need to replace fossil fuels with renewable biofuels, and a need to feed and clothe the extra two to three billion people who will be added to the world's population by mid-century. With C_3 -based agriculture now approaching its maximum yield potential, the higher productive potential and resource use efficiency of C_4 photosynthesis may provide additional food supplies to forestall a global food crises in coming decades, while also producing enough biomass to economically justify a developing biofuel industry (Heaton et al., 2008; Hibberd et al., 2008). The recent advances in molecular biology and genomics now allow plant scientists to discover the genetic controls over C_4 photosynthesis, allowing for rapid improvements in C_4 productivity, and potentially, the engineering of the C_4 pathway into C_3 crops. In recognition of this potential, the International Rice Research Institute (IRRI) in Manila, The Philippines has recently spearheaded an international research effort to engineer C_4 photosynthesis in rice (Sheehy et al., 2007; Hibberd et al., 2008), and many of the industrialized nations of the earth have invested in improving the productivity of C_4 grasses, namely *Miscanthus* and switchgrass (*Panicum virgatum*), to provide large amounts of biomass for a liquid fuel industry based on cellulosic ethanol (Jones, Chapter 19).

While C_4 photosynthesis has much potential for increased production of food and fuel for a rapidly expanding global society, the changes in global conditions associated with human population and economic growth threaten the natural diversity of the planet through climate and land use change, terrestrial eutrophication, and the encroachment of invasive species. C_4 species could be among the most threatened species, due to their relative insensitivity to rising atmospheric relative to C_3 species (Sage and Kubien, 2003; Leakey, 2009). Already, much C_4 -dominated grassland and savanna habitat has been lost due to encroachment, conversion to managed rangeland, or invasion by exotic grasses (Scholes and Archer, 1997; Sage and Kubien, 2003).

Abbreviations: GDC – Glycine decarboxylase complex; IRRI – International Rice Research Institute; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; NUE – Nitrogen use efficiency; PEPCK – Phosphoenolpyruvate carboxykinase; PPDK – Pyruvate phosphate dikinase; PPDK RP – Pyruvate phosphate dikinase regulatory protein; Rubisco – Ribulose 1,5-bisphosphate carboxylase oxygenase; WUE – Water use efficiency

This loss of natural grassland habitat threatens many of the native C_4 species of the Earth through the destruction of their habitat. Some C_4 species are also pernicious invaders who are responsible for biodiversity loss in warm regions. These grasses accelerate fire cycles and reduce soil fertility, thereby allowing them to completely displace both C_3 and C_4 native species (Fig. 1; D'Antonio and Vitousek, 1992). How these invaders will respond in a future, higher CO_2 world is uncertain, but it is probable that they will increase with climate change due to enhanced probability of fire and drought. If grasslands of exotic species continue to expand, the consequence could be far reaching, because the shift from forests to fire-prone C_4 grasslands leads to reduced atmospheric quality, altered precipitation patterns and hydrology, and less buffering of climate within the affected region (D'Antonio and Vitousek, 1992). Increasingly, regions affected by C_4 grass invasions are growing in size such they affect large areas of low-latitude continents and oceanic islands, with consequences for the global environment.

With these issues in mind, we asked many of the leading experts in C_4 plant biology to contrib-

ute their perspectives on diverse topics ranging from the regulation of C_4 enzymes to the evolutionary divergence of the C_4 pathway in recent geological time. Unlike past volumes, such as *C₄ Plant Biology* (1999, R.F. Sage and R.K. Monson, eds. Academic Press) a decade ago, this volume adds perspectives on issues that have been rarely covered in reviews or edited volumes. We begin with tributes to Sir Jagadish Chandra Bose (by Raghavendra and Govindjee, Chapter 1) and Dr Constance Endicott Hartt (by Benson and Maretzki, Chapter 2). Bose was the first to note (in 1924) that the aquatic species *Hydrilla* may exhibit distinct modes of photosynthesis, one in summer, and a second in winter. The significance of this observation would not be known until decades later, when single-celled C_4 photosynthesis was identified in both land and aquatic plants, as is reviewed by Bowes in Chapter 5. Constance Hartt's research on sugarcane proved to be important to the eventual discovery of C_4 photosynthesis. Beginning in the 1947 and continuing into the 1950s, her work with radio-labeled ^{14}C first demonstrated that malate was involved in sucrose synthesis in sugarcane, more than a decade before the first official reports of C_4 photosynthesis in the



Fig. 1. A photograph illustrating the conversion of Hawaiian forest cover to a grassland dominated by a few invasive C_4 grass species. Once established, the invasive grasses promote frequent fire events which suppress the native woodland and eventually lead to a C_4 dominated-grassland or savanna. See D'Antonio and Vitousek (1992) for a detailed review of the process (Photo taken on July 20, 2009 on the east side of the Waianae Mountains, northern Oahu, Hawaii USA by R. Sage).

mid-1960s. These two remembrances highlight the need for a historian of science to comprehensively document the early work that enabled the discovery of the C_4 metabolic cycle in the 1960s, so that the contributions of the early pioneers of C_4 photosynthesis can receive just recognition.

II. New Physiological and Developmental Perspectives

The first section of the book provides new physiological and structural perspectives on C_4 plant biology. Edwards and Voznesenskaya (Chapter 4) provide an overview of the latest developments in our understanding of the two types of single celled C_4 photosynthesis observed in land plants. They also review the many versions of Kranz anatomy in C_4 species, showing that the C_4 structural forms are far more diverse than the three biochemical subtypes that have been recognized for over three decades. How Kranz anatomy arose from the pre-existing anatomy of the C_3 leaves has not been identified, but will need to be understood if a Kranz-based system of C_4 photosynthesis is to be engineered into C_3 crop plants. Nelson (Chapter 9) assesses the controls over vein patterning in C_3 plants, and discusses some of the possible mechanisms by which the C_3 anatomy may be altered. C_4 anatomy does not appear to result from the evolution of novel genes, but rather, through re-regulation within existing developmental networks. One potential change altering leaf development during C_4 evolution is the distribution of polar auxin transporters, which regulates vascular patterning in C_3 species and affects the timing of cell division. Elucidating how polar auxin transporters are altered between C_3 and C_4 species will be complicated, and will likely require comprehensive systems biology approaches to unravel the complex web of genetic controls that affect leaf development.

In chapter 5, Bowes reviews the physiology and molecular biology of C_4 photosynthesis in aquatic species. Bowes' research group first demonstrated *Hydrilla* and other aquatic species were able to conduct C_4 photosynthesis, thus providing a mechanism to explain the observations made by Bose six decades earlier. Many of the aquatic C_4 species exhibit facultative expression of C_4 metabolism, inducing it only when warm,

low CO_2 conditions in the water column favor photorespiration. On land, no species is known to exhibit facultative C_4 metabolism, although many C_4 species can produce C_3 tissues, for example, in the husk leaves of maize, or the cotyledons of some C_4 species such as *Haloxylon persicum* (Pyankov et al., 1999).

The ability of warm, low CO_2 conditions to induce C_4 photosynthesis in certain aquatic plants highlights the importance of photorespiration as a factor in the establishment of C_4 photosynthesis, whether through a plastic response in individuals, or as an evolutionary response within populations. On land, reductions in atmospheric CO_2 may favor the evolutionary origin of C_4 photosynthesis in plants from warm regions of the earth (Ehleringer et al., 1991, 1997; Osborne, Chapter 17). The mechanism linking the CO_2 reduction, warm climates and the rise of C_4 photosynthesis is an excess of photorespiration in the C_3 ancestors of the C_4 lineages. Recent work supports the low CO_2 hypothesis by indicating that the first C_4 species evolved about the time when earth's atmospheric CO_2 content declined to below current levels some 25 million years ago (Tippie and Pagani, 2007; Osborne, Chapter 17). Hermann Bauwe (Chapter 6) describes the key role played by photorespiration in the evolution of C_4 photosynthesis, and reviews how scavenging of photorespiratory CO_2 in C_3 - C_4 intermediates can promote the origin of the C_4 pathway. Photorespiratory CO_2 scavenging occurs in C_3 - C_4 species by localizing the photorespiratory enzyme glycine decarboxylase complex (GDC) to the bundle sheath through mutations in either the mesophyll promoter gene or one of the structural genes for GDC that are specific to the mesophyll (Sage, 2004; Berry et al., Chapter 12). Once GDC is localized in the bundle sheath, the CO_2 released from photorespiration can accumulate to high levels that enhance the efficiency of bundle-sheath Rubisco, thereby improving photosynthesis in hot, low CO_2 conditions promoting photorespiration. After the establishment of the photorespiratory CO_2 pump based on GDC localization to the bundle sheath, place the superior CO_2 concentrating system based on a C_4 metabolic cycle can evolve. The ubiquity of this mechanism for C_4 evolution is supported by the observation that all known C_3 - C_4 intermediate species appear to

localize GDC to an internal compartment that is homologous to the compartment where Rubisco is localized in C_4 plants.

C_4 plants have greater nitrogen and water use efficiencies than C_3 species, which has important consequences for their ecological success. Ghanoun et al. (Chapter 8) review the mechanisms for the enhanced nitrogen use efficiency (NUE) and water use efficiency (WUE) of C_4 relative to C_3 species, and some of the ecological consequences of these differences. Of particular note, they discuss the differences in NUE and WUE observed between the different subtypes of C_4 photosynthesis. C_4 plants of the NADP-malic enzyme (ME) subtype have a greater NUE than plants of the NAD-ME subtype, due in large part to a greater catalytic efficiency of Rubisco in the NADP-ME type species. NAD-ME species have greater WUE than NADP-ME types during drought, and this may explain the greater frequency of NAD-ME relative to NADP-ME grasses in dry regions. Sage et al. (Chapter 10) provide an environmentally-themed chapter that emphasizes temperature responses of C_4 photosynthesis. They review the relationship between temperature and C_4 species distributions, noting the well-described pattern where C_4 species are common in warm to hot environments, but are rare to absent in cold environments. However, dozens of C_4 species represent exceptions to the general pattern in that they have evolved cold tolerance and persist in alpine tundra and boreal habitats – areas thought to be free of C_4 species. Despite being cold-adapted, these C_4 species still require warm periods during the day in order to exist in generally cold habitats. Recent work highlighted by Sage et al. in Chapter 10 indicates that C_4 photosynthesis is limited by Rubisco capacity at low temperature, and this may explain poor performance of C_4 species in cold climates. Where Rubisco capacity strongly limits C_4 photosynthesis, the C_4 species appear to be poorly competitive against C_3 species, possibly because the limitation imposed by Rubisco prevents C_4 species from fully acclimating to low temperature (Sage and McKown, 2006).

C_4 species have evolved spatially separated compartments – the mesophyll where the C_4 cycle reactions predominate, and an inner compartment, commonly called the bundle sheath, where Rubisco and the C_3 cycle reactions are localized (Edwards and Voznesenskaya, Chapter 4). Often,

the separation of the C_3 and C_4 cycle reactions leads to different energy demands within the various cell types, which has consequences for the spatial segregation of other metabolic processes, such as nitrogen and sulfur assimilation. Segregation of energy production is most apparent in the NADP-ME subtype of C_4 species, because photosystem II activity is depleted in bundle sheath chloroplasts. Thus, either reducing power has to be imported into these chloroplasts, typically via malate reduction and oxidation, or metabolites have to be shuttled out to the mesophyll cells. In the case of the energy intensive processes of nitrogen and sulfur reduction and assimilation, Kopriva (Chapter 7) outlines the spatial segregation of key steps in these two pathways within C_4 species. In C_4 grasses, nitrogen reduction is generally localized to the mesophyll cells as demonstrated by the exclusive presence of nitrate and nitrite reductase in mesophyll tissue. This presumably allows for easy access to the relative abundance of reducing power in the mesophyll cells compared to the bundle sheath cells. Nitrogen assimilation enzymes such as glutamine synthetase and glutamate synthase (GS/GOGAT) are not always localized to the mesophyll cells, as they often occur in bundle sheath tissues, possibly because they are needed for localized assimilation of any photorespiratory ammonia produced in the bundle sheath. Sulfur reduction and assimilation shows an opposite pattern in C_4 grasses, with the bundle sheath being the predominant compartment for sulfur assimilation. This pattern is not apparent in the dicot species of *Flaveria*, so it remains uncertain whether the distribution of sulfur metabolism is related to the spatial segregation of energy transduction in C_4 leaves.

Spatial segregation and high photosynthetic capacity creates a need for rapid transport of metabolites and energy between the two compartments. This area has not been a mainstream focus for C_4 studies until recently when Andreas Weber and colleagues developed powerful molecular tools to elucidate the behavior of important transport proteins. Brautigam and Weber (Chapter 11) review C_4 transport systems and some of the molecular tools used to identify the specific transporters. Intercellular transport requires two sets of transporters – at organellar membranes within cells and between the cells of the two tissues types. In C_4 photosynthesis, most of the transport

between mesophyll and bundle sheath cells occurs symplastically via plasmodesmata. Compared to C_3 species, the walls between mesophyll and bundle sheath cells are rich with plasmodesmata. Intracellular transporters for C_4 metabolites must be numerous, given the number of metabolites that rapidly move between the organelles of mesophyll and bundle sheath cells. Few of the C_4 -specific transporters have been characterized, although based on analogous carriers studied in C_3 plants, many of their properties can be inferred. Transport physiology of C_4 photosynthesis will be a research area of increasing importance, for the high capacity mesophyll to bundle sheath transport systems will need to be identified in order to engineer a Kranz-based C_4 pathway into C_3 species.

III. Molecular Basis of the C_4 Pathway

The molecular biosciences have revolutionized our capacity to dissect enzymes, metabolic systems and developmental pathways. Molecular work on the major C_4 enzymes has revealed many new insights on how expression of the genes encoding C_4 cycle enzymes are regulated and compartmentalized. Berry et al. (Chapter 12) provide an overview of the regulatory controls governing the expression of the major metabolic enzymes in C_4 plants. Rubisco mRNAs, for example, are expressed in both mesophyll and bundle sheath tissue of very young leaves in what they term as the C_3 default pattern. As the leaf becomes photosynthetically competent, expression of Rubisco mRNAs is restricted to the bundle sheath by unknown C_4 activation factors. In *Amaranthus*, the activation factors are associated with the source to sink transition, while in maize, exposure to light initiates the bundle sheath-specific expression of Rubisco. With respect to the bundle sheath expression of other C_4 pathway enzymes, Berry et al. raise the interesting point that the bundle sheath is the site of numerous stress responses, and that numerous enzymes such as NAD-ME that are incorporated into the C_4 pathway of some species may be coupled to stress responses in the vascular bundles of their C_3 ancestors.

The best studied of the C_4 enzymes is PEP carboxylase (PEPC, Gowik and Westhoff,

Chapter 13), which has evolved from a C_3 isoform in each of the independent evolutionary lineages of C_4 photosynthesis. Despite this independent evolution, C_4 PEPCs show convergence on similar kinetic properties and metabolite sensitivity driven by a common number of amino acid substitutions, the best described of which is a serine replacing an alanine at about position 774 in the *ppc* gene. This substitution raises the K_m for PEP of PEPC. To enable tissue specific expression, there also has to be changes in the promoters of C_4 enzymes such as PEPC. In the case of PEPC from *Flaveria*, the C_4 -specific PEPC is targeted to the mesophyll by a “mesophyll expression module” on the distal portion of the *ppcA* promoter.

Biochemically, C_4 plants are classified into three subtypes based upon the predominant decarboxylating enzyme used in the C_4 cycle; however, this classification blurs in many species which often have substantial activities of two decarboxylating enzymes (E). Drincovich et al. (Chapter 14) discuss the biochemistry and molecular biology of the three decarboxylating enzymes – NADP-ME, NAD-ME and PEPC. Of these, NADP-ME is the best studied due to its presence in maize and other economically valuable C_4 grasses, and in the model species of *Flaveria*. In *Flaveria*, the expression of NADP-ME is directly correlated with the strength of the C_4 metabolic cycle (Drincovich et al., 1998). NADP-ME expression is specific to the chloroplasts of bundle sheath cells, NAD-ME is localized into the mitochondria of bundle sheath cells, and PEPC expression is cytosolic in the bundle sheath cells. To date, it is not known why evolution selected any particular decarboxylating enzyme as the predominant form in a given C_4 lineage. Plastidic forms of NADP-ME in C_3 plants apparently provide a burst of NADPH for protein and lipid biosynthesis. In plastids of vascular bundle, C_3 NADP-ME may also aid in synthesizing organic acids to assist ion uptake and transport. NAD-ME has been derived from ancestral C_3 forms involved in respiration of malate. The prior localization of NAD-ME in the bundle sheath may facilitate its selection as a primary decarboxylase in certain types of C_4 species. The least commonly used decarboxylase is PEPC, which is utilized in some C_4 grasses and is not known to be a major decarboxylase in dicots and sedges. C_4 isoforms of PEPC evolved from C_3 forms which were important in metabolizing

lipids to sugars during germination of seeds and amino acid biosynthesis during seed maturation and fruit ripening.

A third critical enzyme in the C_4 metabolic cycle is pyruvate phosphate dikinase (PPDK), which regenerates PEP using the equivalent of two ATP in the NADP-ME and NAD-ME subtypes. In Chapter 15, Chastain reviews the biochemistry of PPDK, with particular emphasis on a newly discovered PPDK regulatory protein (PPDK RP) that activates and inactivates PPDK by reversibly phosphorylating a threonine residue near the catalytic site. The PPDK RP type of protein is uncommon in plant biology because of its ability to activate PPDK by dephosphorylating it in light and deactivate it by phosphorylating it in the dark. Most activation/deactivation mechanisms by reversible phosphorylation depend upon separate kinase and phosphatase enzymes. ADP concentration in the chloroplast appears to exert the critical regulatory control over PPDK RP, and in doing so, couples PPDK activity to photophosphorylation and energy status in the cell. ADP inhibits the phosphatase activity of PPDK. At high light, ADP levels in the chloroplast are depressed, allowing for great RP phosphatase activity and greater PPDK activity. At lower light, ADP levels rise, inhibiting phosphatase activity and allowing the inactive, phosphorylated form of PPDK to predominate. Throughout this action of the PPDK RP, one of the critical steps in C_4 photosynthesis is closely coordinated with activity of both the C_3 and C_4 metabolic cycles in C_4 plants.

IV. Systematics, Diversity and Evolution

Most C_4 species are monocots. Grasses account for about 50% of the total number of C_4 species, while sedges account for about a fourth (Sage et al., 1999a). Grasses and sedges have great significance for humanity as they provide the grain consumed by humanity, the bulk of the refined sugar and syrups, and a large majority of the fodder used for meat production by grazing animals (Brown, 1999). Thus, understanding the distribution of C_4 photosynthesis in the grass and sedge family is critical to understanding when and how these important species diversified in recent geological time. Roalson (Chapter 16) reviews the

phylogenetic status of the grasses and sedges. Recent years have seen great strides in the phylogeny of both grasses and sedges. Where just a few years ago it was uncertain how many times C_4 photosynthesis arose in these two species-rich families, it can now be claimed with some confidence that there are at least 22 independent origins of the C_4 pathway in the grasses and sedges, with 17 estimated for the grasses, and 5 for the sedges. Species level phylogenies are not completed yet for the two subfamilies with the greatest number of C_4 species, the Chloridoideae and the Panicoideae. As better phylogenetic resolution is obtained in these groups, it will be possible to clarify the exact number of origins and any reversion from C_4 to C_3 that may be present.

Osborne (Chapter 17) discusses the latest views on the diversification of C_4 species following the origin of the C_4 pathway. Isotopic and fossil evidence indicates C_4 grasses were not very common on the landscape for some 10–20 million years after they first evolved. Instead, they were ecological subdominants until the late-Miocene, when there was a widespread expansion of C_4 -dominated grasslands between 10 and 6 million years ago. This expansion occurred at a time when the Earth's climate was changing for the worse, with increasing aridity and seasonality leading to conditions such as increased fire frequency which favor grasses over woodland species. Osborne proposes the novel hypothesis that increased grazing pressure from newly-evolved species of large herbivores may have selected for herbivore resistance in the C_4 grasses, and the traits conferring herbivore resistance may have promoted grass flammability. Greater flammability would have interacted with increased seasonality to stimulate the intensity and frequency of wildfire, which would then have restricted the occurrence of woody species on the landscape. Osborne makes the critical point that there is rarely one ecological factor that determines changes in vegetation structure, and thus to understand the driving forces for the ecological expansion of C_4 photosynthesis, we have to take a broad view that considers a range of factors and their interactions. To do this, it is likely that the phylogenetic approach outlined by Roalson in chapter 16 will have to be coupled with paleoecology, climate modeling, isotopic studies and current physiological understanding.

V. New Uses of C₄ Photosynthesis

The final section of the book turns to the promise of exploiting C₄ photosynthesis in novel ways. In Chapter 18, Burnell reviews the efforts to engineer C₄ photosynthesis into rice. These efforts, largely in the 1990s, produced some notable transformants, such as rice and tobacco plants expressing one or more C₄ cycle enzymes from a C₄ plant. None of these efforts proved successful in terms of producing a functional C₄ cycle in C₃ plants; however, the experience produced valuable lessons that are being incorporated into renewed efforts at C₄ engineering underway at IRRI and elsewhere. Jones (Chapter 19) reviews the prospects for the utilization of C₄ grasses for the production of biofuels, notably bioethanol. Currently, there are two leading C₄ crops used in bioethanol production, sugarcane stems (largely in Brazil) and maize grain (in the USA). These are the leading first generation biofuel crops. Both crops are controversial, as they require significant subsidies to be profitable, and have huge input requirements in terms of water and fertilizers. In the case of maize grain, there is concern whether the energy produced significantly exceeds the energy invested in growing the crop and processing it to ethanol. Jones describes the effort to develop better alternatives to first generation biofuel crops such as maize and sugarcane, which were originally bred for food production and have been co-opted as a feedstock for bioethanol. The next, or second generation of biofuel crops would be specifically selected from wild-species for the ability to produce large amounts of biomass that is easily converted to a liquid fuel. Second generation bioenergy crops are largely perennial C₄ grasses such as Miscanthus and switchgrass that exhibit superior production characteristics and high water, light and nitrogen use efficiencies. As perennials, the C₄ grasses effectively recycle their above ground nutrients into roots and rhizomes over the winter. This produces a carbon rich shoot biomass for fuel production and reduces fertilizer costs. It will be important for the biofuel industry to contain production and environmental costs, which may be possible with the highly productive C₄ perennials. Key to the success of the new, second generation crops will be the development of markets for the biomass. Currently, the hope is that

Agepati S. Raghavendra and Rowan F. Sage

advances in cellulose degradation will provide an economical means to degrade the wall fraction of the harvested biomass into easily fermentable hexoses. Such a development would greatly enhance the fraction of plant mass that could be exploited for fuel production relative to that of first generation biofuel species.

VI. Conclusions

In summary, this volume in the *Advances in Photosynthesis and Respiration* series reviews some of best work on C₄ plant biology produced in recent years, and in doing so highlights the significant contributions of the many researchers whose efforts now allow us to consider novel ways to improve photosynthetic productivity to meet the critical needs of humanity in the coming century. While there is much still to be learned about the C₄ pathway, there is much we already know, and with the new tools of genomics, bioinformatics and systems biology, we are in a strong position to exploit current understanding to direct groundbreaking research into the improvement of C₄ photosynthesis for the benefit of all people on planet earth.

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Part II

New Physiological and Developmental Perspectives

Chapter 4

C₄ Photosynthesis: Kranz Forms and Single-Cell C₄ in Terrestrial Plants

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Summary	30
I. Introduction	30
A. What Does It Take to Be C ₄ ?	30
B. Occurrence of C ₄ Among Terrestrial Plants	30
II. Structural and Biochemical Diversity in Kranz Type Anatomy	31
A. Structural Diversity	31
1. Poaceae	31
2. Family Cyperaceae	37
3. Dicotyledons	39
B. Biochemical Diversity: C ₄ Cycles and Energy Requirements for C ₄ Subtypes	45
1. Chloroplasts and Mitochondria	45
2. Illustration of Energetics for NADP-ME Type Species	46
3. Illustration of Energetics for NAD-ME Type Species	48
4. Illustration of Energetics for PEP-CK Type Species	48
5. Additional Energy Requirements in C ₄ Photosynthesis	48
III. Single-Cell C ₄ Photosynthesis in Terrestrial Plants	48
A. Occurrence (Family and Phylogeny)	49
B. Biogeography of Single-Cell C ₄ Species	49
C. Overview of Two Types of Single-Cell C ₄ Photosynthesis in Terrestrial Plants	50
D. Biochemical Evidence for Function of C ₄ Photosynthesis in Single-Cell C ₄ Plants	51
1. General Features Characteristic of C ₄	51
2. Spatial Compartmentation Enabling Function of NAD-ME Type C ₄ Photosynthesis	53
E. Development of Spatial Compartmentation and Dimorphic Chloroplasts	54
F. Form of Photosynthesis in Different Photosynthetic Organs in Single-Cell C ₄ Species	54
G. How Did Single-Cell C ₄ Evolve?	55
IV. Future Perspectives	55
Acknowledgments	56
References	56

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Summary

Plants identified as having C_4 photosynthesis have a C_4 metabolic cycle with phosphoenolpyruvate carboxylase as the initial catalyst for fixation of atmospheric CO_2 , and a C_4 acid decarboxylase (NADP-malic enzyme, NAD-malic enzyme, or phosphoenolpyruvate carboxykinase), which releases CO_2 for fixation by the C_3 cycle. Effective donation of CO_2 to Rubisco minimizes competition by O_2 and photorespiration, and thus increases photosynthesis under conditions where CO_2 is limiting. To achieve this, fixation of atmospheric CO_2 in the cytosol by phosphoenolpyruvate carboxylase must be separated from the donation of CO_2 to Rubisco by the decarboxylation of C_4 acids. In most documented C_4 plants, this is accomplished through evolution of various forms of Kranz anatomy, with fixation of atmospheric CO_2 in mesophyll cells and donation of CO_2 from C_4 acids to Rubisco in bundle sheath cells. In the family Chenopodiaceae, two alternative means of accomplishing this spatial separation evolved within individual photosynthetic cells, whereby one cytoplasmic compartment specializes in fixation of atmospheric CO_2 in the carboxylation phase of the C_4 cycle, and the other cytoplasmic compartment specializes in donating CO_2 from C_4 acids to Rubisco. In this chapter, biochemical and structural variations of Kranz anatomy in three major C_4 -containing families, Poaceae, Cyperaceae, and Chenopodiaceae, as well as other known forms for dicots, are summarized. Then, the phylogeny, biogeography, development, and structure-function relationships of the single-cell C_4 systems are discussed in comparison to Kranz type C_4 plants.

I. Introduction

A. What Does It Take to Be C_4 ?

This question was posed in a short commentary on the history of C_4 by (Edwards et al., 2001) who noted that the minimum requirements for the CO_2 concentrating mechanism in C_4 photosynthesis are “(a) cell-specific amplification of enzymes of C_4 photosynthesis; i.e. phosphoenolpyruvate carboxylase (PEPC) in mesophyll, and C_4 acid decarboxylases and Rubisco in bundle sheath cells, with complementary adjustments of photosystem and electron transport activities; (b) novel cell-specific organelle metabolite translocators; (c) symplastic connections of the spatially separated sources and sinks of 4C-dicarboxylic acid transport metabolites; and (d) barriers to

CO_2 diffusion between the site of CO_2 fixation by PEPC in mesophyll cells and sites of CO_2 release and refixation by Rubisco in bundle-sheath cells”. These requirements have been met through the multiple, independent evolution of C_4 photosynthesis in different groups of terrestrial plants. Until the recent discovery of two alternative means of performing C_4 photosynthesis within individual chlorenchyma cells (single-cell C_4), all terrestrial C_4 plants were presumed to have Kranz anatomy.

B. Occurrence of C_4 Among Terrestrial Plants

The earliest studies which led to the identification of C_4 plants were on maize and sugarcane, members of family Poaceae (see review by Hatch, 1999). Since then, C_4 plants have been found in 19 families with the largest number of species appearing in families Poaceae, Cyperaceae and Chenopodiaceae. C_4 is estimated to have evolved independently over 50 times (Muhaidat et al., 2007), resulting in three biochemical subtypes (see Chapter 14 by Drincovich et al.) based on the mechanism of C_4 acid decarboxylation: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEP-CK).

Abbreviations: BS – Bundle sheath(s); Kranz cells – An inner layer of chlorenchyma cells specialized for C_4 photosynthesis, irrespective of whether there is contact with vascular bundles (sometimes referred to as BS cells in C_4 plants); M – Mesophyll; MS – Mesophyll sheath(s); NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PEPC – Phosphoenolpyruvate carboxylase; PEP-CK – Phosphoenolpyruvate carboxykinase; PGA – 3-Phosphoglyceric acid; PPDK – Pyruvate, Pi dikinase; PSI – Photosystem I; PSII – Photosystem II; RuBP – Ribulose 1,5-bisphosphate; SL – Suberin lamella

II. Structural and Biochemical Diversity in Kranz Type Anatomy

The occurrence of Kranz anatomy (Kranz means wreath in German) has been known since its initial characterization by Haberlandt (1884). In a broad sense, Kranz anatomy can now be functionally defined to accommodate all known structural variants of Kranz type C₄ plants. A double concentric layer of chlorenchyma cells together form the Kranz tissue with the outer layer capturing atmospheric CO₂ in the C₄ cycle, and the inner layer donating CO₂ from C₄ acids to Rubisco in the C₃ cycle. The outer layer is commonly referred to as mesophyll (M) cells (usually consisting of palisade parenchyma) and the inner layer as specialized bundle sheath (BS) cells or Kranz cells. The M cells are always closer to the atmosphere than the BS cells, and the BS cells, as a rule, have limited contact with intercellular air space. The cells of chlorenchymatous M and BS layers are usually adjacent to one another, but in some cases they are separated by an additional layer of cells. The ratio of M/BS cells is lower than in C₃ plants, and most of the M cells are in direct contact with BS cells. There are two common structural forms, a double concentric layer of chlorenchyma around individual veins, and a double concentric layer which surrounds all the vascular tissue in the leaf. Since C₄ evolved multiple times from different C₃ leaf anatomies, there are various structural types. Specific types are described below which represent striking examples of evolutionary convergence on a common suite of anatomical features.

A. Structural Diversity

Among C₄ plants, there is considerable variation in features relevant to the C₄ mechanism (Carolin et al., 1973, 1975, 1977, 1978; Laetsch, 1974; Brown, 1975, 1977; Ellis, 1977; Edwards and Walker, 1983; Dengler et al., 1985; Voznesenskaya and Gamaley, 1986; Prendergast and Hattersley, 1987; Prendergast et al., 1987; Ueno et al., 1988a; Hattersley and Watson, 1992; Dengler and Nelson, 1999; Sage, 2004; Muhaidat, et al., 2007). While distinct biochemical and anatomical types have been catalogued since C₄ photosynthesis was first described more than three decades ago (see Hatch, 1971), new structural subgroups continue

to be discovered, providing further insight into the evolution of the syndrome. Usually, several characteristics are taken into account to distinguish between different structural and biochemical subtypes. The most important among them are: (1) number of BS layers; (2) presence or absence of a mestome sheath (MS) and its positioning in relation to other parenchyma sheaths (notably in grasses); (3) presence or absence of a suberin lamella (SL) in BS cell walls (in grasses); (4) position of BS organelles (mainly chloroplasts); and (5) chloroplast differentiation between M and BS: BS cells in NADP-ME species have grana-deficient chloroplasts with a few, small mitochondria, while NAD-ME species have chloroplasts with well-developed grana and numerous, large mitochondria with specialized cristae. In both subtypes, M chloroplasts have a reversed pattern of grana development to that expressed in the BS, with abundant, large grana in NADP-ME species, and a deficiency of grana in chloroplasts of M cells in NAD-ME species. Some other features, such as the shape (outline) of the outer parenchyma BS, have been mentioned as being useful in characterizing certain subtypes, for example an uneven outline of the outer BS in PEP-CK type grasses; however, this does not seem to be especially important phylogenetically in relation to C₄ photosynthetic subtypes, as there are many exceptions (Prendergast et al., 1987). Only features of organelle differentiation provide an easy means to predict biochemical subtypes, while other structural characters only give additional information for considering evolutionary development of Kranz anatomy. The main structural forms of Kranz that are known to occur among C₄ species are illustrated in Figs. 1, 2 and 3. Historically, different forms of Kranz anatomy have been referred to either by taxonomic names, or by names descriptive of their structure. We have used taxonomic names to be consistent and concise, recognizing that they are used descriptively and do not always imply phylogenetic identity. An exception is description of the three classical forms of Kranz anatomy associated with the three biochemical subtypes in family Poaceae, which accounts for many C₄ grasses.

1. Poaceae

For C₄ grasses, important distinguishing characteristics include the presence or absence of a

MS, and, when present, the size of MS cells and thickness of their cell walls, the positioning of chloroplasts in BS cells, the presence or absence of a SL and, when present, its distribution in the Kranz cell walls (Carolin et al., 1973; Brown, 1975, 1977; Ellis, 1977; Hattersley and Browning, 1981; Hattersley, 1992; Hattersley and Watson, 1992). At least nine structural subtypes have been distinguished on the basis of these features, and most of the known C_4 grasses fit into these subtypes (described below and illustrated in Fig. 1 and Table 1). It is suggested from phylogenetic analyses that C_4 evolved from C_3 a minimum of 17 times in family Poaceae (Christin et al., 2008, 2009). Certain forms of Kranz anatomy evolved multiple times in the family (Table 1).

In the Poaceae, C_4 species occur in subfamilies Panicoideae, Chloridoideae, Aristidoideae, and Micrairoideae (Sanchez-Ken et al., 2007; Vicentini et al., 2008; Christin et al., 2008, 2009). While most C_4 species in subfamily Panicoideae are NADP-ME type, NAD-ME and PEP-CK type species also occur in the subfamily as discussed below. In subfamily Chloridoideae, most C_4 species are NAD-ME type, while a few genera have PEP-CK type species. C_4 species identified in subfamilies Aristidoideae and Micrairoideae are NADP-ME type (Table 1). Structural forms of Kranz anatomy among these biochemical subtypes are discussed below.

Classical NADP-ME Type Anatomy

This type of anatomy was originally the so-called Panicoid (Carolin et al., 1973). Among the three major biochemical C_4 subgroups first identified in Poaceae (Gutierrez et al., 1974; Hatch et al., 1975; Brown, 1977), classical NADP-ME type species have a single parenchyma BS (the Kranz BS which is derived from provascular tissue and, thus, lacks a MS), with BS chloroplasts in a centrifugal/peripheral position and with a deficiency in their grana development, whereas, M chloroplasts have well-developed grana (Fig. 1)

(Voznesenskaya and Gamaley, 1986; Hattersley, 1992; Yoshimura et al., 2004). C_4 species with classical NADP-ME type anatomy in subfamily Panicoideae occur in tribes Paniceae, Arundinelleae, and Andropogoneae (Hattersley and Watson, 1992; Sage et al., 1999; GPWG, 2001; Vicentini et al., 2008). The degree of grana reduction in chloroplasts in the BS cells varies, from having nearly agranal BS chloroplasts (representatives of tribe Andropogoneae), to having numerous, small grana (tribe Paniceae), to having a few rather large grana (*Panicum obseptum* or *Rhynchelytrum repens*). This subtype has fewer mitochondria in BS cells than in the NAD-ME and PEP-CK subtype (Yoshimura et al., 2004). The SL is present in the outer tangential wall, and partly in the radial cell wall of the BS cells (Hattersley and Browning, 1981).

Classical NAD-ME Type Anatomy

This structural type of C_4 grasses has a double sheath: a MS with thick cell walls and a few plastids, surrounded by a Kranz type chlorenchyma sheath (derived from ground tissue). Bundle sheath chloroplasts are in a centripetal position and have well-developed grana, while M chloroplasts show different degrees of grana reduction according to species (Fig. 1). A SL is usually absent in BS cells; but if present, is only in BS cell walls adjacent to sclerenchyma cells which do not contain chloroplasts (Hattersley and Browning, 1981). This subgroup also has abundant, large specialized mitochondria in BS cells, the site of the C_4 acid decarboxylase (Gutierrez et al., 1974; Hatch et al., 1975; Brown, 1977; Hattersley and Watson, 1992; Yoshimura et al., 2004). This form of anatomy was originally named Eragrostoid (Carolin et al., 1973). It occurs particularly in subfamily Chloridoideae (the core Chloridoideae and *Centropodia* lineages); but, also in subfamily Panicoideae, tribe Paniceae, evolving once in the *Panicum*, *Urochloa*, *Setaria* clade, Table 1 (Sage et al., 1999; GPWG, 2001; Aliscioni et al., 2003; Christin et al., 2008, 2009).

in *Arundinella hirta* for Arundinelloid type, of large veins in *Aristida adscensionis* for Aristidoid type, of *Stipagrostis pennata* for Stipagrostoid type, of *Eriachne aristidea* for Eriachneoid type, of *Alloteropsis semialata* ssp. *semialata* for Neurachneoid type, and of *Triodia scariosa* for Triodioid type (Pictures are adapted from Voznesenskaya and Gamaley, 1986; Prendergast and Hattersley, 1987; Dengler and Nelson, 1999). B, biochemical subtype; BS, bundle sheath; Chl, chloroplast; M, mesophyll; Mito, mitochondria; MS, mestome sheath; OP BS, outer parenchymatous BS; SL, suberin lamella; VB, vascular bundle.

Poaceae

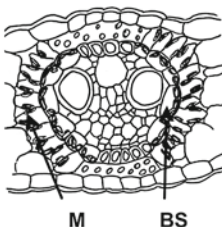
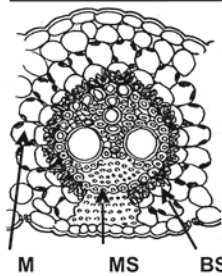
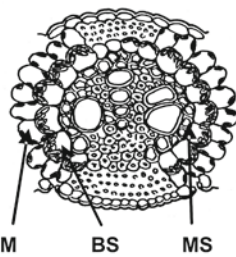
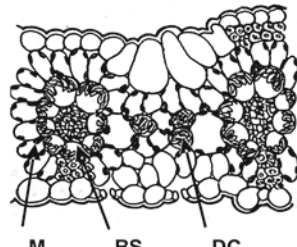
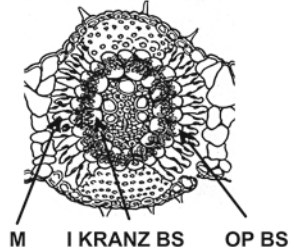
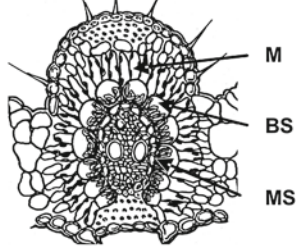
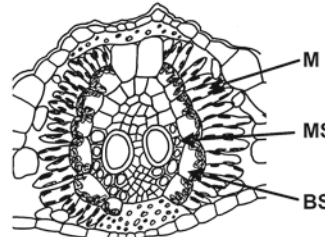
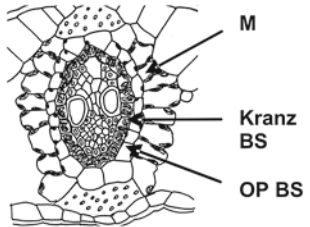
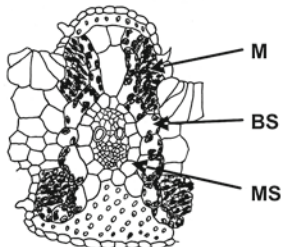
Classical NADP-ME  MS:(-). Chl: BS with reduced grana in centrifugal position, M with well-developed grana. Mito: small and few in BS. SL: usually in outer tangential BS cell wall.	Classical NAD-ME  MS:(+). Chl: BS granal, in centripetal position, M with reduced grana. Mito: numerous specialized in BS cells. SL: Absent in BS cell wall.	Classical PEP-CK  MS:(+). Chl: have well-developed grana in M and BS. Centrifugal or scattered/peripheral in BS cells. Mito: BS a little larger than in M, with more abundant cristae. SL: present.
Arundinelloid  B: NADP-ME. MS:(-). Chl: agranal in BS and centrifugal. SL: present. Presence of distinctive cells (DC) similar to BS cells without VB.	Aristidoid  B: NADP-ME. BS: two parenchymatous BS, the inner Kranz. Chl: agranal, peripheral or centrifugal in Kranz BS, centripetal and granal in outer parenchymatous BS. SL: absent.	Stipagrostoid  B: NADP-ME. MS:(+) enlarged thin-walled cells. Chl: BS cells have reduced grana and are centripetal. SL: absent.
Eriachneoid  B: NADP-ME. MS:(+) enlarged, thin-walled cells. Chl: BS centrifugal with well-developed grana, but stacking lower than in M. SL: absent.	Neurachneoid  MS:(-). BS: double parenchyma sheath, inner is Kranz. SL: present. B: PEP-CK. Chl: BS with well-developed grana, peripheral. B: NADP-ME. Chl: BS centrifugal, with grana reduced in comparison with M.	Triodioid  B: NADP-ME. MS: (+) thin-walled. BS: extensions towards chlorenchyma. Chl: BS centrifugal or peripheral with well-developed grana. SL: absent.

Fig. 1. Illustrations of the forms of Kranz anatomy in family Poaceae. Sketch of vascular bundles in maize for classical NADP-ME, of a large vein in *Eragrostis* sp. for classical NAD-ME, and of species representing PEP-CK type. Sketches of leaf structure

Table 1. Structural and biochemical forms of Kranz anatomy in relation to the number of independent C₄ lineages in family Poaceae. The number of each lineage is according to Christin et al., (2008, 2009)

Subfamily	Lineage	Type	
		Structural	Biochemical
Aristidoideae	1 <i>Stipagrostis</i>	Stipagrostoid	NADP-ME
	2 <i>Aristida</i>	Aristidoid	NADP-ME
Chloridoideae	3 Core Chloridoideae	Classical	NAD-ME
		Classical	PEP-CK
		Triodoid	NAD-ME
Micrairoideae	4 <i>Centropodia</i>	Classical	NAD-ME
	5 <i>Eriachne</i>	Eriachneoid	NADP-ME
Panicoideae	6 Arundinelleae	Arundinelloid	NADP-ME
		Classical	NADP-ME
Panicoideae (tribe Paniceae)	12 Andropogoneae	Classical	NADP-ME
	7 <i>Panicum</i>	Classical	NAD-ME
	<i>Urochloa</i>	Classical	PEP-CK
	<i>Setaria</i> clade	Classical	NADP-ME
	9 <i>Echinochloa</i>	Classical	NADP-ME
	11 <i>Digitaria</i>	Classical	NADP-ME
	13a <i>Paspalum</i> clade	Classical	NADP-ME
	13b <i>Ophichloa</i> clade	Classical	NADP-ME
	14 <i>Anthraenantia</i>	Classical	NADP-ME ^a
	15 <i>Oncorachis ramose</i> (= <i>Streptostachys</i>)	Classical	NADP-ME ^b
	17 <i>Mesosetum</i> clade	Classical	NADP-ME
	8 <i>Neurachne munroi</i>	Neurachneoid	NADP-ME
	10 <i>Alloteropsis</i>	Neurachneoid	PEP-CK
		Neurachneoid	PEP-CK
		Neurachneoid	NADP-ME
	16 <i>Panicum prionitis</i> clade	Neurachneoid	NADP-ME

^aDr. Osvaldo Morrone, personal communication, 2009 Argentina

^bSede et al., 2009

Classical PEP-CK Type Anatomy

The classical PEP-CK type has a double chlorenchyma sheath similar to the NAD-ME type, with an inner MS and outer Kranz chlorenchyma sheath with grana-containing BS chloroplasts in a centrifugal position, or scattered peripherally around the cell, see Fig. 1 (Gutierrez et al., 1974; Brown, 1977; Dengler and Nelson, 1999; Yoshimura et al., 2004). The level of grana development is very similar in BS and M chloroplasts, and the BS mitochondria are quite small (generally comparable in size to M mitochondria) and are usually more numerous than in NADP-ME species, but less abundant than in NAD-ME species (Yoshimura et al., 2004; Voznesenskaya et al., 2006). Suberin lamella is present in the outer tangential BS cell walls and extends approximately to the middle of the radial cell walls (Hattersley

and Browning, 1981). This subtype has been found in subfamily Chloridoideae in *Bouteloua*, *Eleusine*, *Muhlenbergia*, *Spartina*, *Sporobolus*, and *Zoysia*, and in subfamily Panicoideae, evolving once in the *Panicum/Urochloa/Setaria* clade, e.g. in *Brachiaria*, *Chaetium*, *Eriochloa*, *Melinis*, and *Urochloa*, see Table 1 (Sage et al., 1999; Guissani et al., 2001; Aliscioni et al., 2003; Christin et al., 2008).

Arundinelloid: Biochemical Subtype NADP-ME

This type of anatomy was studied in detail in genus *Arundinella* (tribe Arundinelleae) subfamily Panicoideae. Like the classical NADP-ME type, species in this genus have NADP-ME type biochemistry, with BS chloroplasts having reduced grana and in the centrifugal position; MS is absent in all vascular bundles. However,

the Kranz anatomy in veins is widely spaced, and there is the unusual occurrence of a row, or rows, of Kranz assemblies between the veins, which sometimes are referred to as distinctive cells because these bundle sheath-like cells are not associated with vascular tissue (Tateoka, 1958), see Fig. 1. A SL is usually present and continuous in the distinctive cells, or interrupted in the radial cell walls in BS cells surrounding the vascular tissue (Hattersley and Browning, 1981). Distinctive cells have structural and biochemical characteristics similar to the BS cells (Crookston and Moss, 1973; Dengler et al., 1990, 1996; Dengler and Dengler, 1990; Wakayama et al., 2002, 2003, 2006). Distinctive cells have also been found in genera *Arthraxon* and *Microstegium* (tribe Andropogoneae), where they have ultrastructural characteristics similar to those shown for *Arundinella* (Ueno, 1995), and in genus *Garnotia*; but, there is no additional biochemical or ultrastructural data for species of this genus (Tateoka, 1958).

Aristidoid: Biochemical Subtype NADP-ME

The Kranz anatomy of species in genus *Aristida*, tribe Aristideae in subfamily Aristidoideae, is unusual in having three distinct layers of chlorenchyma cells surrounding the vascular tissue: an inner BS, an outer BS, and the M cells (Brown, 1958; Johnson, 1964; Bisalputra et al., 1969), see Fig. 1. *Aristida* species have NADP-ME type biochemistry, based on analyses of several species in the genus (Gutierrez et al., 1974; Hattersley, 1987; Prendergast et al., 1987; Voznesenskaya, et al., 2005b). Mestome sheath is absent; both the inner and outer sheaths are chlorenchymatous. However, in the inner sheath, chloroplasts are nearly agranal, while the outer BS contains chloroplasts with well-developed grana similar to the M chloroplasts. In this type, only the inner sheath functions as Kranz cells, while the outer BS functions mainly for storage of starch and, possibly, for refixation of photorespired CO₂ (Voznesenskaya et al., 2005b). In the inner BS, chloroplasts are scattered around the cell or tend to be centrifugal, while in the outer parenchyma BS, chloroplasts are located in a centripetal position. The SL is absent in cell walls of both types of BS (Hattersley and Browning, 1981).

Stipagrostoid: Biochemical Subtype NADP-ME

The genus *Stipagrostis* belongs to tribe Aristideae in subfamily Aristidoideae. Like *Aristida*, *Stipagrostis* species also evolved NADP-ME type photosynthesis, but they have a different type of Kranz anatomy, named Stipagrostoid (Voznesenskaya et al., 2005a). This subtype has an inner MS consisting of enlarged cells with thinner cell walls and few chloroplasts, and an outer layer of Kranz cells with chloroplasts in the centripetal position (Brown, 1975). In the Kranz cells of *Stipagrostis*, mitochondria are few and small, and the chloroplasts are deficient in grana compared to M chloroplasts, which have well-developed grana. In contrast, the classical NADP-ME subtype grasses lack a MS and they have Kranz cells with chloroplasts in a centrifugal position. Also, the Kranz cells of *Stipagrostis* lack a SL in the walls, whereas the classical NADP-ME type grasses have SL in the Kranz cells, which are thought to have originated from the MS.

Eriachneoid: Biochemical Subtype NADP-ME

This subtype, which occurs in genera *Pheidochloa* and *Eriachne* (subfamily Micrairoideae, Christin et al., 2008, 2009), has NADP-ME type biochemistry (Prendergast et al., 1987) and an inner MS with an outer Kranz sheath, like the Stipagrostoid type. However, unlike *Stipagrostis*, which has chloroplasts in a centripetal position in Kranz cells, in *Pheidochloa* and in most *Eriachne* species (18 of 21) the chloroplasts in Kranz cells are in a centrifugal position (Hattersley, 1987; Prendergast and Hattersley, 1987; Prendergast et al., 1987; Taniguchi et al., 2003). The BS chloroplasts have well-developed grana with numerous, long intergranal thylakoids; in *Eriachne aristidea*, the degree of grana stacking is lower compared to the M chloroplasts (Taniguchi et al., 2003). For other species, the situation is not very clear. Bundle sheath chloroplasts of *E. glabrata*, *E. obtusa* and *P. gracilis* have an unexpectedly large number of grana (up to 21 thylakoids in a stack) for an NADP-ME subtype (Prendergast et al., 1987); however, there is no data about the degree of grana differentiation in M chloroplasts of these

species. The SL is absent in cell walls of Kranz cells (Prendergast et al., 1987).

Neurachneoid: Biochemical Subtypes NADP-ME and PEP-CK

Species with this type of anatomy have a double parenchymatous sheath and the MS is absent; however, in this case the inner sheath is Kranz and the outer sheath is a non-specialized parenchyma BS containing only a small number of chloroplasts (see Dengler et al., 1985; Hattersley et al., 1986; Prendergast et al., 1987; Ueno and Sentoku, 2006). It was suggested that the inner Kranz BS in all species having this type of anatomy originated from the MS of C_3 grasses (Brown, 1975, 1977; Dengler et al., 1985). Species with this type of anatomy which perform NADP-ME type photosynthesis are *Neurachne munroi*, *Paraneurachne muelleri* in the Neurachne clade, and also *Panicum petersonii* and *P. prionitis* in section Prionita; all belong to the subfamily Panicoideae. In both *N. munroi* and *P. muelleri*, thick cell walls of the Kranz inner sheath have a SL, which is only continuous in the outer tangential walls and outer parts of radial walls. The outer parenchyma sheath has relatively thin cell walls without SL. Chloroplasts in Kranz cells are distributed evenly in *N. munroi*, but are in a centrifugal position in *P. muelleri*. Kranz cell chloroplasts have granal stacks which are less pronounced than in the M chloroplasts (Hattersley et al., 1986).

Alloteropsis semialata, subfamily Panicoideae, represents a very unique case, where diversity in the form of photosynthesis occurs among subspecies, with ssp. *semialata* being C_4 and ssp. *eckloniana* being C_3 (Frean et al., 1983; Prendergast et al., 1987; Ueno and Sentoku, 2006; Ibrahim et al., 2009). An Australian accession of spp. *semialata* biochemically is PEP-CK type with Neurachneoid type anatomy (Prendergast et al., 1987). The Kranz sheath, unlike the classical PEP-CK type species, is considered to be derived from the MS sheath of C_3 plants. Most anatomical characteristics are similar to those cited above: cell walls of Kranz cells are thicker than in the parenchymatous BS and they have a SL. There are abundant chloroplasts and mitochondria in Kranz cells which do not have a special orientation,

and chloroplasts have well-developed grana like those in the M cells (Ueno and Sentoku, 2006). The ssp. *semialata* has high PEP-CK activity and variable amounts of NADP-ME which may be influenced by growth conditions (Prendergast et al., 1987; Ueno and Sentoku, 2006).

Other Forms of NAD-ME Type Anatomy

There are several NAD-ME C_4 species in family Poaceae having some anatomical features that are not characteristic of the classical NAD-ME type C_4 species. Like the classical NAD-ME and PEP-CK types, these species have a double parenchyma sheath, with an inner MS and outer Kranz chlorenchyma sheath with grana-containing BS chloroplasts. Also, the BS cells have abundant mitochondria characteristic of NAD-ME type species. However, the BS chloroplasts are not arranged in the centripetal position, but are located in a centrifugal or peripheral position like the PEP-CK species. Also, the BS cell walls generally have a SL which is usually absent in the classical NAD-ME type species. This includes some species in genera *Eragrostis*, *Enneapogon*, *Triraphis*, and some *Panicum* species of the section Dichotomiflora (Ohsugi and Murata, 1980; Ohsugi et al., 1982; Prendergast et al., 1986). Interestingly, different cultivars of one NAD-ME type species, *P. coloratum*, were found to have different positions of chloroplasts in the Kranz cells, centripetal versus centrifugal (Ohsugi et al., 1982), which further shows that this feature cannot be taken as a criterion for distinguishing between different biochemical types.

A more extreme structural variant of NAD-ME type species is the Triodioid type anatomy. Species with Triodioid anatomy have two BS: an inner, thin-walled MS and an outer, chlorenchymatous Kranz BS which lacks SL in the cell walls. There are two variants of Kranz anatomy in this genus: Kranz BS form ("drape") extensions between adjacent vascular bundles, as in *Triodia pungens* (Hattersley and Watson, 1992), or BS extensions towards patches of M cells on both the abaxial and adaxial sides of the leaf, which are not associated with vascular bundles, for example *T. irritans* and *T. scariosa*, as illustrated in Fig. 1 (Dengler and Nelson, 1999). The species

which have been studied have NAD-ME type biochemistry; the appearance of mitochondria in Kranz cells is typical for NAD-ME species (Craig and Goodchild, 1977). The chloroplasts in Kranz cells have well-developed grana; but, unlike classical NAD-ME species, they, are in a centrifugal position or peripherally scattered around the cytoplasm, as in PEP-CK type species (Craig and Goodchild, 1977; Prendergast et al., 1987).

2. Family Cyperaceae

In family Cyperaceae, there are four types of Kranz anatomy (Fig. 2). As in family Poaceae, C₃ Cyperaceae species have an inner MS and an outer parenchyma sheath around the vascular tissue. In C₄ Cyperaceae species, the Kranz cells are considered to have evolved either internal to the MS (Fimbristyloid, Chlorocyperoid and Eleocharoid) or from the MS (Rhynchosporoid) (Brown, 1975; Carolin et al., 1977; Gilliland and Gordon-Gray, 1978; Bruhl et al., 1987; Bruhl and Perry, 1995; Soros and Dengler, 1998, 2001; Dengler and Nelson, 1999). In the first case, the MS, which is situated between M and Kranz cells, is nonphotosynthetic, thick-walled and generally suberized (Carolin et al., 1977; Ueno et al., 1988b; Ueno and Samejima, 1989; Bruhl and Perry, 1995; Soros and Dengler, 2001). This sheath may contribute to diffusional resistance of gases and help to minimize leakage of CO₂ generated from decarboxylation of C₄ acids in the Kranz cells. Most C₄ representatives in the family are NADP-ME type; NAD-ME species have only been found in genus *Eleocharis* (Bruhl et al., 1987; Ueno et al., 1988a; Murphy et al., 2007).

Fimbristyloid: Biochemical Subtypes NADP-ME and NAD-ME

This type of anatomy was found in C₄ species of the tribe Fimbristylideae, for example in *Bulbostylis* and *Fimbristylis* (see Carolin et al., 1977; Ueno et al., 1988a) and more recently in genus *Eleocharis* (Bruhl et al., 1987; Ueno, 1998a; Murphy et al., 2007). The Kranz cells originated internal to the MS and do not form a continuous wreath; rather, they are interrupted by metaxylem elements. In this type, there are

three BS layers around all vascular bundles, even small ones: Kranz BS surrounded by the MS, and parenchymatous BS (external to the MS) having fewer chloroplasts than in M cells. Both NADP-ME biochemical subtype in genera *Fimbristylis* and *Bulbostylis* (Ueno et al., 1986; Ueno, 1998a), and NAD-ME subtype in some species of *Eleocharis* (Ueno, 1998b; Murphy et al., 2007), have been reported to have Fimbristyloid anatomy. The species of *Fimbristylis* have Kranz cells with chloroplasts that are centrifugally located and nearly agranal having numerous small, short grana; mitochondria are small and few, consistent with NADP-ME type photosynthesis (Carolin et al., 1977; Gilliland and Gordon-Gray, 1978). One population of *Eleocharis vivipara* (type 1) and *E. retroflexa* ssp. *chaetaria* are NAD-ME type C₄ species with Fimbristyloid-like anatomy; the latter has Kranz cell chloroplasts with well-developed grana and large mitochondria, typical of NAD-ME type C₄ species (Ueno and Samejima, 1989; Ueno et al., 1989; Ueno, 1996a). Immunolocalization studies show M and parenchymatous BS cells of *E. vivipara* type I (Ueno, 1996b) and *Fimbristylis dichotoma* (Ueno, 1998a) have PEPC and pyruvate, Pi dikinase (PPDK), indicating both cell types function to capture CO₂ by PEPC, with delivery of C₄ acids to the Kranz cells, where Rubisco is located.

Chlorocyperoid: Biochemical Subtype NADP-ME

The Chlorocyperoid type, as a rule, has two layers of BS, with the Kranz cells internal to the MS. Chlorenchyma cells external to the MS include a partial parenchymatous chlorenchyma sheath (occurring in large vascular bundles, it is less developed than in the Fimbristyloid, and may be completely absent in some species) and palisade-like M cells, both of which are considered to function in the carboxylation phase of the C₄ cycle. Kranz cells contain few large centrifugally-arranged chloroplasts having mostly single stroma thylakoids, convoluted in loops. The degree of grana reduction varies in different species. The SL is usually discontinuous in the radial cell wall of the MS and absent from Kranz BS (Ueno et al., 1988a, b; Bruhl and Perry, 1995). In some species (as with the Fimbristyloid), both

Cyperaceae

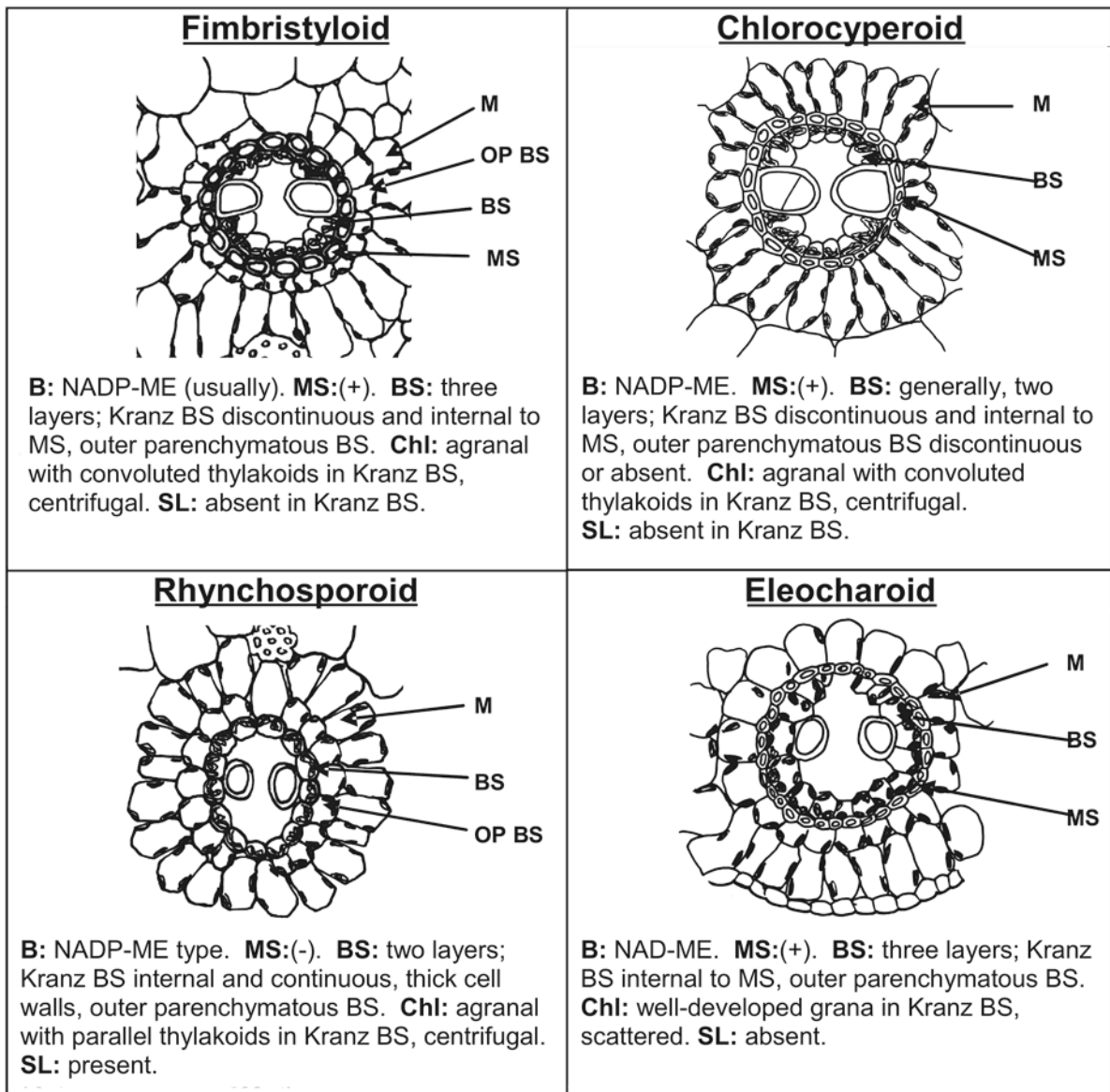


Fig. 2. Illustrations of the forms of Kranz anatomy in family Cyperaceae. Sketches of vascular bundles in *Fimbristylis* sp. for Fimbristyloid type, in *Cyperus* sp. for Chlorocyperoid type, in *Rhynchospora* sp. for Rhynchosporoid type and in *Eleocharis retroflexa* for Eleocharoid type (Drawings to illustrate the anatomy were made from light micrographs, Dengler and Nelson, 1999). For abbreviations, see Fig. 1.

M and parenchymatous BS cells may function to capture CO₂ by PEPC, with delivery of C₄ acids to the Kranz cells, where Rubisco is exclusively localized (Ueno, 1998a). This type of anatomy has been found in genera *Cyperus*, *Kyllinga*, *Pycneus* and *Torulinium* of the tribe Cyperaeae and *Lipocarpha* in the Lipocarphaeae (Carolin et al., 1977; Gilliland and Gordon-Gray, 1978;

Ueno et al., 1986, 1988a). Representative species having Chlorocyperoid type anatomy have NADP-ME type C₄ photosynthesis (Ueno et al., 1986; Bruhl et al., 1987). *Eleocharis baldwinii*, which has NAD-ME biochemistry and ultrastructure, has an intermediate type of anatomy called sub-Chlorocyperoid (Ueno and Samejima, 1989; Ueno, 2004).

*Rhynchosporoid: Biochemical Subtype
NADP-ME*

Unlike the other forms of Kranz anatomy in Cyperaceae, in Rhynchosporoid type the Kranz cells evolved from the MS (Takeda et al., 1980). This thick-walled sheath is surrounded by an incomplete chlorenchymatous parenchyma sheath and palisade-like M cells which are considered to function in fixation of atmospheric CO₂ into C₄ acids. Both M and outer parenchyma sheath chloroplasts have similar thylakoid structure with large grana. Kranz cells have numerous, centrifugally-arranged agranal chloroplasts but, unlike the convoluted thylakoids in the previous types, here the thylakoid membranes usually have a parallel arrangement (Gilliland and Gordon-Gray, 1978; Ueno et al., 1988a; Bruhl and Perry, 1995). Mitochondria are comparable in size and number in M and BS cells. The SL is mostly continuous around the cell but can be discontinuous in the radial cell walls. Biochemical analysis indicates NADP-ME type photosynthesis (Ueno et al., 1986; Bruhl et al., 1987).

Eleocharoid: Biochemical Subtype NAD-ME

The Eleocharoid type anatomy was named after C₄ species of *Eleocharis* which have three types of BS, with the innermost Kranz cells forming a continuous wreath. As in Chlorocyperoid and Fimbristylloid types, the Kranz cells originate internal to the MS. The outer parenchyma BS contain some chloroplasts, while the middle MS lacks, or contains only a few, chloroplasts filled with starch; Kranz cells contain numerous organelles typical of NAD-ME species, with no strict orientation in the cell (scattered around the periphery) or tending slightly towards centrifugal. Chloroplasts of Kranz cells have well-developed grana and store starch; chloroplasts of parenchyma BS are smaller than those in M cells, but in both types of cells there are well-developed grana. Usually, a SL is present on both the inner and outer tangential cell walls in the MS, but sometimes it is absent on the radial cell walls (Ueno and Samejima, 1989; Bruhl and Perry, 1995). The Kranz cells have

abundant and large mitochondria, typical of NAD-ME type C₄ grasses and dicots (Ueno and Samejima, 1989; Bruhl and Perry, 1995; Ueno, 2004; Ueno and Wakayama, 2004). The genus *Eleocharis* is very diverse in forms of photosynthesis between species (C₃, C₃-C₄, C₄-like). It includes amphibious species which change their mode of photosynthesis between submerged and terrestrial growth; and both Eleocharoid and Fimbristylloid type anatomy with NAD-ME type photosynthesis has been found among its C₄ species (Bruhl et al., 1987; Bruhl and Perry, 1995; Ueno, 2004; Ueno and Wakayama, 2004; Murphy et al., 2007).

3. *Dicotyledons*

Among dicot families, it is well-established that family Chenopodiaceae has the largest number of C₄ species and also the greatest diversity in leaf anatomy, including C₃, C₄ Kranz and C₄ single-cell types (Carolin et al., 1975; Pyankov et al., 1992; Sage et al., 1999; Edwards et al., 2004; Voznesenskaya et al., 2007). This family has been studied most extensively, resulting in classification of six types of Kranz anatomy (Fig. 3) which has been extended to several other families (Carolin et al., 1975, 1982; Jacobs, 2001). The C₄ types of leaves vary in the structure and arrangement of chlorenchyma tissue, in arrangement of water storage and vascular tissue, and by the presence, or absence, of various specialized hypodermal cells. Within these six main types of Kranz anatomy, additional anatomical differences have been recognized, indicating potential for further subdivision of structural types of Kranz in the family (Kadereit et al., 2003). The C₄ structural types in family Chenopodiaceae are named after the corresponding taxonomic names, as indicated below. These main structural forms were also given descriptive names (Vasilevskaya and Butnik, 1981; Voznesenskaya and Gamaley, 1986) which are also referred to in the descriptions below. In addition to the six Kranz types in the Chenopodiaceae, other forms have been recognized in dicot lineages found in *Cleome* (Cleomaceae), *Isostigma* and *Glossocardia* (Asteraceae) and *Portulaca* (Portulacaceae).

Chenopodiaceae

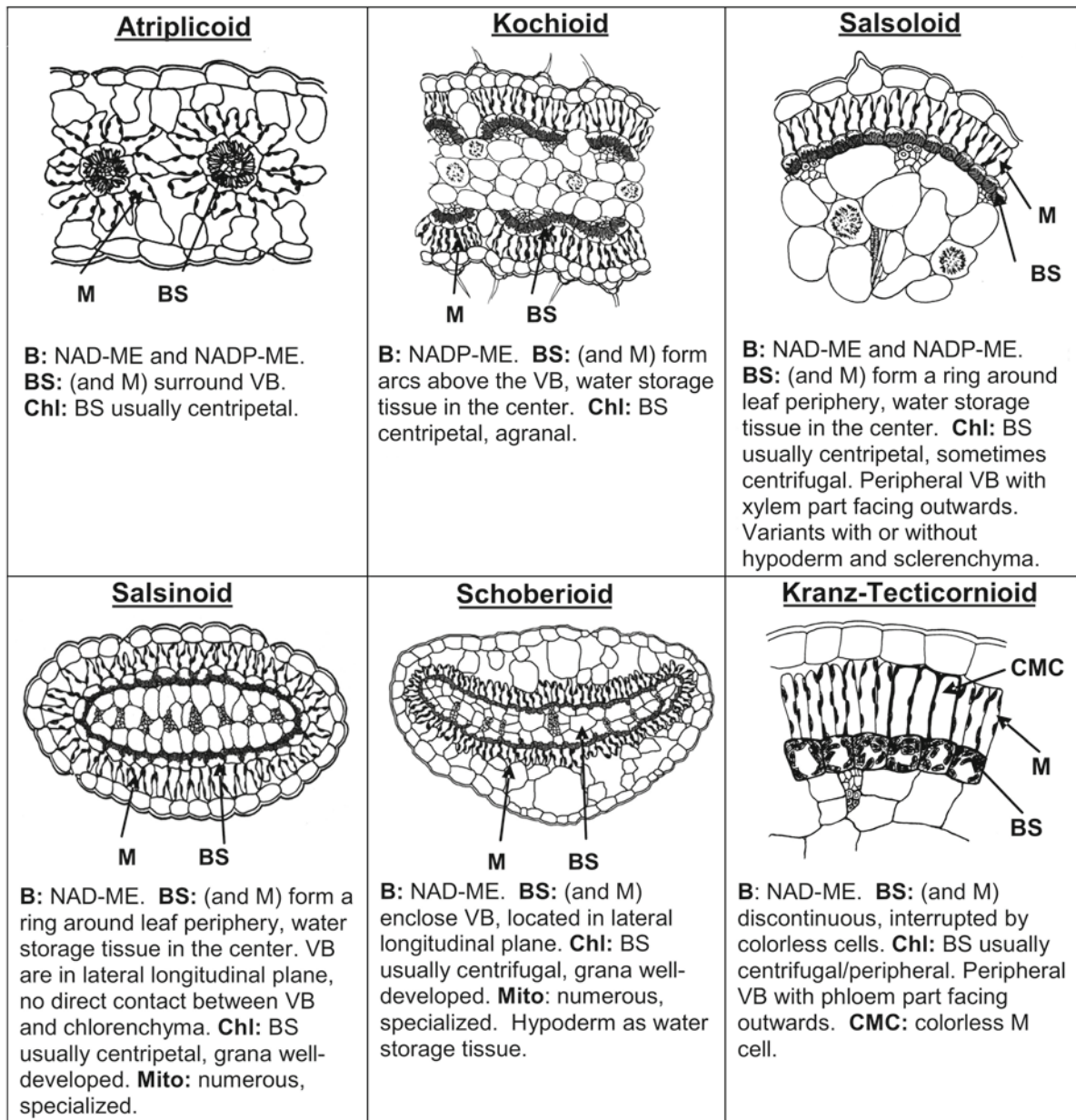


Fig. 3. Illustrations of the forms of Kranz anatomy in family Chenopodiaceae. Sketches of leaf structure in *Atriplex* sp. for Atriplicoid type (tribe Atripliceae), *Bassia hyssopifolia* for Kochioid type (tribe Camphorosmeae), *Salsola collina* for Salsoloid type (tribe Salsoleae), *Suaeda taxifolia* for Salsinoid type (tribe Suaedeae), *Suaeda eltonica* for Schoberoid type (tribe Suaedeae), and *Tecticornia* (=Halosarcia) *indica* for Kranz-Tecticornioid type (tribe Salicornieae) (Some pictures are adapted from Voznesenskaya and Gamaley, 1986). For abbreviations, see Fig. 1.

Atriplicoid: Biochemical Subtypes NAD-ME and NADP-ME

In C_3 dicots, all the vascular bundles are surrounded by a parenchyma sheath which is more

or less distinguishable from M tissue; this sheath becomes a specialized Kranz BS in C_4 species. In Atriplicoid type of anatomy which occurs in some dicot species having laminate leaves, the Kranz tissue forms a classical wreath-like

structure with concentric layers of chlorenchyma around each vascular bundle. The Kranz BS encloses vascular bundles; although it can become disrupted on the phloem side in larger bundles. There is structural diversity and potential for recognition of additional subtypes where Kranz encloses individual veins in C₄ dicots having flattened leaves. In this type, hypodermal tissue when present usually fulfills the role of water storage tissue. For example, *Portulaca oleracea* has extensively developed water storage hypoderm with variable positioning of the veins between the abaxial and adaxial sides of the leaf. In Atriplicoid type, palisade M cells are usually arranged radially; but this can vary in different species (Rathnam et al., 1976; Dengler and Nelson, 1999; McKown et al., 2005; Muhaidat et al., 2007). For species of families Chenopodiaceae and Amaranthaceae, Kadereit et al. (2003) distinguished four different types of anatomy in laminate leaves within the Atriplicoid type, which differ in the presence or absence of hypoderm, the occurrence of parenchyma cells between M cells, or the occurrence of additional layers of spongy parenchyma on the abaxial side of the leaf. Nevertheless, similar features may be found in other taxons. Two biochemical subtypes, NADP-ME and NAD-ME, have been found in species having this leaf structure, each having differences in chloroplast ultrastructure (Laetsch, 1968; Kennedy and Laetsch, 1974; Carolin et al., 1975, 1978; Rathnam et al., 1976; Gamaley and Voznesenskaya, 1986; Voznesenskaya and Gamaley, 1986; Sage et al., 1999; Marshall et al., 2007; Muhaidat et al., 2007; Akhiani et al., 2008). In the NADP-ME subtype, BS chloroplasts have reduced grana, as is typical for this subtype. The NADP-ME subtype is present in Acanthaceae, Aizoaceae, Amaranthaceae, Asteraceae, Boraginaceae, Cariophyllaceae, Chenopodiaceae, Euphorbiaceae, Nyctaginaceae, Portulacaceae and Zygophyllaceae. The opposite variant, NAD-ME type, which has well-developed grana in BS chloroplasts and reduced grana in M chloroplasts, is found in Acanthaceae, Aizoaceae, Amaranthaceae, Chenopodiaceae, Cleomaceae, Euphorbiaceae, Gisekiaceae, Molluginaceae and Portulacaceae. As a rule, Kranz BS have thickened cell walls, but the thickness varies; they are usually thinner in NAD-ME species. Organelles

are usually arranged centripetally in BS cells, except for *Trianthema triquetra* (Aizoaceae), which has centrifugal positioning of organelles (Carolin et al., 1975).

Kochioid: Biochemical Subtypes NAD-ME and NADP-ME

Kochioid type species, also referred to as Semi-Wreath type, have laminate or semi-terete to terete succulent leaves with water-storage tissue underneath the chlorenchyma. The main vascular bundle is in the center, and the remaining vascular bundles are located in two paradermal planes on the leaf periphery or around the periphery in terete leaves. The chlorenchyma tissue is distributed along the peripheral veins; BS and M cells form arcs above the vascular bundles. Bundle sheath cells have relatively thick cell walls, and organelles are located in the centripetal position. Kadereit et al. (2003) recognized three different types of anatomy with such distribution of chlorenchyma tissues, differing in the presence or absence of hypoderm in two *Kochia* species, while in *Kirilowia* species, vascular bundles with arcs of chlorenchyma are distributed in the lateral plane only on the adaxial side of the leaf, with spongy parenchyma on the abaxial side. Species with this type of anatomy have been found to have NADP-ME type biochemistry and chloroplast ultrastructure (reduction in grana in BS cells is highly pronounced, up to having totally agranal chloroplasts) in genera *Bassia* and *Kochia* of family Chenopodiaceae (Gutierrez et al., 1974; Carolin et al., 1975; Gamaley, 1985; Voznesenskaya and Gamaley, 1986; Pyankov et al., 2000a; Jacobs, 2001), and NAD-ME type biochemistry in C₄ species of the genus *Zygophyllum* with respective granal chloroplasts and numerous specialized mitochondria in BS cells (Crookston and Moss, 1972; Muhaidat et al., 2007). In the latter case, the leaf is cylindrical with the main vascular bundle in the center of water storage tissue. Two layers of Kranz tissue form arcs outside of the small peripheral veins.

Salsoloid: Biochemical Subtypes NAD-ME and NADP-ME

Species with Salsoloid type anatomy, also referred to as Kranz-Central type, have cylindrical or

terete leaves (or stems in aphyllous species) with two concentric layers of chlorenchyma, typical of C_4 Kranz anatomy, located around the periphery of assimilating organs. The central part is occupied by water storage tissue with the main vein in the middle. The net of secondary vascular bundles penetrates into the water storage tissue; and the small peripheral veins contacting with BS cells are facing toward the chlorenchyma by their xylem. In some desert species, a scleromorphous variant of this type has been found which has a high volume of sclerenchymatous tissue in the center around the main vein and/or in the peripheral bundles, with only a small amount of water storage tissue, for example, in reduced leaves of *Nanophyton erinaceum*, in the leaves and stems of *Arthrophytum lehmannianum* from family Chenopodiaceae, or in some *Calligonum* species of family Polygonaceae (see Butnik et al., 2001). Kadereit et al. (2003) distinguished five different types of anatomy within this type: *Salsola* type with or without hypoderm, *Nanophyton* type with sclerenchyma, *Climacoptera* type having no contact of peripheral veins with chlorenchyma and *Halothamnus auriculus* type with flattened leaves and several secondary veins distributed in the water storage parenchyma in lateral plane and the net of small peripheral veins adjacent to BS cells. Two biochemical subtypes, NADP-ME (in genera *Salsola*, *Halothamnus*, *Haloxylon*, *Horaninovia* and some others in family Chenopodiaceae) and NAD-ME (for example in genera *Salsola*, *Climacoptera*, *Halocharis* in Chenopodiaceae and *Calligonum* in Polygonaceae), with their respective ultrastructural chloroplast subtypes, have been found in species with this anatomy (Winter et al., 1977; Voznesenskaya and Gamalety, 1986; Pyankov and Vakhrusheva, 1989; Sage et al., 1999; Pyankov et al., 2000c; Muhaidat et al., 2007). Variants occur with or without hypodermal tissue, which, if present, plays the role of additional water storage tissue. Usually BS chloroplasts are in the centripetal position, but species of the genus *Halothamnus* (previously named *Aellenia*) have centrifugally-arranged chloroplasts, see Edwards et al. (2004).

Salsinoid: Biochemical Subtype NAD-ME

Species with Salsinoid type anatomy (also referred to as Kranz-Isopalisade Circular type)

occur in genus *Suaeda*, section *Salsina* (Kadereit et al., 2003; Schütze et al., 2003). They have terete leaves with two concentric layers of chlorenchyma, palisade M and Kranz cells, around the leaf periphery, and water storage tissue in the center of the leaf. The vascular tissue forms a network in the lateral longitudinal plane; there are no peripheral vascular bundles and only the lateral veins may have contact with chlorenchyma. Only one biochemical subtype has been found, NAD-ME, and structural characteristics are typical for this subtype: numerous specialized mitochondria and chloroplasts with well-developed grana in BS cells, and reduced grana in M chloroplasts. Unlike other C_4 subtypes, the Kranz cells have a large vacuole with less abundant organelles which occur in a centripetal position in a relatively thin layer of cytoplasm. This type was originally called Kranz-Suaedoid (Carolin et al., 1975; Jacobs, 2001). However, subsequently this form of Kranz was recognized as *Salsina* type after the section of *Suaeda* in which it occurs (Schütze et al., 2003), and is called Salsinoid here for consistency in nomenclature. For a description of the structural and functional features, see (Shomer-Ilan et al., 1975, 1979, 1981; Fisher et al., 1997; Voznesenskaya et al., 2007).

Schoberoid: Biochemical Subtype NAD-ME

This is another form of Kranz anatomy in genus *Suaeda* which recently was called Schoberia after the section in which it occurs (Kadereit et al., 2003; Schütze et al., 2003); it is called Schoberoid type here for consistency in nomenclature (also referred to as Kranz-Isopalisade type). Before more recent phylogenetic analyses of the Suaedoideae subfamily, it was referred to as Conospermoid type anatomy by Freitag and Stichler (2000). It is found in semi-terete leaves with positioning of vascular bundles in a lateral plane. This subtype is unique in having the vascular bundles enclosed by two layers of Kranz type chlorenchyma in the central part of the leaf, with continuous BS extensions between the veins. Large hypodermal cells, which are located between the chlorenchyma and epidermis, function as water storage tissue. These are NAD-ME type species with typical ultrastructural features for this subtype: BS cells have

granal chloroplasts and specialized mitochondria, and M cells have reduced chloroplasts with less grana development. Unlike the Salsinoid type, Schoberoid type species have BS chloroplasts located in the centrifugal position. A variant of this type of anatomy has been found in *Suaeda cochlearifolia*, which has only one layer of BS cells between the vascular bundles (Voznesenskaya et al., 2007).

Kranz-Tecticornioid: Biochemical Subtype NAD-ME

This unique structural subtype of Kranz anatomy is found in the genus *Halosarcia* (*H. indica*, Saliicornieae, family Chenopodiaceae) (Carolin et al., 1982; Jacobs, 2001), which is now included in the broadly circumscribed genus *Tecticornia* (Shepherd and Wilson, 2007). In general appearance, it is similar to the Kranz-Central (Salsoloid) type of leaf anatomy, with peripheral distribution of two chlorenchyma layers in cylindrical assimilating stems and with a net of small peripheral vascular bundles adjoining BS cells. However, in the Kranz-Tecticornioid type, these small veins are oriented with the phloem side facing towards the chlorenchyma. A striking feature of this type is the presence of bands of thick-walled colorless parenchyma cells between groups of chlorenchymatous M cells. Also, in the Kranz cells granal chloroplasts tend to be located centrifugally, or occasionally scattered around the periphery of the cell. Western blot analysis for C₄ acid decarboxylases and immunolocalization studies indicate NAD-ME type C₄ photosynthesis. There are numerous mitochondria in the Kranz cells, which compared to other NAD-ME species in the family are smaller, but they have a similar specialized structure (Voznesenskaya et al., 2008). Mesophyll chloroplasts have reduced grana, characteristic of this biochemical subtype.

Pilosoid: Biochemical Subtype NADP-ME

An interesting variant of Kranz anatomy occurs in species of the clade Pilosa, genus *Portulaca*, family Portulacaceae (for example, in *P. grandiflora*, *P. pilosa*, *P. villosa*, *P. sclerocarpa*) which have terete cylindrical leaves with a circular arrangement of the small vascular bundles around the

leaf periphery, with main vein and water storage cells in the center. Each peripheral vein is surrounded by BS cells (sometimes less developed on the inner side), with M cells forming a wreath-like structure only on the outer and lateral sides of the vascular bundles as illustrated in Nishioka et al. (1996) and Kim and Fisher (1990). Thus, the structure of the mesophyll-bundle sheath-vascular bundle complex of each vein is similar to one of the variants of Atriplicoid type anatomy, but differs in having a peripheral arrangement of veins around the leaf (Fig. 4). Flattened leaves of *P. amilis* have similar arrangement of VB but only about four layers of water storage tissue in the middle part of the leaf. The whole leaf anatomy can be considered to represent an intermediate stage of evolution from laminate Atriplicoid anatomy to Kochioid or directly to Salsoloid. NADP-ME type of biochemistry is well known for *P. grandiflora* (Gutierrez et al., 1974; Guralnick et al., 2002). It was recently also shown for two other species of this clade, *P. pilosa* and *P. amilis* (Voznesenskaya et al., 2010); and, all other studied species with this type of anatomy have similar ultrastructural features of BS and M chloroplasts characteristic of this biochemical subtype. A similar distribution of vascular bundles was found in *Zygophyllum simplex*; but, with Kranz tissue forming open arcs typical for Kochioid type of anatomy, showing similar evolutionary trends in different families.

Portulacelloid: Biochemical Subtype NADP-ME

Species of the clade/section Portulacella, genus *Portulaca* (Portulacaceae) have vascular bundles surrounded by two concentric layers of Kranz anatomy distributed only on the adaxial side of the leaf; there are 4–5 layers of water storage tissue on the abaxial side (Voznesenskaya et al., 2010). As in Pilosoid type, palisade M cells are better developed on the upper and lateral sides of vascular bundles. It is NADP-ME biochemical type with centripetal position of grana-deficient chloroplasts in bundle sheath cells.

Glossocardioid: Biochemical Subtype NAD-ME and NADP-ME

Kranz anatomy similar to Salsoloid type was reported for representatives of family Asteraceae *Glossocardia bosvallia* (Das and Raghavendra,

Dicotyledonae

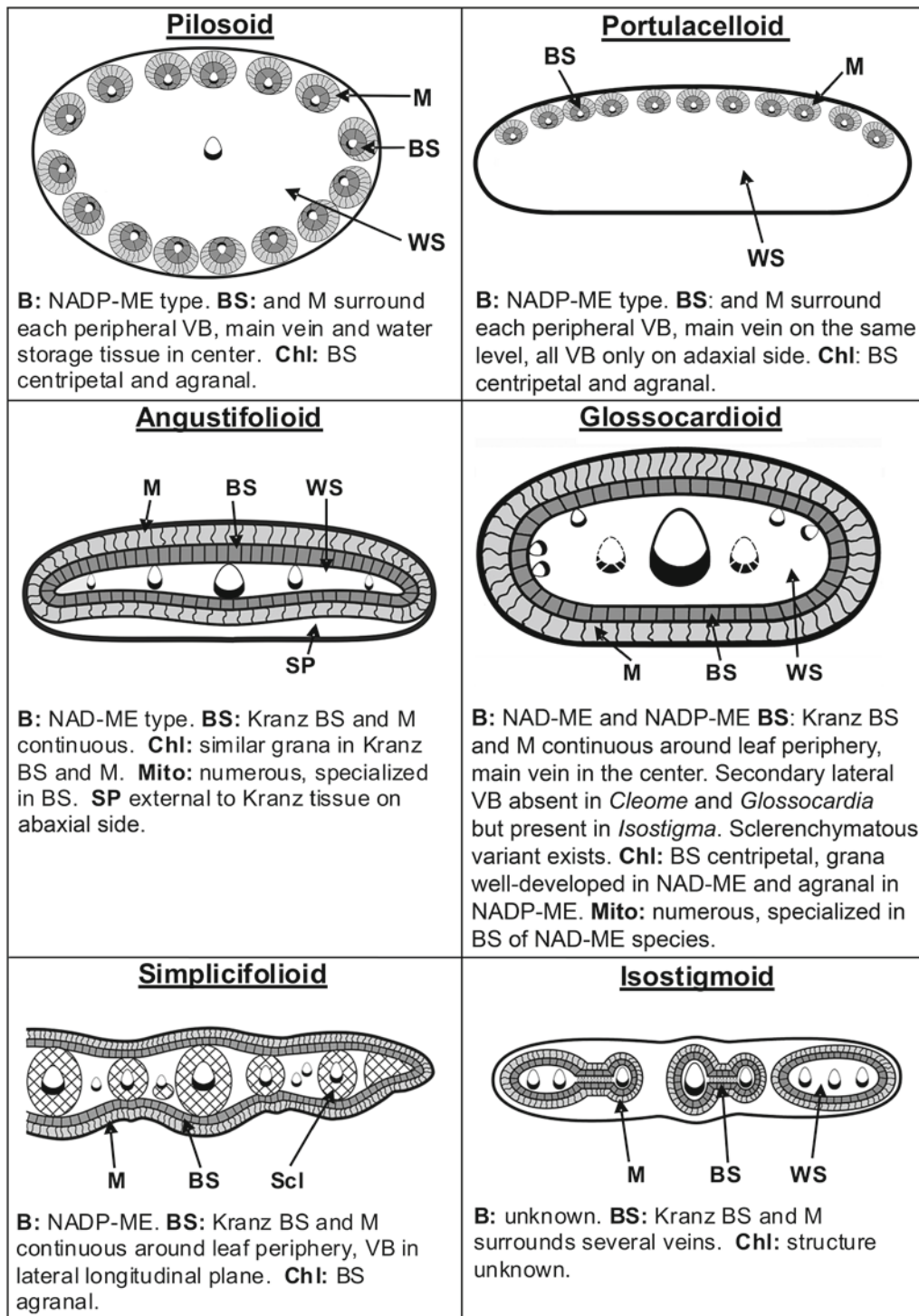


Fig. 4. Illustration of other forms of Kranz anatomy among Dicotyledonae. Sketches of vascular bundles in *Portulaca grandiflora* for Pilosoid type, *Portulaca cf. bicolor* for Portulacelloid type, *Cleome angustifolia* cotyledon for Angustifolioid type, *Glossocardia bosvallia* and/or *Cleome angustifolia* leaf for Glossocardioid type, *Isostigma simplicifolium* for Simplicifolioid type, and Isostigmoid type (adapted from Fig. 2, Peter and Katinas, 2003). SP, spongy parenchyma tissue; for other abbreviations, see Fig. 1.

1976) and some *Isostigma* species (Peter and Katinas, 2003), and also for *Cleome angustifolia* leaf (Cleomaceae); all these species have semiterete to terete leaves with concentric layers of Kranz type chlorenchyma surrounding the leaf on the periphery. Leaf venation consists of the central main vein with or without lateral secondary veins embedded in the water storage parenchyma, with sclerenchyma tissue being either present around veins or absent depending on the species. Small peripheral bundles are in contact with BS cells with their xylem side, characteristic of Salsoloid type anatomy. The main difference from the classic Salsoloid anatomy is the absence of even distribution of small veins around the leaf periphery. The biochemical subtype of *G. bosvallia* based on western blot analysis is NADP-ME, while *C. angustifolia* is NAD-ME, the biochemical subtype for *Isostigma peucedanifolium* is NADP-ME based on deficiency of grana in BS chloroplasts (Voznesenskaya, Koteyeva and Edwards, unpublished). This type of structure was designated as *Eryngiophyllum* (= *Chrysanthellum*) in Peter and Katinas (2003); but it has only been observed in *Isostigma* and *Glossocardia* in family Asteraceae. Therefore, according to the genus where it was first described (Das and Raghavendra, 1976), we define this as Glossocardioid type anatomy.

Simplicifolioid: Biochemical Subtype NADP-ME

Isostigma simplicifolium (family Asteraceae) has a form of Kranz anatomy in which the chlorenchyma tissue forms a continuous layer around flattened leaves (Fig. 4). The major veins, which run in parallel with rare anastomoses, are enclosed in the well-developed sclerenchyma sheaths, with only very small bundles lacking sclerenchyma tissue. There is some water storage parenchyma between major veins. The biochemical subtype is NADP-ME based on deficiency of grana in BS chloroplasts (E. Voznesenskaya, N. Koteyeva and G. Edwards, unpublished).

Isostigmoid: Biochemical Subtype unknown

An unusual form of Kranz anatomy, called Isostigmoid, was reported for several species of the genus *Isostigma* in family Asteraceae having flattened leaves (Peter and Katinas, 2003). In Isostigmoid type, instead of Kranz anatomy encircling

individual veins, as occurs in Atriplicoid, the two chlorenchyma layers surround several veins together (illustrated in Fig. 4). It is considered an intermediate form between Atriplicoid type and anatomical forms having a continuous ring of Kranz anatomy around the leaf. The biochemical subtype is unknown.

Angustifolioid: Biochemical Subtype NAD-ME

This form of Kranz anatomy occurs in cotyledons of the C₄ species *Cleome angustifolia* (family Cleomaceae). Kranz tissue continuously surrounds the central part of the blade with vascular bundles distributed in the lateral longitudinal plane and with the main vein in the center of the leaf. Vascular bundles are separated by water storage parenchyma. Chlorenchyma layers, consisting of palisade M and BS cells, are located under the epiderm on the adaxial side; whereas, on the abaxial side the M cells adjacent to BS are small and rounded, and they are separated from the epiderm by two layers of spongy M cells (Fig. 4). Bundle sheath and M cells have ultrastructural features characteristic of NAD-ME biochemistry with granal BS chloroplasts and numerous mitochondria in BS cells; but, there is no difference in the granality between BS and M chloroplasts. This type of anatomy differs from all other known C₄ types. The concentric layer of Kranz tissue around veins has some features of Salsinoid type, but this type is unusual in having spongy parenchyma on the abaxial side of the leaf. The biochemical subtype of this species is NAD-ME (Voznesenskaya, Koteyeva and Edwards, unpublished).

B. Biochemical Diversity: C₄ Cycles and Energy Requirements for C₄ Subtypes

1. Chloroplasts and Mitochondria

Despite C₄ photosynthesis having evolved multiple times in different families and subfamilies with structural variations on Kranz anatomy, species belonging to each biochemical subtype tend to have features in common in terms of the structure of chloroplasts in M and BS cells, and the occurrence of mitochondria in BS cells. In NADP-ME type C₄ species, BS chloroplasts are deficient in grana compared to M chloroplasts, whereas in NAD-ME type species, the

M chloroplasts tend to be more deficient in grana development (Gamaley, 1985; Voznesenskaya and Gamaley, 1986; Fisher et al., 1997; Voznesenskaya et al., 1999). In PEP-CK type C_4 species, the granal development is similar for M and BS chloroplasts (Yoshimura et al., 2004; Voznesenskaya et al., 2006). This has been quantified in a number of studies on different photosynthetic types by determining the granal index (the length of all appressed thylakoid membranes as a percentage of the total length of all thylakoid membranes in a chloroplast). Early studies indicate that the degree of grana development in BS chloroplasts correlates to the capacity for photosystem II (PSII) activity, linear electron flow and capacity for generation of NADPH, with low grana-containing chloroplasts being richer in photosystem I (PSI)-mediated cyclic electron flow producing ATP (Edwards and Walker, 1983; Anderson, 1999). Thus, differences in grana development are associated with differences between M and BS cells in the need for NADPH relative to ATP to support C_4 photosynthesis.

Mitochondria are most abundant, and often larger, in BS cells of NAD-ME type species (where the decarboxylase is located in mitochondria), and least abundant in BS cells of NADP-ME type species (where the decarboxylase is located in chloroplasts). Bundle sheath mitochondria in PEP-CK species also function to provide ATP to support decarboxylation via PEP-CK in the cytosol (Burnell and Hatch, 1988), and they are generally intermediate in size and number compared to NAD-ME or NADP-ME type species (Yoshimura et al., 2004; Voznesenskaya et al., 2006).

The basal energy required per CO_2 assimilation in C_4 photosynthesis is the sum of energy to support the C_4 and C_3 cycles. Analyses to date on biochemical subtyping (by Western blots, enzyme assays) indicate each species has a major form of delivery of CO_2 to Rubisco through one type of C_4 acid decarboxylase. For each biochemical subtype, the amount of energy required to support the C_4 cycle for delivery of CO_2 and the C_3 cycle for fixation of CO_2 can be calculated. This is shown in Fig. 5, with illustrations of how the provision of energy can be met cooperatively by M and BS chloroplasts. This demonstrates how the photochemical demands for energy can be

shared between the cell types, and the differences between the three types of C_4 cycles. However, the exact photochemical demands for energy within each subgroup may vary between species. This is most evident in NADP-ME type C_4 species, as discussed below.

2. Illustration of Energetics for NADP-ME Type Species

Summary

1. 5 ATP, 2 NADPH required per CO_2 assimilated (2 ATP for the C_4 cycle, 3 ATP, 2 NADPH for the C_3 cycle).
2. The C_4 cycle delivers primarily malate to BS cells (NADP-ME species are mainly malate formers).
3. In NADP-ME type C_4 species, BS chloroplasts have fewer grana than do M chloroplasts, but there is variation in the degree of deficiency of grana in the BS chloroplasts. The extremes range from the BS chloroplasts being agranal (in sugarcane, sorghum), to BS chloroplasts having granal indices about half that of M chloroplasts, observed in members of family Chenopodiaceae (see Voznesenskaya et al., 1999). The illustration in Fig. 5a is based on the granal index and linear electron flow to generate NADPH being two fold higher in M than in BS chloroplasts, as observed in some NADP-ME type chenopods (Voznesenskaya et al., 1999). The two fold higher use of reductive power in BS cells could vary due to the amount of 3-phosphoglyceric acid (PGA) shuttled from BS to M chloroplasts for reduction (in the current scheme, one sixth of the PGA). Alternatively, this balance in reductive power could be modified in NADP-ME dicots by a partial shuttle of aspartate from M to BS cells through NADP-ME, see Moore et al. (1984) and Meister et al. (1996). Also, the degree of grana development in BS chloroplasts of NADP-ME type species appears to correlate with the development of a secondary aspartate PEP-CK shuttle (Gutierrez et al., 1974; Wingler et al., 1999; Voznesenskaya et al., 2006).

The deficiency in PSII in BS chloroplasts in this subtype is thought to reduce production of O_2 in BS cells and help maintain a high CO_2/O_2 ratio, which is favorable for limiting ribulose 1,5-bisphosphate (RuBP) oxygenase activity and photorespiration. This results in an increased

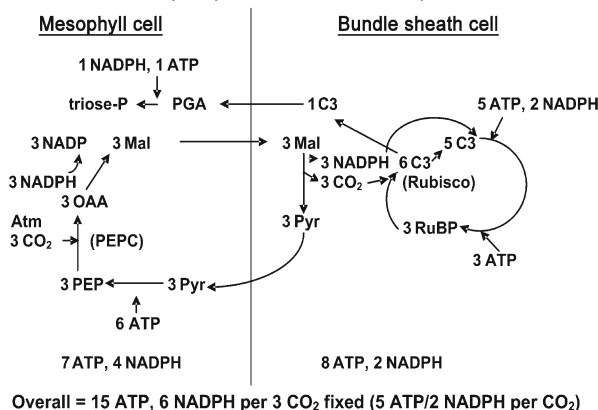
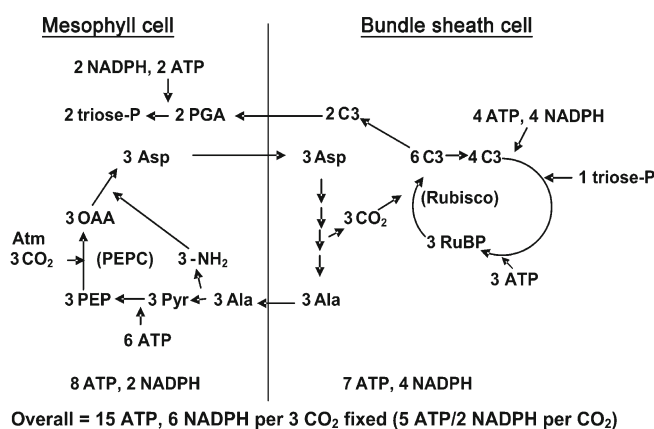
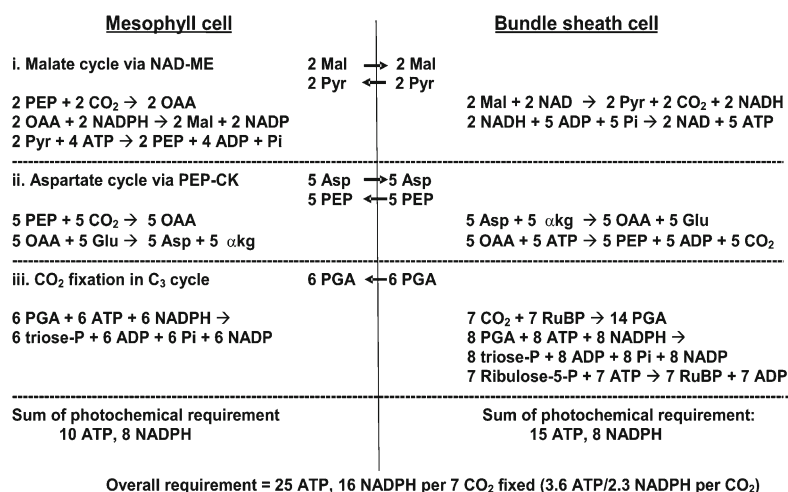
a Illustration of NADP-ME type (assimilation of 3 CO₂)**b Illustration of NAD-ME type (assimilation of 3 CO₂)****c Illustration of PEP-CK type (assimilation of 7 CO₂)**

Fig. 5. Illustrations of the three types of C₄ cycles and their bioenergetics: **a**, NADP-ME, **b**, NAD-ME and **c**, PEP-CK subtypes. *αkg*, α-ketoglutarate; *Ala*, alanine; *Asp*, aspartate; *Atm*, atmospheric; *Glu*, glutamate; *Mal*, malate; *OAA*, oxaloacetate; *PEP*, phosphoenolpyruvate; *PEPC*, PEP carboxylase; *PGA*, 3-phosphoglyceric acid; *Pi*, inorganic phosphate; *pyr*, pyruvate; *RuBP*, ribulose 1,5-bisphosphate; *triose-P*, triose phosphate. Panels a and b, adapted from Voznesenskaya et al., 1999, Oxford University Press.

need for photochemically-produced NADPH in M chloroplasts, see (Edwards and Walker, 1983).

3. *Illustration of Energetics for NAD-ME Type Species*

Summary

1. 5 ATP, 2 NADPH required per CO₂ assimilated (2 ATP for the C₄ cycle, 3 ATP, 2 NADPH for the C₃ cycle).
2. The C₄ cycle delivers primarily aspartate to BS cells (NAD-ME species are primarily aspartate formers). The aspartate cycle requires only production of ATP (but not reductive power) to drive PEP regeneration from alanine by the M chloroplasts (Edwards and Walker, 1983; Voznesenskaya et al., 1999). This ATP may be provided via PSI cyclic electron flow in the M chloroplasts. Extensive studies of NAD-ME-type species in family Chenopodiaceae have shown that M chloroplasts are deficient in grana compared to BS chloroplasts (Gamaley, 1985; Gamaley and Voznesenskaya, 1986; Voznesenskaya and Gamaley, 1986; Glagoleva et al., 1991).
3. In the illustration of Fig. 5b, the use and generation of reductive power in BS chloroplasts is two-fold higher than in M chloroplasts. This occurs with the granal index and linear electron flow to generate NADPH being two fold higher in BS than M chloroplasts, as observed in some NAD-ME type chenopods (Voznesenskaya et al., 1999). Again, this partitioning of reductive power can be regulated by the amount of PGA shuttled from BS to M chloroplasts for reduction to triose-P (in the current scheme, one third of the PGA is shuttled to M cells).

4. *Illustration of Energetics for PEP-CK Type Species*

Summary

1. 3.6 ATP, 2.3 NADPH required per CO₂ assimilated (0.6 ATP and 0.3 NADPH per CO₂ for the C₄ cycles; 3 ATP, 2 NADPH for the C₃ cycle). The PEP-CK type requires less ATP, but more NADPH per CO₂ fixed than the malic enzyme type species.
2. There are two C₄ cycles, one shuttling aspartate, the other shuttling malate (Fig. 5c). Aspartate is utilized in the cytosol to generate oxaloacetate for the PEP-CK reaction. Malate is used by the mitochondria

via NAD-ME, which generates CO₂ and NADH; the NADH is then utilized by the mitochondria to generate ATP to support the PEP-CK decarboxylase reaction (Burnell and Hatch, 1988; Walker and Chen, 2002; Voznesenskaya et al., 2006). In this scheme, the aspartate cycle generates about 70%, and the malate cycle about 30%, of the total CO₂ delivered to the BS cells.

3. In this illustration (Fig. 5c), an equal amount of reductive power is required by M and BS cells. This is consistent with M and BS chloroplasts of PEP-CK species having a similar granal index, suggestive of equivalent capacity for PSII activity for generating NADPH (Voznesenskaya et al., 2006). Again, this partitioning of reductive power can to some extent be regulated by the amount of PGA shuttled from BS to M chloroplasts for reduction (in the scheme, about 40%).

5. *Additional Energy Requirements in C₄ Photosynthesis*

Besides the basic requirements for energy to support the C₃ and C₄ cycles, additional energy will be consumed by over-cycling of the C₄ cycle (CO₂ leakage from BS cells, that is 2 ATP per CO₂ lost from BS cells), and due to the occurrence of limited photorespiration because of some O₂ reacting with RuBP, see Kanai and Edwards (1999). The cost of photorespiration is illustrated in Fig. 6. The requirement for reductive power per O₂ reacting with RuBP (eq. to 2 NADPH) is the same as for CO₂ reacting with RuBP, and the scheme illustrates how this can be shared equally between M and BS chloroplasts.

III. *Single-Cell C₄ Photosynthesis in Terrestrial Plants*

For decades following the discovery of C₄ photosynthesis in the 1960s, it was considered that the requirements for C₄, as summarized in the Introduction, could only be met in terrestrial plants by the presence of Kranz type anatomy. Thus, it was surprising to find species in family Chenopodiaceae that undergo traditional C₄ photosynthesis, but have a unique anatomy that does not consist of the Kranz dual-cell system. Instead, the single-cell C₄ system functions in individual

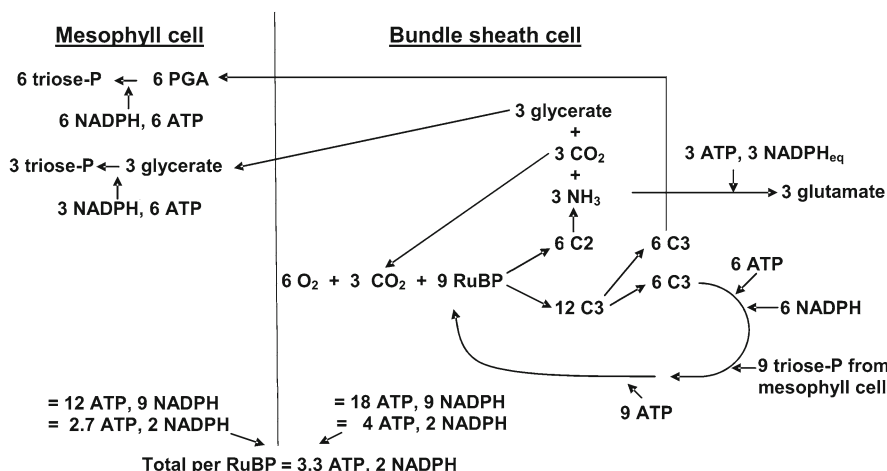


Fig. 6. Scheme of energetics of photorespiration in C₄ plants. PGA, 3-phosphoglyceric acid; RuBP, ribulose 1,5-bisphosphate; triose-P, triose phosphate.

chlorenchyma cells by means of intracellular biochemical and organelle compartmentation. Two very novel means of accomplishing this evolved in subfamily Suaedoideae. These systems function by spatial development of two cytoplasmic domains in chlorenchyma cells, which contain dimorphic chloroplasts. The arrangement of M and BS cells that so long has defined terrestrial forms of C₄ plants has now been joined by single-cell C₄ systems as functional anatomical alternatives (Voznesenskaya et al., 2001, 2002; Sage, 2002; Edwards et al., 2004; Akhani et al., 2005; Park et al., 2010).

A. Occurrence (Family and Phylogeny)

In family Chenopodiaceae, which has C₃ and C₄ species, all C₄ genera except for subfamily Chenopodioideae (including genus *Atriplex*) occur in a succulent clade made up of subfamilies Salicornioideae/Suaedoideae/Salsoloideae. The single-cell C₄ type species occur in subfamily Suaedoideae (Fig. 7), in two species in genus *Bienertia* (*B. cycloptera* Bunge ex Boiss., *B. sinuspersici* Akhani sp. nov.) and in one species in genus *Suaeda*, *S. aralocaspica* (Bunge) Freitag & Schütze (Kadereit et al., 2003; Schütze et al., 2003; Akhani et al., 2005; Kapralov et al., 2006). *Suaeda aralocaspica*, originally named *Borszczowia aralocaspica* Bunge, was subsequently classified in the monotypic *Suaeda* section *Borszczowia*, with a leaf type called *Borszczowoid* (Freitag and Stichler, 2000; Schütze et al., 2003; Kapralov et al., 2006).

There are four independent origins of C₄ photosynthesis in subfamily Suaedoideae: two parallel origins of Kranz C₄ anatomy (Salsinoid and Schoberoid in genus *Suaeda*) (see Section II A 3), and two independent origins of single-cell C₄. The single-cell C₄ plant *Suaeda aralocaspica* is in tribe Suaedeae. In this tribe, the veins are invariably located in one plane, with the primary vein in the center of the leaf and all deviating bundles in lateral positions. However, unlike other *Suaeda* species, *S. aralocaspica* has a primary vein in the center with peripheral veins adjacent to the chlorenchyma tissue (Freitag and Stichler, 2000; Schütze et al., 2003). This species is positioned between the C₃ section Schanginia and a C₃ shrubby section *Suaeda*, which suggests this type of photosynthesis evolved from C₃ ancestors rather than from a C₄ ancestor with Kranz anatomy (Kapralov et al., 2006). The two *Bienertia* species occur in an isolated tribe, Bienertiaeae, with a leaf type called Bienertoid, and the species have no known close relatives. We will use the common names Bienertia and Borszczowia to refer to these two types of single-cell C₄ taxa which are classified as C₄ structural forms called Bienertoid and Borszczowoid, respectively.

B. Biogeography of Single-Cell C₄ Species

The single-cell C₄ species grow in desert conditions. *Borszczowia* grows in central Asia from northeast of the Caspian lowlands east to Mongolia

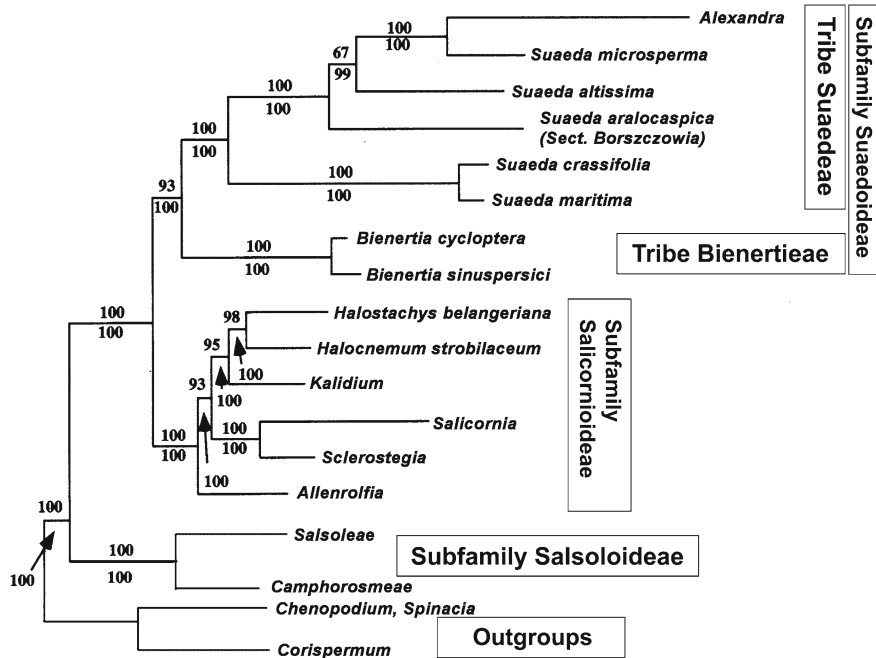


Fig. 7. Phylogenetic position of “single-cell” C_4 in Chenopodiaceae. The single maximum likelihood phylogram based on combined complete sequence information on nuclear ITS and five chloroplast DNA regions. Numbers above branches refer to bootstrap percentages and those below branches refer to Bayesian inference posterior probabilities (adapted from Kapralov et al., 2006).

and western China (Fig. 8). It is a hygro-halophyte that grows in temperate salt deserts with low night temperatures. The habitat consists of a high water table in salt marshes, which can support continuous leaf development and growth (Freitag and Stichler, 2000; Boyd et al., 2007).

Bienertia cycloptera grows from east Anatolia eastward to Turkmenistan and Pakistani Baluchestan. Its leaves are very succulent and sensitive to extended drought, which can cause wilting and leaf drop. It grows on dryer soils and is confronted with drought stress during the summer (Akhani et al., 2003).

Bienertia sinuspersici occurs in hot climates, and at lower latitudes and elevations than *B. cycloptera*. It is in a natural biogeographic range occurring from the westernmost coasts of Pakistan and extending westward along the coastal areas in southern Iran and countries surrounding the Persian Gulf. It shows an arc-like, latitudinal range that is separated from the range of *B. cycloptera* populations by the Zagros and Makran Mountains (Fig. 8). *Bienertia sinuspersici* differs anatomically by having mostly one to two layers of chlorenchyma cells, versus

two to three layers in *B. cycloptera*. Furthermore, *B. sinuspersici* is distinguished from *B. cycloptera* in having longer cotyledon leaves and leaves proper, larger seeds, larger flowers, and larger chromosomes, together with a set of micro-morphological features (Akhani et al., 2005).

C. Overview of Two Types of Single-Cell C_4 Photosynthesis in Terrestrial Plants

Two means of partitioning the function of C_4 photosynthesis between two cytoplasmic compartments evolved in family Chenopodiaceae. *Borszczowia* produces elongated palisade chlorenchyma cells with dimorphic chloroplasts polarized towards opposite ends of the cell. This is somewhat analogous to having Kranz anatomy, with the M and BS arrangement, without the intervening cell walls. Surprisingly, a completely different solution to performing C_4 photosynthesis in a single cell is found in *Bienertia*. The chlorenchyma cells of the two *Bienertia* species have a peripheral, chloroplast-containing, thin layer of cytoplasm (peripheral compartment) and a very unusual

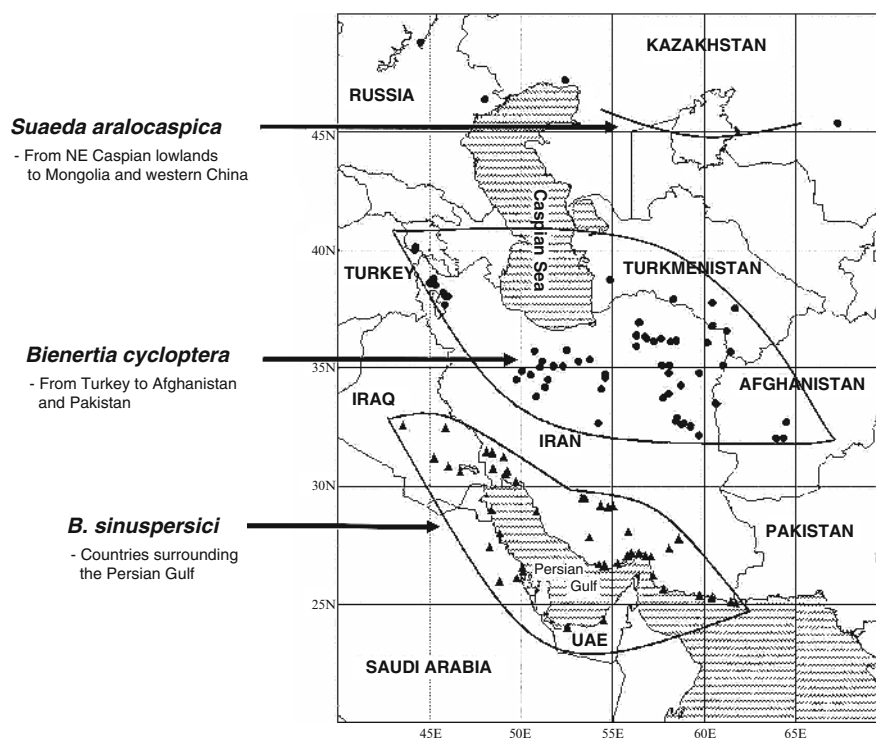


Fig. 8. Map showing the wide-spread distribution of single-cell C₄ species, *Suaeda* (= *Borszczowia*) *aralocaspica*, *Bienertia cycloptera*, and *B. sinuspersici*. The lines on the figure show the range for each species. Further research is needed to determine whether there is more diversity among these populations (subtypes or new species).

chloroplast-containing central cytoplasmic compartment, which are proposed to function like M and BS cells, respectively, in Kranz type C₄. In both systems, the partitioning of biochemically distinct organelles into discrete compartments is considered to result in concentration of CO₂ around the Rubisco-containing chloroplasts, causing inhibition of Rubisco oxygenase activity and photorespiration, as occurs in the typical Kranz system. Models of how these systems operate C₄ photosynthesis have been proposed (Fig. 9, Edwards et al., 2004).

D. Biochemical Evidence for Function of C₄ Photosynthesis in Single-Cell C₄ Plants

1. General Features Characteristic of C₄

Western Blots and Analysis of C₄ Enzymes

Analyses of photosynthetic enzymes by Western blots and enzyme assays show that the single-cell C₄ species have high levels of C₄ cycle

enzymes PEPC and PPDK, similar to Kranz type *Suaeda*, and in contrast to very low levels in the C₃ type *Suaeda* species (Fig. 10, also see Voznesenskaya et al., 2002). Assays for C₄ acid decarboxylases show that these single-cell C₄ species are NAD-ME type (Fig. 10), as are all Kranz type C₄ species which have been examined in subfamily Suaedoideae, see Voznesenskaya et al., 2007. Also, the single-cell C₄ species have a C₄ type PEPC similar to that in Kranz type species in subfamily Suaedoideae (Lara et al., 2006). This includes having high specific activities, a serine residue near the amino-terminus which undergoes phosphorylation/dephosphorylation, and light/dark regulation by phosphorylation with differential sensitivity to malate (Lara et al., 2006).

C₄ Type Carbon Isotope Composition

Reports on carbon isotope values in *Bienertia* (Winter, 1981; Akhani et al., 1997, 2005; Freitag and Stichler, 2002; Voznesenskaya et al., 2002) and *Borszczowia* (Freitag and Stichler, 2000;

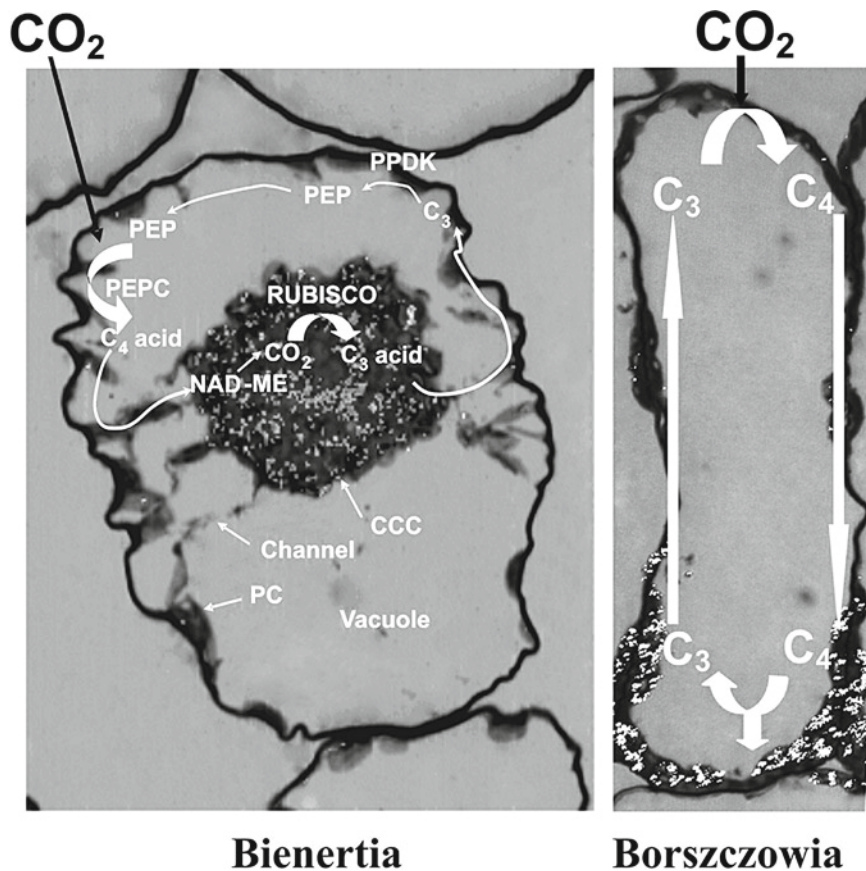


Fig. 9. General models of proposed function of C₄ photosynthesis in the two types of single-cell C₄ systems. The left panel for *Bienertia* illustrates atmospheric CO₂ entering the peripheral cytoplasm where it is fixed by PEPC, and a scheme which shows the path of carbon through the NAD-ME type C₄ cycle. The C₄ cycle delivers CO₂ to Rubisco (this immunogold-treated section for Rubisco shows label appearing as light deposits in the central cytoplasmic compartment). The right panel for *Borszczowia* illustrates atmospheric CO₂ entering the proximal end of the cell where it is fixed by PEPC; CO₂ is donated to Rubisco in the proximal end of the cell (immunolabeling for Rubisco shows light deposits in chloroplasts in the proximal end) via an NAD-ME C₄ cycle (as in *Bienertia*). PC, peripheral chloroplast; CCC, central cytoplasmic compartment; Channel, cytoplasmic channel connecting the PC and CCC; PDK, pyruvate, Pi dikinase; PEPC, PEP carboxylase; NAD-ME, NAD-malic enzyme.

Voznesenskaya et al., 2001) indicated that they have C₄/CAM (Crassulacean acid metabolism) type carbon isotope composition. Although succulent, subsequent studies showed no evidence for performance of CAM (Voznesenskaya et al., 2001, 2002, 2003). Various collections of the two *Bienertia* species and *Borszczowia*, from natural habitats and from plants grown under controlled conditions in high light, show that they have C₄ type carbon isotope composition. Analysis of the carbon isotope composition during a growing season in Iran showed *B. cycloptera* performs C₄ photosynthesis during its life cycle in nature similar to Kranz type C₄ species (Akhani et al., 2009). In *Bienertia* species, more negative values (−16% to −19%) have been observed in young

leaves and during growth under low light (100–200 photosynthetic photon flux density) (Freitag and Stichler, 2002; Voznesenskaya et al., 2002, 2005c). In young leaves, C₄ type chlorenchyma have not fully developed (Voznesenskaya et al., 2005c); growth under low light may limit the developmental transition from C₃ to a fully functional C₄ system (possibly due to incomplete development of dimorphic chloroplasts, or ability to concentrate CO₂ around Rubisco).

Physiological Response

The single-cell C₄ species and the Kranz type *Suaeda* species have low sensitivity of photosynthesis to O₂ under atmospheric levels of CO₂, and

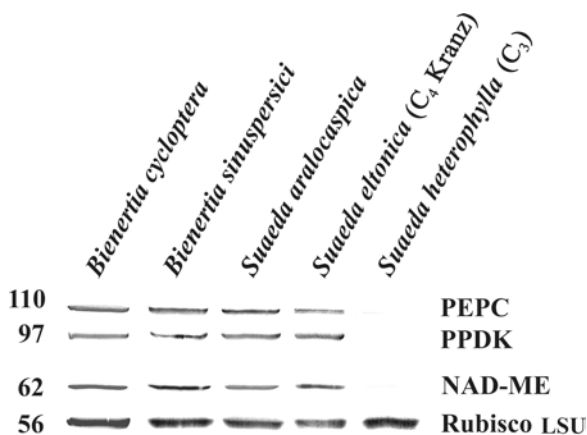


Fig. 10. Western blots of Rubisco and three C₄ cycle enzymes, PPKK, PEPC, and NAD-ME in the single-cell C₄ species *Suaeda* (= *Borszczowia*) *aralocaspica*, *Bienertia cycloptera* and *B. sinuspersici*, Kranz type *S. eltonica* and the C₃ *S. heterophylla* (see Chuong et al., 2006). Copyright American Society Plant Biologists, www.plantcell.org

low CO₂ compensation points, typical of C₄ plants (Voznesenskaya et al., 2001, 2002, 2007; Edwards et al., 2007). Also, the water use efficiency ($\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ water}$) is about two fold higher in the single-cell C₄ species and the Kranz type C₄ species than in representative C₃ species in subfamily Suaedoideae (Edwards et al., 2007).

Resistance to CO₂ Loss

For C₄ photosynthesis to function, high efficiency in trapping of the CO₂ generated by the C₄ pump, and in refixation of photorespired CO₂, are required. This means that the diffusive resistance in C₄ plants for CO₂ from Rubisco to the intercellular air space must be substantially higher than that in C₃ plants. Analyses of the diffusive resistance in the single-cell C₄ species show it is about 50-fold higher than that in C₃ species, and that it is of the same order of magnitude of Kranz type C₄ plants (Edwards et al., 2007).

2. Spatial Compartmentation Enabling Function of NAD-ME Type C₄ Photosynthesis

Critical experiments showing how C₄ photosynthesis functions in these single-cell C₄ species were performed by studying the structural organization by microscopy, by immunolocalization of several photosynthetic enzymes, and by localization of starch. In each single-cell C₄ system, the mechanism of photosynthesis and spatial

compartmentation of function are analogous to those of NAD-ME type Kranz species (Fig. 9, Voznesenskaya et al., 2001, 2002; Edwards et al., 2004). In the *Bienertia* species, the peripheral cytoplasm functions analogous to Kranz type C₄ M cells in fixation of atmospheric CO₂ into C₄ acids, while the central cytoplasmic compartment functions analogous to BS cells in donation of CO₂ from C₄ acids to Rubisco (Voznesenskaya et al., 2001, 2002; Edwards et al., 2004). As illustrated in the model in Fig. 9, CO₂ is fixed by PEPC in the peripheral cytoplasm leading to formation of C₄ acids (aspartate and malate) and their transport via cytoplasmic channels to the central compartment and decarboxylation in mitochondria via NAD-ME, with CO₂ donation to Rubisco. C₃ acids formed from decarboxylation are transported to the peripheral compartment and used for regeneration of PEP from pyruvate in the peripheral chloroplasts. In *Borszczowia*, the carboxylation phase of the C₄ pathway, with fixation of atmospheric CO₂, functions in the distal part of the cell (analogous to M cells in Kranz type), while donation of CO₂ to Rubisco from decarboxylation of C₄ acids occurs in the proximal part of the cell (analogous to BS cells in the Kranz type NAD-ME species).

Dimorphic Chloroplasts (Structure, Enzymes and Starch)

In each structural type of single-cell C₄, C₄ photosynthesis is accomplished in part by the partitioning of two biochemically and ultrastructurally distinct chloroplast types into separate compartments within the cell (Voznesenskaya et al., 2001, 2002, 2005c). These chloroplasts are dimorphic in biochemistry of photosynthesis, in ability to store starch, and in ultrastructure. In addition to immunolocalization studies by confocal microscopy, immunolocalization in *Bienertia* and *Borszczowia* by transmission electron microscopy shows rather strong selective labeling of PPKK in one chloroplast type, and Rubisco in the other chloroplast type (E. Voznesenskaya, N. Koteyeva, G. Edwards, unpublished results). The outer chloroplasts supporting the carboxylation phase of the C₄ cycle to fix atmospheric CO₂ have PPKK, which generates the substrate for PEPC by converting pyruvate to PEP, they store little or no starch, and they have a deficiency in grana development. The inner chloroplasts, which fix CO₂ generated from decarboxylation of C₄ acids, have Rubisco, they store starch

(and have ADPG pyrophosphorylase, the first committed step for starch biosynthesis), and they have well-developed grana. These features are the same as the respective M and BS chloroplasts in related Kranz type NAD-ME species. In C_4 plants, BS chloroplasts usually store larger amounts of starch than M chloroplasts. In NAD-ME type C_4 species, the grana-deficient chloroplasts are thought to be associated with a lower requirement for reductive power to support the C_4 carboxylation phase in this subgroup (see Section B2).

Mitochondria and Peroxisomes

In the single-cell C_4 species, the mitochondria are partitioned to the cytoplasmic compartment, where the Rubisco-containing chloroplasts are located (the proximal end of the cell in *Borszczowia* and in the central cytoplasmic compartment in *Bienertia* species). The mitochondria perform two important functions relative to C_4 photosynthesis: generation of CO_2 by decarboxylation of C_4 acids via NAD-ME and decarboxylation of glycine as a result of any photorespiration. Also, peroxisomes are predominantly located in the cytoplasmic compartment with Rubisco-containing chloroplasts (based on transmission electron microscopy and immunolocalization of catalase), which are presumably associated with metabolism of glycolate to glycinate in the glycolate pathway (Voznesenskaya et al., 2001, 2002; Chuong et al., 2006). While the C_4 cycle concentrates CO_2 around Rubisco and suppresses photorespiration, some photorespiration does occur. The selective localization of glycine decarboxylase in BS mitochondria makes photorespired CO_2 available for refixation by Rubisco.

E. Development of Spatial Compartmentation and Dimorphic Chloroplasts

An intriguing aspect of single-cell C_4 photosynthesis is the development of spatial compartmentation of functions and dimorphic chloroplasts. There is evidence that very young chlorenchyma cells have a single type of chloroplast (monomorphic) which is in a C_3 default mode, with all chloroplasts containing low levels of Rubisco without PPDK, and without spatial

separation of organelles into two compartments (Voznesenskaya et al., 2005c). Since enzymes of the C_4 cycle like PPDK and the small subunit of Rubisco are nuclear encoded, there must be posttranscriptional regulation for selective expression of certain proteins in chloroplasts (see chapter 12 by Berry et al. for selective expression in Kranz type C_4). In mature chlorenchyma cells that have formed two cytoplasmic compartments and dimorphic chloroplasts, there is intricate development of the cytoskeleton, which consists of actin and microtubules. Cytoskeleton-disrupting drugs show that microtubules are important in maintaining the two cytoplasmic compartments (Chuong et al., 2006).

F. Form of Photosynthesis in Different Photosynthetic Organs in Single-Cell C_4 Species

Plants are usually characterized by photosynthetic type according to the mechanism of carbon assimilation in the leaf, which is generally the main photosynthetic organ. Among chenopods having C_4 photosynthesis in leaves, there is variation between species as to the type of photosynthesis in cotyledons (Butnik, 1979, 1984, 1991; Pyankov et al., 1999, 2000b, c; Voznesenskaya et al., 1999, 2004; Akhiani and Ghasemkhani, 2007). There are C_4 chenopods, for example *Salsola richteri* (Salsoloid type), which have the same type of Kranz anatomy in leaves and cotyledons. There are also C_4 chenopods having C_4 photosynthesis in leaves and cotyledons, but different types of Kranz anatomy, for example *Salsola laricina*, which has Salsoloid anatomy in leaves and Atriplicoid type anatomy in cotyledons. Finally, there are dicots which have C_4 photosynthesis in leaves, but C_3 type anatomy and photosynthesis in cotyledons; for example *Salsola gemmascens* has Salsoloid type anatomy in leaves and C_3 type anatomy in cotyledons (Pyankov et al., 2000c).

Not all C_4 plants have leaves as the primary photosynthetic organ during vegetative growth. For example, in family Chenopodiaceae, C_4 species of *Anabasis*, *Haloxylon*, *Halosarcia*, *Hammada* and some species of *Halothamnus* have reduced leaves with stems as the primary

photosynthetic organ (Ocallaghan, 1992; Akhani et al., 1997; Pyankov et al., 2000b).

With respect to single-cell C₄ species, *Borszczowia* and both *Bienertia* species have green cotyledons, leaves, and flowers. Studies of the respective organs, including structure of chlorenchyma, immunolocalization of photosynthetic enzymes (Rubisco and PEPC), and Western blots of Rubisco and C₄ cycle enzymes, show they all have unique chlorenchyma cells which are structurally and biochemically developed to perform single-celled C₄ photosynthesis (Voznesenskaya et al., 2001, 2002, 2004; Edwards et al., 2004; Akhani et al., 2005; Boyd et al., 2007). Thus, these species perform single-cell C₄ photosynthesis throughout their life cycle. Leaves make up the majority of the green tissue during vegetative growth of these species, although younger branches have green stems. However, the flowers have green tepals which become a major photosynthetic organ during the latter reproductive phase of growth. Photosynthesis in flowers may have adaptive value for survival of these desert plants. In *Bienertia*, under increasing temperatures as flowers develop, lower leaves wither and senesce and are replaced by smaller floral leaves consisting of green tepals (Boyd et al., 2007). Also, the stems of *B. cycloptera* have single-cell C₄ type chlorenchyma cells beneath the epidermis. This, combined with the presence of stomata on the stems, starch in stem chlorenchyma, and light-dependent fixation of atmospheric CO₂ by the stems, suggests they contribute to carbon assimilation in *Bienertia*. In contrast, the stems of *Borszczowia* have C₃ type chlorenchyma cells scattered throughout the cortical tissue. They likely function to refix respired CO₂ in stems, since light dependent fixation of external CO₂ is only observed under high atmospheric levels (Boyd et al., 2007).

G. How Did Single-Cell C₄ Evolve?

In considering how single-cell C₄ plants might have evolved, an analogy can be made to the proposed evolutionary development of Kranz type C₄ plants from C₃ plants (see chapter 6 by Bauwe). In C₃ plants, M cells are the main photosynthetic tissue in the leaf, since the BS cells have few, or no, chloroplasts. It has been suggested that evolution of C₄ has occurred multiple times by a step-wise progression of structural and biochemical

changes which were induced by CO₂-limiting conditions (Monson et al., 1984; Edwards and Ku, 1987; Monson and Moore, 1989; Rawsthorne and Bauwe, 1998; Sage, 2004). The occurrence of intermediates between C₃ and C₄ plants, particularly in the genus *Flaveria* (Asteraceae), has provided a basis for suggesting how C₄ may have evolved, from C₃, to intermediates which reduce photorespiration without a C₄ cycle, to intermediates having a partially-functioning C₄ cycle, to full development of C₄. In the first type of intermediate, normal C₃ type photosynthesis occurs in M cells; however, the release of CO₂ in photorespiration occurs in mitochondria in BS cells (by selective localization of glycine decarboxylase in BS mitochondria), where a degree of refixation by chloroplasts occurs. This increases the efficiency of photosynthesis under limiting CO₂ (von Caemmerer, 1989).

By analogy, under CO₂-limiting conditions a single-cell C₃-C₄ intermediate species may develop by spatial separation of the fixation of atmospheric CO₂ by the C₃ cycle and the refixation of photorespired CO₂ (see illustration in Fig. 11). This would generate a form of CO₂ concentrating mechanism and reduce loss of CO₂ by photorespiration. It could provide the initial spatial separation with chloroplasts in one cytoplasmic compartment fixing atmospheric CO₂, and chloroplasts and mitochondria in another cytoplasmic compartment functioning to minimize CO₂ loss by photorespiration. The spatial separation of chloroplasts and mitochondria in this hypothetical C₃-C₄ intermediate is like that in the single-cell C₄ plants. This would provide the initial spatial separation of organelles, with subsequent differentiation of chloroplasts and expression of C₄ cycle enzymes leading to development of a functional C₄ system.

IV. Future Perspectives

The discovery of C₄ plants among terrestrial species, approximately 40 years ago, and their association with Kranz anatomy, has led to numerous studies on the occurrence, photosynthetic mechanism, structural and biochemical diversity, molecular control of development of two photosynthetic cells, and evolution (Edwards and Walker, 1983; Hatch, 1987; Sage and Monson,

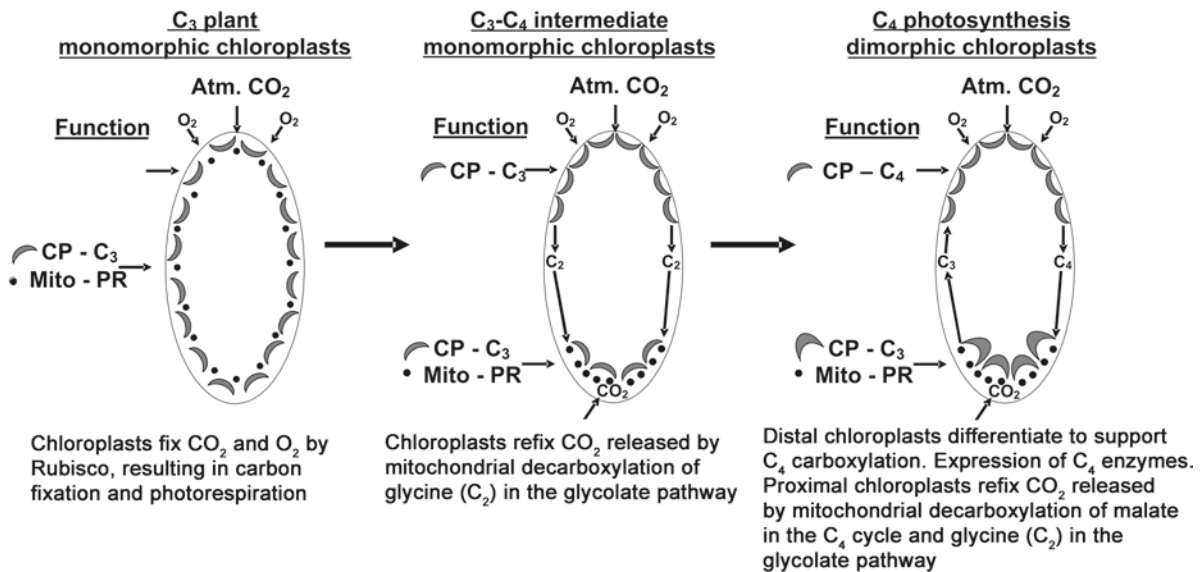


Fig. 11. Scheme illustrating a hypothetical intermediate stage in evolution of single-cell C₄ photosynthesis via a C₃-C₄ intermediate which reduces photorespiratory loss of CO₂ by refixation of CO₂ via Rubisco. *Atm.*, atmospheric; *mito*., mitochondria; *PR*, photorespiration; *CP*, chloroplast.

1999). With the paradigm that this occurred in land plants via development of Kranz anatomy, the finding that terrestrial species can conduct C₄ photosynthesis within individual chlorenchyma cells provides a very different system to study C₄. This includes stages in evolution of C₄, the genetic control of development of the requisite spatial separation of functions, the mechanism of chloroplast differentiation, biochemical and biophysical requirements for the function of C₄ photosynthesis, and development of strategies for engineering C₄ photosynthesis into selected C₃ crops.

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Single-Cell C₄ Photosynthesis in Aquatic Plants

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Summary	63
I. Introduction.....	64
II. Unraveling the Single-Cell C ₄ System	64
A. Some Early Intriguing Observations	64
B. Single-Cell C ₄ Photosynthesis in <i>Hydrilla</i>	65
C. Other Submersed Single-Cell C ₄ Species.....	71
D. Which Originated First: Aquatic or Terrestrial C ₄ Photosynthesis?.....	75
III. HCO ₃ ⁻ -Use Mimics C ₄ Photosynthetic Gas Exchange Characteristics	76
IV. Concluding Thoughts.....	76
Acknowledgments	77
References	77

Summary

In water with low free [CO₂] a common strategy of submersed plants is to use HCO₃⁻, but some species utilize a C₄ photosynthetic system that surprisingly lacks the Kranz dual-cell compartmentation of most terrestrial C₄ plants. Instead, the C₄ and C₃ cycles are in the same cell, with phosphoenolpyruvate carboxylase (PEPC) and ribulose biphosphate carboxylase–oxygenase (rubisco) sequestered in the cytosol and chloroplasts, respectively. Malate decarboxylation by NADP malic enzyme (NADP-ME) in the chloroplasts produces a chloroplastic CO₂ concentrating mechanism (CCM). It occurs in the submersed monocots *Hydrilla verticillata* and *Egeria densa* (Hydrocharitaceae), and in these species it is facultative because low [CO₂] induces a metabolic shift in the leaves from C₃ to single-cell C₄ photosynthesis. Submersed leaves of other species also perform single-cell C₄ photosynthesis, including *Sagittaria subulata* (Alismataceae), the grasses *Orcuttia californica* and *O. viscida* (Poaceae), and the sedge *Eleocharis acicularis*. A marine macroalga (*Udotea flabellum*, Chlorophyta) and a diatom (*Thalassiosira weissflogii*) likewise show evidence of its occurrence, so it is not restricted to higher plants. The change from C₃ to C₄ photosynthetic gas exchange and pulse-chase characteristics is well documented in *Hydrilla*, along with enzyme kinetics and localization; high internal [CO₂], and improved growth. Multiple isoforms of PEPC, NADP-ME and pyruvate orthophosphate dikinase (PPDK) exist in *Hydrilla* and *Egeria*, but specific forms, including *hvpepc4*, *hvmel* and *hvppdk1* are up-regulated in the C₄ leaves of *Hydrilla* and encode proteins with C₄ photosynthetic characteristics. Interestingly, the photosynthetic *hvpepc4* differs from its terrestrial C₄ counterparts in lacking a “C₄-signature” serine near the carboxy terminus. The C₃ leaf must maximize CO₂ conductance to rubisco, but as the C₄ system is induced, chloroplast conductance is probably minimized to reduce leakage from the CCM. Further study of the facultative

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system of *Hydrilla* could determine if down-regulation of chloroplast-envelope aquaporins is involved in reducing CO₂ conductance. *Hydrilla* and *Egeria* are in the ancient Hydrocharitaceae family, and can give insights into early C₄ photosynthesis, which likely originated in water prior to its advent on land.

I. Introduction

C₄ photosynthesis, in its several forms, is essentially a biochemical CO₂ concentrating mechanism that increases the substrate [CO₂] in the vicinity of ribulose biphosphate carboxylase–oxygenase to overcome the competitive inhibition by O₂ and oxygenase activity of this enzyme (Bowes et al., 1971; Ogren and Bowes, 1971). This is achieved, in part, because the initial carboxylating enzyme in C₄ systems is phosphoenolpyruvate carboxylase, which, unlike rubisco, uses HCO₃[−] as substrate and is not inhibited by O₂ (Cooper et al., 1969; Cooper and Wood, 1971; Bowes and Ogren, 1972).

In the 1960s as the biochemical components of the C₄ cycle were being pieced together it was apparent that a specialized anatomical leaf structure, Kranz anatomy, in which mesophyll cells surrounded a chloroplast-containing bundle sheath cell layer, was needed for a functional system (Kortschak et al., 1965; Downton and Tregunna, 1968; Hatch and Slack, 1970). Pains-taking crosses by the geneticist Malcolm Nobs of two *Atriplex* species, one a C₄ (*A. rosea*) and the other a C₃ (*A. patula*), followed by careful anatomical, physiological and biochemical analyses of the subsequent generations of offspring by Olle

Björkman and colleagues provided experimental evidence that the coordination of anatomy and biochemistry were vital (Björkman et al., 1970; Boynton et al., 1970; Pearcy and Björkman, 1970). Sequestering rubisco in the bundle sheath chloroplasts, away from PEPC in the mesophyll cytosol, prevents competition between the two carboxylases for inorganic carbon, and enables CO₂ to be concentrating at the rubisco fixation site (Berry et al., 1970; Andrews et al., 1971). The apparently ubiquitous association between anatomy and biochemistry was thus established.

In addition to Kranz anatomy, the occurrence of C₄ photosynthesis became predominantly associated with high-light requiring species, improved WUE and warmer, drier habitats. Thus, when early reports of C₄ photosynthesis in the submersed plant *Hydrilla verticillata* were published (Holaday and Bowes, 1980; Salvucci and Bowes, 1981; 1983a) it was something of a surprise and the findings were not readily accepted. One can understand the skepticism; the plant did not fit the established dogma in that it lacks specialized anatomy, is a shade species, WUE is not a factor when submersed, and the role of temperature is still uncertain.

II. Unraveling the Single-Cell C₄ System

A. Some Early Intriguing Observations

In the 1950s *Hydrilla* was imported into Florida as a seemingly innocuous freshwater aquarium plant. It inevitably found its way into the environment and was observed in 1960 growing in a canal near Miami and in springs near Crystal River (Blackburn et al., 1969). Initially it was misidentified as American elodea (*Elodea canadensis*) or Brazilian elodea (*Egeria densa*), which it closely resembles. It spread rapidly and by 1967 it was considered a major problem weed in Florida waters. By the 1970s it had spread into lakes and rivers of other southeastern states

Abbreviations: AA – Aminotransferase; Ala – Alanine; Asp – Aspartate; BN-PAGE – Blue native polyacrylamide gel electrophoresis; CAM – Crassulacean acid metabolism; CA – Carbonic anhydrase; CCM – CO₂ concentrating mechanism; Γ-CO₂ compensation point; DW – Dry weight; FW – Fresh weight; Glc – Glucose; MDH – Malate dehydrogenase; NADP-ME – NADP malic enzyme; NAD-ME – NAD malic enzyme; OAA – Oxaloacetate; PEP – Phosphoenolpyruvate; PEPC – Phosphoenolpyruvate carboxylase; PEPCK – Phosphoenolpyruvate carboxykinase; PNUE – Photosynthetic nitrogen use efficiency; PPK – Pyruvate orthophosphate dikinase; PS II – Photosystem II; RT-PCR – Real-time polymerase chain reaction; Rubisco – Ribulose biphosphate carboxylase–oxygenase; Ser – Serine; WUE – Water use efficiency

(Langeland, 1996). Eventually it became clear that at least two genetically-distinct biotypes of *Hydrilla* had been imported into the USA. In the warmer southeastern states a dioecious pistillate biotype occurs, whereas in more northern cooler states a monoecious form is found (Steward et al., 1984; Van, 1989). In both cases the plants reproduce and spread by vegetative means. However, the dioecious female has received by far the most research attention.

During investigations in the 1970s into factors that contributed to the competitive success of dioecious *Hydrilla*, we wondered if it photorepired to the same degree as terrestrial C₃ species (Van et al., 1976, 1977, 1978). There were reports that the photorespiration of submersed plants might be less than that of terrestrial plants in air (Hough and Wetzels, 1972; Hough, 1974). To determine if this was true we measured the Γ values and the O₂ inhibition of photosynthesis, but were frustrated for several months by the inconsistency in the data that varied from high C₃- to low C₄-like values. Eventually it became apparent that the conditions under which the *Hydrilla* plants were incubated had a marked influence on the results. Thus, when they were crowded under high temperatures (30°C) and long photoperiods (14-h) the Γ values declined over a 12- to 14-day period into a C₄ range of <10 μ bar, whereas at 20°C and a 10-h photoperiod they were C₃-like (>40 μ bar). These incubation conditions are like those occurring naturally during summer and winter, and in fact similar variations in Γ values are found with *Hydrilla* growing in Florida lakes (Bowes et al., 1978; Holaday and Bowes, 1980). The major trigger for the decline in Γ values was found to be low [CO₂] in the water, rather than temperature and photoperiod. CO₂-depletion occurs in lakes when vegetation is dense and daytime photosynthetic CO₂ assimilation is high (Van et al., 1976; Holaday et al., 1983). In the summer, plants growing at the center of a dense *Hydrilla* mat have low C₄ Γ values, whereas those at the edge of the mat, where [CO₂] is not depleted, are C₃-like (Spencer et al., 1994).

An initial enzymatic study revealed substantial PEPC activity and a high PEPC/rubisco ratio in low Γ , but not high Γ , leaves, which was consistent with the operation of some form of C₄ acid cycle (Bowes et al., 1978; Holaday and Bowes,

1980; Ascencio and Bowes, 1983). The potential for C₄ acid production in the light in submersed vascular plants was not a novel concept. Bose (1924) showed that *Hydrilla* leaves were more acidic in summer than winter, and had greater malate content. Substantial ¹⁴C-labeling of malate and aspartate in short-term photosynthetic studies was also reported for *Egeria densa* and *Lagarosiphon major*, both monocots in the same Hydrocharitaceae family as *Hydrilla* (Brown et al., 1974). Similarly, DeGroote and Kennedy (1977) found that another member of the family, *Elodea canadensis*, initially incorporated almost 50% of the ¹⁴C-label into C₄ acids during photosynthesis. However, *Egeria* and *Elodea* exhibited little or no turnover of label from the C₄ acids into Calvin cycle products, and as a consequence it was concluded that these plants were not performing C₄ photosynthesis (Browse et al., 1977, 1979a, b, 1980; DeGroote and Kennedy, 1977). Instead, the C₄ acids were variously suggested to function as a pH-stat mechanism, a counter-balance to cation uptake, or an anapleurotic carbon source. In addition, based on leaf anatomy one would not expect these submersed species to exhibit C₄ photosynthesis. *Hydrilla*, *Egeria*, and *Elodea* leaves lack stomata and the lamina is only two photosynthetic cells thick, and thus lacks the chlorenchymatous mesophyll and bundle sheath cells of terrestrial C₄ species (Bowes et al., 2002). Despite these contra-indicators, the substantial up-regulation of PEPC activity as photorespiration and the O₂ inhibition of photosynthesis declined persuaded us to investigate more fully the role of PEPC in *Hydrilla* photosynthesis.

B. Single-Cell C₄ Photosynthesis in *Hydrilla*

Dioecious *Hydrilla* has certain unique features. It is a facultative single-cell C₄ NADP-ME species, but differs from Kranz-type NADP-ME C₄ plants in a number of important respects that go beyond anatomy. Table 1 documents some of the anatomical, physiological and biochemical features of the C₃ and C₄ leaves that provide substantial evidence that *Hydrilla* is a facultative C₄ species. The plant typically exhibits C₃ gas-exchange and biochemical characteristics, but exposure to low [CO₂] induces a C₄-based CCM within a 10–12-day period (Salvucci and

Table 1. A comparison of the anatomical, physiological and biochemical characteristics of C_3 and C_4 *Hydrilla* leaves.

Parameters	C_3 leaf	C_4 leaf
Anatomical		
Stomata present	No	No
Leaf lamina two cell layers thick	Yes	Yes
Bundle sheath (Kranz) present	No	No
Physiological		
Induced by low $[CO_2]$	No	Yes
CO_2 compensation point (μ bar)	>40	<10
O_2 inhibition of photosynthesis (%)	>28	<2
Net photosynthesis, limiting CO_2 (μ mol g^{-1} FW h^{-1})	2	44
^a Net photosynthesis, saturating CO_2 (μ mol g^{-1} FW h^{-1})	N/A	124
^a Carbon gain, limiting CO_2 (μ mol g^{-1} DW day^{-1})	291	407
^a PNUE, limiting CO_2 (μ mol C mg^{-1} N day^{-1})	991	1,241
Ratio leaf internal/external inorganic carbon	0.8	4.2
Estimated chloroplast $[CO_2]$ (μ M)	7	~400
Leaf abaxial surface pH	3.6	4.7
^a $\delta^{13}C$ value (‰)	-27.3	-23.5
Biochemical		
Rubisco activity (μ mol g^{-1} FW h^{-1})	45	40
Rubisco location in all leaf cells	Chloroplast	Chloroplast
PEPC activity (μ mol g^{-1} FW h^{-1})	<10	>150
Major PEPC isoform	HVPEPC3	HVPEPC4
PEPC isoform location in all leaf cells	Cytosol	Cytosol
PEPC isoform light activated and phosphorylated	No	Yes
Recombinant PEPC $K_{0.5}$ (PEP) at pH 7.3 (mM)	0.15	0.30
Recombinant PEPC I_{50} (malate) at pH 7.3 (mM)	0.04	0.15
Ratio PEPC/rubisco activity	0.2	>4.2
NADP-ME activity (μ mol g^{-1} FW h^{-1})	16	44
Major NADP-ME isoform	HVME3	HVME1
Predicted NADP-ME transit peptide (No. amino acids)	None	75
Recombinant NADP-ME k_{cat} (s^{-1})	17.0	46.1
NADP-ME isoform location in leaf cells	Cytosol	Chloroplast
PPDK activity (μ mol g^{-1} FW h^{-1})	3	35
PPDK isoform	Unknown	HVPPDK1
PPDK location in leaf cells	Unknown	Chloroplast
50% ^{14}C -malate + ^{14}C -aspartate turnover (s)	None	<60
CA activity (EU g^{-1} FW h^{-1})	246	1,365
External leaf CA activity	No	No
CA location in leaf cells	Unknown	Unknown
CA isoform induced	None	HVCA1

N/A – not available

^aData for lake-grown plants (Spencer et al., 1994)

Bowes, 1981; Holaday et al., 1983; Magnin et al., 1997). The change from C_3 to C_4 can occur without the production of new leaves, and has been documented in the lake as well as the laboratory (Holaday and Bowes, 1980; Spencer et al., 1994). When the $[CO_2]$ is low, C_4 cycle genes are up-regulated in the leaf and expressed in the original C_3 mesophyll cell background (Rao et al., 2006a).

In a body of water under CO_2 -limiting conditions, this switch to a C_4 system has been shown to provide a number of advantages, including improvements

in carbon gain and photosynthetic nitrogen use efficiency (Table 1; Spencer et al., 1994). Likewise, terrestrial C_4 species may exhibit similar advantages under the present atmospheric $[CO_2]$ that is sub-saturating for terrestrial C_3 species. A schematic of how the C_4 system is believed to operate in a *Hydrilla* cell is presented in Fig. 1.

A micro-electrode study has shown that the abaxial walls of *Hydrilla* leaves are acidified to around pH 4 in a light-dependent process (van Ginkel et al., 2001). Thus it is CO_2 , not HCO_3^- , which enters the cell. Because acidification occurs

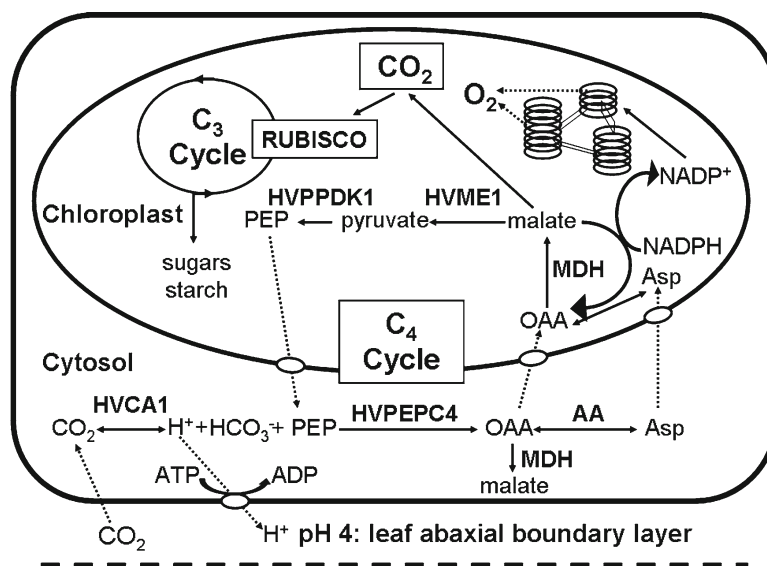


Fig. 1. A proposed scheme of how the single-cell C_4 system operates in a *Hydrilla* leaf cell (modified and updated from Bowes et al., 2007).

in both C_3 and C_4 leaves, and CO_2 entry is by diffusion, this process is not the agent responsible for the CCM of the C_4 leaf. Instead it appears to enhance access to, and diffusion of, CO_2 via the dissolved HCO_3^- that tends to be the more abundant form of inorganic carbon in most water bodies. Other submersed species that do not show evidence of a CCM operate a similar acidification mechanism (Bowes and Salvucci, 1989; Elzenga and Prins, 1989).

After entry into the leaf, a cytosolic carbonic anhydrase, presumed to be HVCA1, catalyzes the equilibration of CO_2 with HCO_3^- , and the latter is used by PEPC (HVPEPC4), to carboxylate phosphoenolpyruvate to oxaloacetate. A cytosolic aminotransferase and a malate dehydrogenase catalyze the production of aspartate and malate, respectively. It is postulated that OAA or Asp are the major C_4 acids transported into the chloroplast, rather than malate. The OAA is converted to malate by a chloroplastic MDH, and NADPH is oxidized to NADP^+ . A chloroplastic NADP malic enzyme (HVME1) catalyzes the oxidative decarboxylation of malate to pyruvate, thereby elevating the $[\text{CO}_2]$ at the rubisco fixation site, and overcoming the competitive effects of O_2 . Thus it is in the chloroplast, not the whole cell, where CO_2 is concentrating. A specific, partially characterized, chloroplastic pyruvate orthophosphate dikinase isoform, believed to be HVPPDK1,

recycles pyruvate to PEP, which returns to the cytosol as substrate for the PEPC reaction.

Much of the recent work with *C₄ Hydrilla* has focused on key regulatory enzymes in the process. During induction of the *C₄* state the activity of what becomes the initial carboxylase, PEPC, increases up to 15-fold. Other *C₄*-cycle enzymes also increase in activity, but PEPC is the first to be up-regulated (Salvucci and Bowes, 1981, 1983a, b; Ascencio and Bowes, 1983; Magnin et al., 1997). Consequently, the decline in Γ in the first day or two is probably the result of photorespiratory CO₂ recapture prior to the CCM becoming fully-operational. Exposure of fully-functional *C₄ Hydrilla* leaves to diethyloxalacetate, a PEPC inhibitor, reduces their photosynthesis rate and increases the O₂ inhibition of photosynthesis by more than twofold (Magnin et al., 1997). By contrast the inhibitor has no effect on *C₃* leaf photosynthesis and its O₂ inhibition. These results are to be expected if the inhibitor curtails carbon fixation through the *C₄* leaf PEPC. Similar findings have been reported in terrestrial *C₄* species with another PEPC inhibitor, 3,3-dichloro-2-(dihydroxyphosphinoylmethyl) propenoate (Jenkins, 1989). The $\delta^{13}\text{C}$ values reported for lake-grown plants are also consistent with a change from rubisco to PEPC as the initial carboxylase (Table 1; Spencer et al., 1994). *Hydrilla* plants at the edge of a dense mat of vegetation have

C₃-like $\delta^{13}\text{C}$ values, whereas those at the center, which have switched to a C₄ system and have high PEPC activity, have less negative values. It should be noted that the C₄ plants at the center were once at the edge of the mat using the C₃ system. As a consequence, the $\delta^{13}\text{C}$ is more negative than a typical terrestrial C₄ value because the plant tissue is the product of both modes of photosynthesis.

Concomitant with the role of PEPC, malate and Asp are among the initial products formed. Two separate ¹⁴C-pulse/¹²C-chase studies show that around 60% of the initial ¹⁴C-label enters malate and Asp, within a 10-s pulse, but it is rapidly “chased” into phosphorylated Calvin cycle compounds and sugars, with around a 50% loss from the acids in 60 s (Holaday and Bowes, 1980; Salvucci and Bowes, 1983a). The demonstration that C₄ acid label is rapidly chased into sugar-P and sugars in the light is perhaps the “gold-standard” for identifying C₄ photosynthesis. Comparable pulse-chase results have been reported for the submersed leaf of the grass *Orcuttia viscida*, which also lacks Kranz anatomy and appears to operate a single-cell C₄ system (Keeley, 1998b).

Three full-length cDNAs encoding PEPC have been sequenced from *Hydrilla* (*hvpepc3*, 4 and 5). Transcript expression of the first two occurs in leaves, and the third in roots. The C₄ photosynthetic form is *hvpepc4*, while *hvpepc3* and *hvpepc5* probably have anapleurotic roles. Although *hvpepc3* and *hvpepc4* are both expressed in the C₃ leaves, *hvpepc4* expression is greatly enhanced in C₄ leaves, especially in the light (Rao et al., 2002, 2006b). Likewise, western blots show that although HVPEPC3 and HVPEPC4 proteins are present in both leaf types, the expression of the latter is induced in the light during the C₃ to C₄ transition (Rao et al., 2006b). HVPEPC4 also undergoes light-dependent, post-translational phosphorylation, but the C₃ isoform (HVPEPC3) shows little evidence of this modification (Rao et al., 2006b). This is consistent with the observation that PEPC activity from C₄ leaves, but not C₃, shows light activation effects that increase its rate and malate inhibition when the leaves are light-incubated (Bowes et al., 2002). PEPC kinetics in C₄ and C₃ leaf extracts differ in other respects, in that they exhibit allosteric and Michaelis–Menten responses, respectively. Glc-6-phosphate is a positive effector of both as it increases activity,

and reduces malate inhibition and the $K_{0.5}(\text{PEP})$ values. The data clearly indicate that a specific isoform (HVPEPC4) with distinct characteristics is required for the photosynthetic C₄ cycle.

Although HVPEPC4 functions kinetically as a C₄ enzyme, its sequence differs in a major feature from all other C₄ photosynthetic isoforms (Rao et al., 2002). It lacks the otherwise ubiquitous “C₄-signature” Ser at the carboxy-terminus; a feature that is found in terrestrial C₄ Kranz and single-cell species, and which is an important determinant of C₄-type kinetics (Bläsing et al., 2000; Lara et al., 2006). Instead, at this site both HVPEPC and HVPEPC4 possess Ala, which is the characteristic residue of C₃ and CAM isoforms. It was thus of interest to generate recombinant *Hydrilla* PEPC isoforms in a PEPC-deficient *E. coli* host F15, and perform site-directed mutagenesis to determine the effect of Ala and Ser substitutions (Rao et al., 2008). Kinetic analyses of the recombinant isoforms indicated that rHVPEPC4 had a $K_{0.5}(\text{PEP})$ of 0.3 mM that is higher than rHVPEPC3, and thus is typical of C₄ isoforms, despite the lack of the signature Ser. Replacing Ala with Ser in rHVPEPC4 increased its $K_{0.5}(\text{PEP})$ to 0.78 mM, but had the detrimental effect of reducing the $V_{\max}$ by 25%. However, a similar Ser substitution in rHVPEPC3 not only increased the $K_{0.5}(\text{PEP})$ to 0.92 mM but also increased the $I_{50}(\text{malate})$, $V_{\max}$ and k_{cat} . Thus, with a single amino acid substitution rHVPEPC3 became kinetically similar to a C₄ PEPC, and may even be superior in this regard to HVPEPC4. Glc-6-phosphate was a positive effector, lowering the $K_{0.5}(\text{PEP})$ and reducing malate inhibition in all cases. Because HVPEPC4 lacks the signature Ser the overall C₄ characteristics must be conferred by other parts of its primary structure.

Fluorescence and immunogold labeling of *Hydrilla* show that PEPC and rubisco are present in all leaf cells, but are segregated in the cytosol and chloroplast, respectively (Reiskind et al., 1989). In contrast to PEPC, rubisco activity is somewhat down-regulated (10–20%) during induction of C₄ photosynthesis, but its kinetic characteristics do not change (Ascencio and Bowes, 1983; Salvucci and Bowes, 1983a). The $K_m(\text{CO}_2)$ values are 26 and 28 μM for the enzyme isolated from C₃ and C₄ leaves, respectively, while the corresponding $K_i(\text{O}_2)$ values are 690 and 734 μM , and the specificity factor is 73 (Bowes and Salvucci, 1984).

These kinetic data have been used to compute the [CO₂] needed in the C₄ leaf chloroplasts to cause the observed value for the O₂ inhibition of photosynthesis of only 2% (Reiskind et al., 1997). The $K_m(\text{CO}_2)$ values for *Hydrilla* rubisco are over twice that of the C₃ plant tobacco (10 μM), but only half those of terrestrial C₄ species that are in the same NADP-ME category, which average 53 μM CO₂ (Bowes and Salvucci, 1984; Yeoh and Hattersley, 1985). A low rubisco affinity for CO₂, as indicated by a higher K_m , has been associated with the ability to maintain a high CO₂ concentration around the enzyme (Yeoh and Hattersley, 1985). The submersed eudicot *Myriophyllum spicatum* exhibits similar $K_m(\text{CO}_2)$ values to those of *Hydrilla*, but although this species can have a low Γ it shows no evidence of a C₄ cycle, instead it has a facultative system that uses HCO₃⁻ (Salvucci and Bowes, 1983b; Bowes and Salvucci, 1984).

Two decarboxylases that could potentially operate in C₄ photosynthesis are present in *Hydrilla* leaves, a mitochondrial NAD-ME and NADP-ME, but phosphoenolpyruvate carboxykinase activity is absent (Salvucci and Bowes, 1981; Magnin et al., 1997). Both increase in activity during induction of C₄ photosynthesis, but the evidence points to a chloroplastic NADP-ME as the principle decarboxylating enzyme. Direct measurements of dissolved inorganic carbon in C₃ and C₄ leaves, similar to those made by Hatch (1971) on terrestrial C₃ and C₄ leaves, demonstrated that the C₄ leaf of *Hydrilla* achieves over a fourfold concentrating effect for total inorganic carbon (CO₂ + HCO₃⁻), that results in a [CO₂] in the chloroplasts of around 400 μM (Reiskind et al., 1997). In contrast, the C₃ leaf is below equilibrium with the external concentration, having a chloroplastic [CO₂] of only about 7 μM , which is on the order of that observed in terrestrial C₃ leaves. The concentrating location is confined to the C₄ leaf chloroplasts, rather than the whole cell (Reiskind et al., 1997). This is consistent with a chloroplastic decarboxylase, NADP-ME, releasing CO₂ directly in the vicinity of rubisco.

Three full-length cDNA sequences of NADP-ME, *hvme1*, *hvme2* and *hvme3* were obtained from *Hydrilla* plants, but only one, *hvme1*, with a molecular mass of 64 kD is up-regulated in C₄ leaves (Estavillo et al., 2007). Northern blots and semi-quantitative RT-PCR indicated that *hvme3* is constitutively expressed in C₃ and C₄ leaves,

while *hvme2* predominates in C₃ leaves. In contrast, *hvme1* expression is up-regulated in the light during C₄ induction, and this isoform has a putative 75-residue transit peptide. Concomitant cell fractionation studies indicate an NADP-ME isoform that associates as a tetramer is localized in the chloroplasts. Unlike terrestrial C₄ species that have two plastidic NADP-ME isoforms, *Hydrilla* and *Egeria* possess only one that is probably multifunctional and presumably performs house-keeping functions in the C₃ leaf but is up-regulated as the photosynthetic decarboxylase in the C₄ leaf.

The possibility that a complex between NADP-ME and rubisco might facilitate the refixation of CO₂ and limit leakage in this system that lacks Kranz anatomy was investigated (Estavillo et al., 2007). It has been hypothesized that it might operate in a similar manner to the CA and rubisco association in cyanobacterial carboxysomes (Reiskind et al., 1997; Kaplan and Reinhold, 1999). However, a BN-PAGE analysis produced no evidence of a complex.

Recombinant proteins were generated for the two major NADP-ME isoforms in C₄ leaves, *hvme1* (chloroplastic) and *hvme3* (cytosolic), using the host *E. coli* BL21(DE3). The photosynthetic isoform rHVME1 showed intermediate kinetic characteristics between isoforms in terrestrial C₃ and C₄ species (Estavillo et al., 2007). The catalytic efficiency of rHVME1 was fourfold higher than the cytosolic rHVME3, but lower than reported for plastidic forms of maize. The $K_m(\text{malate})$ of 0.6 mM for rHVME1 was higher than that of C₄ maize plastid isoforms, but fourfold lower than found with C₃ rice. These data point to trade-offs in effectiveness by using the same NADP-ME isoform for different functions such as house-keeping and photosynthesis. The presence of a single plastidic, and apparently multifunctional, NADP-ME decarboxylase, together with a photosynthetic PEPC that lacks the ubiquitous C₄-signature Ser, are consistent with origins of the *Hydrilla* C₄ system that differ from terrestrial groups.

A comprehensive phylogenetic analysis of 46 NADP-ME sequences from 25 taxa, which included a basal angiosperm, *Amborella trichopoda*, as well as those of *Hydrilla*, indicated that NADP-ME photosynthetic isoforms have different origins in eudicots and monocots (Estavillo et al., 2007).

In eudicots, the C_4 photosynthetic isoform appears to have arisen from a plastidic non-photosynthetic NADP-ME. In the case of *Hydrilla* the sequences formed a clade with all the monocots. The monophyly of this clade suggests that *hvme1* was derived from the cytosolic isoform *hvme3*, but this was independent of the origin of the C_4 isoforms in grasses. The report of an independent origin for the three *Hydrilla* PEPC sequences is also consistent with this pattern (Rao et al., 2002). Thus, the inclusion of the *Hydrilla* sequences shows that monocot chloroplastic isoforms are a polyphyletic group. The *Hydrilla* sequences share the closest identity with a cytosolic NADP-ME found in leaves of *Aloe arborescens*, a CAM plant, which may reflect the close phylogenetic relationship between the Alismatales and Liliales families to which these two plants belong.

We have obtained two partial cDNA sequences encoding two PPDK proteins (Rao et al., 2006a). A Blast search indicated that one, *hvppdk1*, is likely a chloroplastic isoform and cell fractionation studies are consistent with the presence of a chloroplastic PPDK (Magnin et al., 1997). Northern and western analyses confirmed that its transcript and protein expression, although evident in C_3 leaves, was up-regulated in C_4 leaves.

CA activity also increases with C_4 induction (Salvucci and Bowes, 1983b; Rao et al., 2005). When C_4 leaves were incubated with ethoxzylamide, an inhibitor of CA, net photosynthesis was reduced by 40%, while O_2 inhibition and Γ values were increased (Salvucci and Bowes, 1983b). In contrast, C_3 leaves exhibited only slight effects of the inhibitor. These data point to an important role for CA in the C_4 cycle. A partial cDNA sequence for CA (*hvcsl*) has been obtained from the 3'-end that gives a deduced amino acid peptide of 131 residues (V.S. Rao and G. Bowes, 2004, unpublished data). Northern analysis indicates it is only induced in C_4 leaves in the light. Alignments with β -type sequences from various species show it shares 85% identity with a rice CA. However, it is unclear where this CA is located, though we hypothesize it is cytosolic and its up-regulation provides the HCO_3^- substrate to keep pace with the up-regulated PEPC.

Despite the progress that has been made in understanding how the single-cell C_4 system of *Hydrilla* functions there remain some important unresolved issues. The $[CO_2]$ in the chloroplasts

of C_4 *Hydrilla* leaves is estimated to be $\sim 400 \mu M$, which would suppress the O_2 inhibition and oxygenase activity of *Hydrilla* rubisco (Reiskind et al., 1997). However, the source of this chloroplastic CO_2 pool, in terms of which C_4 acid(s) are imported and exported, has not been experimentally demonstrated. We have postulated a role for OAA and/or Asp, rather than malate. This is based in part on the fact that C_3 and C_4 *Hydrilla* leaf chloroplasts have well-developed grana, and therefore differ from the agranal bundle sheath chloroplasts of terrestrial C_4 NADP-ME species. The decarboxylation of malate by NADP-ME, without a way to recycle the NADPH produced, could lead to photoinhibition in a chloroplast that contains grana and PS II activity. In this regard, overexpression of maize NADP-ME in the granal chloroplasts of rice causes substantial photoinhibition due to a high NADPH/NADP⁺ ratio that is probably a by-product of malate import (Takeuchi et al., 2000; Chi et al., 2004). Despite well-developed grana, *Hydrilla* is not particularly susceptible to photoinhibition, even though its canopy in the top 0.3 m of the lake surface can be exposed to near full-sun irradiance (White et al., 1996). This suggests that malate is not the major imported acid, but rather Asp or OAA. Such a scenario would enable MDH to recycle the NADPH produced by NADP-ME and avoid the depletion of NADP⁺ that can restrict Photosystem II activity and result in photoinhibition.

Dicarboxylic acid transporters are associated with the inner membrane of chloroplast envelopes, and orthologs have been reported that encode general dicarboxylate transporters with broad substrate specificities (including OAA), and show close (>70%) homologies within plant species but differing expression patterns (Hatch et al., 1984; Weber and Flügge, 2002; Renne et al., 2003; Taniguchi et al., 2004). It would be of interest to monitor transcript and protein expression for specific transporters in chloroplasts from C_3 and C_4 *Hydrilla* leaves to determine if differences exist. One might expect to see up-regulation in C_4 leaf chloroplasts of transporters capable of importing OAA and/or Asp. In a differential display study some 65 cDNA sequences were up-regulated or unique in the C_4 *Hydrilla* leaf. Of particular interest is the finding of a clone that exhibits 50% identity with the C-terminal segment of the rice pleiotropic drug resistant ATP binding cassette

(ABC) transporter, which has a suggested role in the compartmentation of endogenous metabolites (Rao et al., 2006a). On the export side, PPK activity in the C_4 leaf chloroplasts points to PEP, not pyruvate, transport to the cytosol to complete the C_4 cycle.

A second unresolved issue is what may be termed *Hydrilla*'s conductance conundrum. In terrestrial C_3 plants, a large chloroplast surface area adjacent to intercellular air spaces facilitates CO_2 influx, as it minimizes the path length and diffusion resistance. This chloroplast surface area can be 15–20 times the C_3 leaf area (Evans et al., 1994). In terrestrial C_4 plants, however, CO_2 permeability of the bundle sheath must be restricted to reduce leakage, and the cytosol and chloroplast arrangement, and in some cases a suberized cell wall, appear to be important factors (von Caemmerer et al., 2007). However, the facultative nature of the *Hydrilla* leaf poses a conductance problem. In the C_3 condition the leaf must maximize conductance of CO_2 from the boundary layer directly to rubisco in the chloroplasts. The many small chloroplasts that actively move around the circumference of the *Hydrilla* cells undoubtedly aid conductance by exposing a large surface area close to the entry point of CO_2 . When the leaf changes to C_4 , CO_2 conductance into the cytosol must still be maintained, but leakage from the high CO_2 pool in the chloroplasts must be minimized, and C_4 acid import maximized, if the pumping system is to be effective. The many small chloroplasts, not one large entity as in some algae, would then appear to be a CO_2 leakage liability for the C_4 leaf. No pyrenoid- or carboxysomal-like structures, as occur in some algae and cyanobacteria, have been observed in *Hydrilla* chloroplasts.

The high $[CO_2]$ generated in the C_4 leaf chloroplasts points to an effective system that does not have a high leakage rate (Reiskind et al., 1997). However, field-measurements of apparent quantum yield showed that C_4 plants from the center of the mat had half the efficiency of the C_3 edge plants, which might indicate high CO_2 leakage and recycling rates (Spencer et al., 1994; von Caemmerer, 2003; von Caemmerer et al., 2007). Unfortunately, the conditions under which the measurements were made are unclear, and due to the additional energy demands of the C_4 cycle even terrestrial C_4 species are inherently

less “efficient” than C_3 when photorespiration is minimized at high $[CO_2]$ and low $[O_2]$. This is clearly an area that requires further study.

The location where CO_2 leakage is restricted is intracellular. The C_4 leaf surface cannot be the site restricting CO_2 leakage or concentrating the CO_2 because in both C_3 and C_4 leaves it is acidified in the light to pH ~4 (van Ginkel et al., 2001). Thus, as in terrestrial mesophyll cells where the cell wall is also acidified, dissolved CO_2 diffuses into the cytosol (Fig. 1), and this step could not produce net influx if it were against a cytosolic CO_2 gradient.

We have hypothesized that aquaporins may be involved in the CO_2 conductance changes as the C_3 *Hydrilla* leaf is transformed into a C_4 system (Bowes et al., 2007). Aquaporins are membrane intrinsic proteins of 26–29 kD mass that form water-permeable complexes (Weig et al., 1997). Evidence has accumulated that they also facilitate CO_2 transport and play a role in photosynthesis. In tobacco, for example, the plasma membrane aquaporin NtAQP1 facilitates CO_2 membrane transport, possibly in a unidirectional manner (Uehlein et al., 2003; Flexas et al., 2006). Suppression of NtAQP1 expression reduces CO_2 permeability and the photosynthesis rate. Aquaporins are associated with the outer membrane of chloroplasts, which has a higher CO_2 permeability (P_{CO_2}) than the inner membrane (Raven et al., 2002; Tetlow et al., 2005). If aquaporins are a factor in the CO_2 conductance of *Hydrilla* leaves we postulate that their abundance and expression should be down-regulated in the chloroplast envelope during the shift from C_3 to C_4 photosynthesis, but not in the cell membrane. The facultative *Hydrilla* system is uniquely positioned to investigate a potential role for aquaporins in restricting CO_2 leakage that may apply to C_4 plants in general, and could provide valuable information for attempts to engineer a C_4 -based CCM in a rice plant (Sheehy et al., 2007).

C. Other Submersed Single-Cell C_4 Species

In addition to *Hydrilla*, approximately a dozen aquatic species to date show some evidence of single-cell C_4 photosynthesis, and they are not restricted to vascular plants. The marine diatom *Thalassiosira weissflogii* has been reported to perform C_4 photosynthesis in a single cell

(Reinfelder et al., 2001, 2004). This conclusion is based on the induction of PEPC activity at low $[\text{CO}_2]$ whose inhibition reduces photosynthesis by 90%, the presence of the decarboxylase PEPCK, and the ^{14}C -labeling of C_4 acids in the light with rapid transfer to Calvin cycle components. However, it is not unequivocally established that the C_4 cycle is a CCM, and other labeling studies point to *T. weissflogii* being more like a C_3/C_4 intermediate, with both C_3 and C_4 properties (Roberts et al., 2007). The genome of a related species, *Thalassiosira pseudonana*, also shows that the genetic components for a C_4 system are present (Armbrust et al., 2004). But despite what might be hypothesized from its genome, *T. pseudonana* shows no experimental evidence of functional C_4 photosynthesis (Roberts et al., 2007). Terrestrial genera can also exhibit diverse photosynthetic pathways among different species, the genus *Panicum* being a classic example, so it is not surprising that variation occurs in a diatom genus. Diatoms are a taxonomically diverse assemblage and even more variation in photosynthetic mechanisms may occur within this group.

There is substantial evidence for a C_4 -based CCM in the marine macroalga *Udotea flabellum* including C_4 -like gas exchange characteristics, C_4 cycle enzymes, ^{14}C -labeling of C_4 acids in the light, and inhibitor studies (Reiskind et al. 1988; Reiskind and Bowes 1991). The *Udotea* system is unusual in that it uses PEPCK, rather than PEPC, as the initial carboxylase, and being a siphonaceous alga in the Chlorophyta it lacks Kranz anatomy. Incubation of the thallus with β -mercaptopycolinic acid, which inhibits PEPCK activity, reduces photosynthesis and causes C_3 -like gas exchange and ^{14}C -labeling characteristics (Reiskind and Bowes, 1991). Unlike *Hydrilla* however, the alga shows no signs of being facultative, but appears to be an obligate C_4 . In contrast to *Udotea*, a related siphonaceous marine macroalga, *Codium fragile*, exhibits only C_3 photosynthetic characteristics, even though both species are found in the same marine habitats around the coast of Florida (Reiskind et al., 1988).

Among freshwater monocots, four species in the Hydrocharitaceae family (order: Alismatales) have very similar shoot morphology and leaf anatomy (Fig. 2). They are easily confused as their shoot morphologies are very plastic and depend

on the growth conditions (Bowes and Salvucci, 1989). Of the four, *Hydrilla* is by far the best documented, but there is good evidence that *Egeria* is also a facultative, single-cell C_4 NADP-ME plant. It was originally reported for *Egeria* that C_4 acids, especially malate, were labeled in the light, but a C_4 photosynthetic system was largely discounted because the label did not readily turnover (Brown et al., 1974; Browse et al., 1977, 1979a, b, 1980). However, it appears that the plants were probably in the C_3 (high Γ) state (Brown et al., 1974). We found that under CO_2 -depleted conditions the Γ value of *Egeria* decreases and the PEPC activity increases, becoming twofold higher than rubisco activity (Salvucci and Bowes, 1981). Subsequently, the Andreo group investigated this phenomenon in more detail (Casati et al., 2000; Lara et al., 2001, 2002). Their research shows that *Egeria* responds very similarly to *Hydrilla* in that Γ values decline in low $[\text{CO}_2]$, and PEPC and NADP-ME activities are up-regulated. In western analyses of the leaves two cytosolic PEPC isoforms are observed, one constitutive and the other inducible. Likewise, *Hydrilla* leaves have only two PEPC isoforms, as the third is a root isoform. The inducible PEPC isoform of *Egeria* is phosphorylated in the light and shows a degree of light-activation. It also differs kinetically from the constitutive form and exhibits a very low $K_m(\text{HCO}_3^-)$ of around $8\text{ }\mu\text{M}$ that indicates it has a much greater affinity than reported for terrestrial C_4 species (Lara et al., 2002). Only one NADP-ME isoform is localized in the chloroplast, which presumably has to multitask, as in *Hydrilla*. Kinetically the *Egeria* NADP-ME is similar to terrestrial C_3 forms of the enzyme (Casati et al., 2000), but the comparable *Hydrilla* recombinant isoform, rHVME1, has a much lower $K_m(\text{malate})$ than those reported for C_3 rice (Estavillo et al., 2007).

Among this group of Hydrocharitaceans, *Lagarosiphon* and *Elodea* have also been reported to exhibit substantial ^{14}C -labeling of malate and Asp (28–45%) during the first few seconds in the light, but in these studies they did not appear to play a role in C_4 photosynthesis (Brown et al., 1974; DeGroote and Kennedy, 1977). In the case of *Lagarosiphon* the Γ value was high ($56\text{ }\mu\text{bar}$), so it is not known if it is a facultative C_4 plant that was in the C_3 mode. Further studies with *Elodea* indicate that it is not a C_4 plant, even though its Γ values vary



Fig. 2. Four submersed species in the Hydrocharitaceae family with similar shoot morphology and leaf anatomy, but differing photosynthetic systems. *Hydrilla verticillata* and *Egeria densa* are facultative single-cell C_4 species; *Elodea canadensis* is a facultative HCO_3^- -user and not a C_4 species; the photosynthetic system in *Lagarosiphon major* remains to be identified (Photos of *Hydrilla*, *Lagarosiphon* and *Elodea* by Vic Ramey, University of Florida/IFAS Center for Aquatic and Invasive Plants. Used with permission (Photo of *Egeria* by Bradford G. McLane, Florida Aquatic Nurseries. Used with permission).

with the $[CO_2]$ used for growth (Madsen et al., 1996). PEPC is only a fraction of the rubisco activity, irrespective of Γ , and shows no evidence of induction at low growth $[CO_2]$ (Madsen et al., 1996). Some form of HCO_3^- -use capacity is up-regulated in *Elodea* at low growth $[CO_2]$, possibly at the chloroplast membrane, and this enables it to achieve a reduced Γ , but it is not clear if this constitutes a true CCM (Madsen et al., 1996).

It is intriguing that among this group of closely-related submersed species, with almost identical shoot morphology and anatomy, two different facultative strategies have arisen for dealing with periodic CO_2 depletion in the water: C_4 (*Hydrilla* and *Egeria*) and HCO_3^- -use (*Elodea*) mechanisms. Where *Lagarosiphon* fits in this regard has yet to be determined. The relative cost-benefits

of the two systems are unknown. Likewise, it is unclear as to how the different photosynthetic systems influence the competitiveness of the plants. In New Zealand lakes, *Egeria* and *Lagarosiphon* both displace *Elodea* (Brown et al., 1974). However, *Elodea*, with its facultative HCO_3^- -use system can be a major weed problem, for example, after its introduction into European waterways in the eighteenth century (Holm et al., 1969). In Florida the dioecious biotype of *Hydrilla* flourishes in warm summer waters that *Egeria* does not tolerate (Spencer and Bowes, 1990). Monoecious *Hydrilla*, unlike the dioecious biotype, appears to be more suited to the cooler waters of more northerly states, but whether it also has a facultative C_4 system is unknown. It is also not known if the single-cell C_4 system in submersed plants has any

bearing on the optimum temperature regime for growth, as is generally true for terrestrial C_4 species. The evidence from *Egeria* suggests that C_4 characteristics do not guarantee that a plant can tolerate higher temperature habitats (G. Bowes, unpublished data).

Of further interest in this four species comparison is the fact that, despite different photosynthetic mechanisms, *Hydrilla*, *Egeria*, *Elodea*, and presumably *Lagarosiphon*, all acidify the leaf abaxial boundary layer in the light. Consequently, CO_2 , not HCO_3^- , is the inorganic carbon form that diffuses across the plasma membrane (van Ginkel et al., 2001; Lara et al., 2002).

The family Alismataceae also appears to contain at least one single-cell C_4 species. *Sagittaria subulata* (awl-leaf arrowhead) is a submersed monocot that lacks Kranz anatomy, and was originally thought to be CAM, based on diel fluctuations in titratable acidity and malate (Keeley, 1998a). However, the fluctuations are small when compared with a true submersed CAM species such as *Isoetes howellii*, and are more like those of C_4 *Hydrilla* leaves (Holaday and Bowes, 1980; Keeley and Bowes, 1982; Keeley, 1999). The major proportion of ^{14}C -label initially enters malate and other organic acids, but this is in the light rather than in the dark, which is consistent with a C_4 -like system (Bowes et al., 2002; A.M. Farmer and G. Bowes, 1985, unpublished data). The PEPC: rubisco ratio is approximately two, and PEPC activity is equivalent to that in *Hydrilla* C_4 leaves. Its decarboxylase activity is suggestive of a single-cell C_4 NADP-ME species, but it is not known if it is a facultative system. Other *Sagittaria* species also exhibit small diel fluctuations in titratable acidity (Keeley, 1998a), but it is premature to assign them to either the single-cell C_4 or CAM categories. Furthermore, not all *Sagittaria* species are C_4 or CAM. For example, the rubisco activity in *S. latifolia* leaves is over tenfold greater than that of PEPC, and diel malate fluctuations are negligible (Bowes et al., 2002). There are insufficient data to draw firm conclusions about the range of photosynthetic mechanisms within this genus, but the data that exist indicate it is worthy of further investigation.

Several amphibious grasses (Poaceae) show C_4 features, including *Neostapfia colusana*, *Tuctoria greenei*, and two *Orcuttia* species (Keeley, 1998b). The first two have Kranz anatomy in both the

aerial and submersed leaves and thus are not single-cell C_4 species. However, *Orcuttia californica* and *O. viscida* have Kranz anatomy in the aerial leaves, but the submersed leaves lack this anatomy and still show evidence of C_4 photosynthesis. In particular, a pulse-chase study shows that 95% of the initial ^{14}C -labeling occurs in C_4 acids (malate and Asp) in the light and this is followed by rapid (within 15 s) transfer of the label to phosphorylated sugars (Keeley, 1998b). PEPC activity is relatively high, though in most cases it is lower than that of rubisco. All the leaves have considerable PPDK activity and probably use NADP-ME as the decarboxylase. The submersed leaves seem to have a single-cell C_4 system like that of *Hydrilla*, but without more gas-exchange data it is unclear how effectively the CCM operates, or if they are more akin to the C_3/C_4 intermediate amphibious sedges. It is suggested that the submersed *Orcuttia* leaves have lost Kranz anatomy, rather than never developed it, and the loss may represent better adaptation to the aquatic environment (Keeley, 1998b). Because of the paucity of data it is not possible to ascertain whether Kranz anatomy provides a submersed leaf with additional benefits over a single-cell system for concentrating CO_2 or reducing leakage.

Nearly 20% of all known C_4 species are sedges (Cyperaceae), but although many sedge species grow in wetland areas they utilize aerial leaves and culms for photosynthesis (Sage, 2001). Most C_4 sedge species possess Kranz anatomy and fall into the C_4 NADP-ME category, so in regard to photosynthesis they are neither "aquatic" nor single-cell. However, species in the genus *Eleocharis* exhibit some unusual features (Bruhl et al., 1987). Several, including *E. vivipara* and *E. baldwinii*, are facultative in that they are capable of both C_3 and C_4 -like photosynthesis. The facultative mechanism, however, differs from that of *Hydrilla* and *Egeria* in that the plants are amphibious, so C_3 and C_4 -like photosynthesis occur in different leaf types. The submersed leaf has C_3 characteristics but the emergent leaves develop Kranz anatomy and C_4 biochemistry with NAD-ME as the decarboxylase in the mitochondria of the bundle sheath cells (Ueno et al., 1988; Uchino et al., 1995). The aerial leaves of these two species are probably more similar to C_3/C_4 intermediate species than true C_4 , as enzyme localization studies show that rubisco is present in both the mesophyll and

bundle sheath chloroplasts, which suggests segregation of the two carboxylases is incomplete (Brown and Bouton, 1993; Uchino et al., 1995; Ueno, 1996). The C_4 -like nature of the aerial leaves may be triggered by water stress, and not low $[CO_2]$ (Ueno, 1998).

Eleocharis acicularis diverges from the usual sedge pattern of C_4 photosynthesis being restricted to the aerial leaves and culms. It too is amphibious and facultative, but it lacks Kranz anatomy in both the aerial and submersed leaves. The aerial leaves appear to be C_3 , but the submersed leaves show evidence of a C_4 or C_3/C_4 system. It grows submersed in seasonal pools in California where daytime $[CO_2]$ is often low, but it apparently cannot use HCO_3^- for photosynthesis. In the light some 27–55% of the initial label enters C_4 acids in the submersed leaves (Morton and Keeley, 1990; Keeley, 1999). Diel acidity and malic acid variations are small, so it is not a CAM plant. The rubisco:PEPC activity ratio of the submersed leaves is 2.8, which is lower than that of typical C_3 species, but in the emergent leaf the ratio increases to a C_3 -like value of 18.8 (Keeley, 1999). NADP-ME is the only detectable decarboxylase and its activity is greatest in the submersed leaf. Thus *E. acicularis* differs from the amphibious *E. vivipara* and *E. baldwinii*, with respect to the type and location of the decarboxylase. PPK activity is only observed in the submersed leaves. Gas exchange and biochemical data are too sparse to determine conclusively if the submersed leaves have a single-cell C_4 system, but in a pool that becomes CO_2 -depleted during the day, a system that could minimize the CO_2 loss from photorespiration would be advantageous (Keeley, 1999).

Among submersed angiosperms all of the single-cell, or possible single-cell, C_4 systems so far described use NADP-ME as the decarboxylase. Intuitively, the release of CO_2 in the chloroplast, adjacent to rubisco, should be the most effective concentrating method. A mitochondrial NAD-ME, or a cytosolic PEPCK, would potentially incur futile cycling of CO_2 as it passes through the cytosol in order to reach the chloroplast. In fact, it is likely that a decarboxylase in the mitochondria or cytosol could not concentrate CO_2 in a single-cell system, especially if the chloroplasts are small and motile. This is consistent with the observation that species with submersed leaves that use NAD-ME as the C_4 decarboxylase have

Kranz anatomy. Similarly, in the single-cell C_4 macroalga *Udotea* and the diatom *T. weissflogii* the decarboxylating PEPCK should be located in the chloroplast, not in the cytosol (Reiskind and Bowes, 1991; Roberts et al., 2007).

D. Which Originated First: Aquatic or Terrestrial C_4 Photosynthesis?

It has long been recognized that photosynthesis in dense aquatic vegetation, whether composed of cyanobacteria, phytoplankton, seaweeds, seagrasses, or freshwater plants, can drive the pH of the water as high as 10, deplete the free CO_2 and even HCO_3^- to near zero, and produce an $[O_2]$ that is double the air-equilibrium value (Brown et al., 1974; Van et al., 1976; Bowes and Salvucci, 1989). Even under high atmospheric $[CO_2]$, such conditions likely occurred in the water on the early earth, as water bodies are rarely in equilibrium with air. The presence of CCMs in cyanobacteria, which circumvent the negative effects of these conditions on rubisco and photosynthesis, is consistent with this conclusion. Consequently, it is not surprising to find that a diatom and a green macroalga utilize a photosynthetic C_4 acid cycle to achieve the same goal as the cyanobacterial HCO_3^- concentrating system.

In regard to more modern species, the order that contains *Hydrilla* and *Egeria*, Alismatales, has an early Cretaceous lineage of ~100 mya, and the Hydrocharitaceae family appears more ancient than the C_4 grasses that became abundant in the Miocene (Sculthorpe, 1967; Bremer, 2000). Fossil evidence for the *Hydrilla* genus has been reported from the upper Eocene of about 40 mya (Mai and Walther, 1985; Kvaček, 1995). A phylogenetic analysis of PEPC sequences shows that the *Hydrilla* isoforms are in the “ C_3 ” group, with a gymnosperm as a sister clade, which supports a putative ancient origin (Rao et al., 2002). Additional phylogenetic analyses of NADP-ME sequences indicate that *Hydrilla* isoforms differ from those of other monocots because the ancestral species seems to be non-graminaceous and close to the basal angiosperm *Amborella* (Estavillo et al., 2007). The kinetic features of the *Hydrilla* and *Egeria* C_4 isoforms, which tend to fall between C_3 and C_4 values, are also consistent with a relative early advent for their C_4 system (Lara et al., 2002). Thus single-cell C_4 systems

appear to be ancient forms of C_4 photosynthesis that likely predate terrestrial C_4 angiosperms and originated in waters subject to localized CO_2 depletion.

III. HCO_3^- -Use Mimics C_4 Photosynthetic Gas Exchange Characteristics

In addition to *Hydrilla* and *Egeria* other submersed freshwater plants have Γ values that vary in response to the incubation conditions. These include *Cabomba caroliniana*, *Ceratophyllum demersum*, *Myriophyllum brasiliense*, *M. heterophyllum*, *M. spicatum*, *Proserpinaca palustris*, *Ranunculus peltatus*, *Elodea canadensis*, *E. nuttalli*, *Callitriche cophocarpa*, the moss *Fissidens mantleensis*, and the filamentous alga *Nitella* sp. (Salvucci and Bowes, 1981; Eighmy et al., 1991; Madsen et al., 1996). However, they do not appear to possess C_4 photosynthesis. It has been estimated that some 50% of submersed angiosperms have the capacity to use HCO_3^- actively in photosynthesis (Madsen and Sand-Jensen, 1991). This facility is facultative and when up-regulated it probably can function in some species as a CCM (Eighmy et al., 1991; Maberly and Madsen, 2002). Thus, when C_4 photosynthesis is absent, the change from high to lower Γ values may be due to acclimation in some form(s) of HCO_3^- use at low $[CO_2]$. In this regard the dissolved $[CO_2]$ appears to be the effective agent resulting in up-regulation of HCO_3^- -use, more so than $[HCO_3^-]$ in the water (Madsen et al., 1996). However, up-regulation of HCO_3^- use is not the complete answer, as some species, including mosses, appear only able to use CO_2 .

IV. Concluding Thoughts

Even when the amphibious plants and algae are included, only a dozen or so submersed species have been reported to perform some form of C_4 photosynthesis. Given the relatively common occurrence of this pathway among monocots, it raises the question as to why there is such low representation among aquatic species. Several explanations can be postulated.

The notion that the aquatic environment is relatively "benign" for plants and lacks the selection pressure that resulted in terrestrial C_4 CCMs is invalid. Aridity may not be a primary factor (Sage, 2004), but it is not uncommon to find freshwater systems and marine rock pools that are very unfavorable for C_3 photosynthesis, with high day-time $[O_2]$, temperature, pH, and irradiance, and minimal inorganic carbon (Van et al., 1976; Bowes and Salvucci, 1989). The situation is exacerbated by slow dissolution and diffusion of CO_2 in water, which has a diffusion resistance 10^4 times greater than air. These conditions rival any terrestrial extremes, and stress submersed photoautotrophs. It is not unreasonable to conclude that similar environments existed in the past, even when atmospheric CO_2 was high, because water and air are rarely in equilibrium in regard to CO_2 and O_2 . A plant or alga with the ability to minimize photorespiration, or offset CO_2 loss by taking it up at night in a CAM-like mode, would have an advantage in environments where CO_2 availability during the day can be severely limiting.

A more likely scenario to explain the low number of submersed C_4 species is that access to HCO_3^- , which in most fresh and marine waters is many-fold higher in concentration than dissolved CO_2 , obviates the need for a C_4 -based CCM (Bowes and Salvucci, 1989; Madsen and Sand-Jensen, 1991). HCO_3^- -use requires the organism to have a mechanism that can utilize this ion in photosynthesis, either by direct uptake through active transport or by its conversion in the boundary layer to CO_2 in an ATP-dependent acidification process. Not all submersed species can use HCO_3^- ; the submersed eudicot *Callitriche cophocarpa* uses neither a C_4 cycle nor HCO_3^- for photosynthesis and consequently can only survive in freshwaters with a high $[CO_2]$ (Madsen et al., 1996). The presence of HCO_3^- -based CCMs in cyanobacteria suggests that this method has a longer lineage than C_4 or CAM systems. It is interesting to note that a C_4 cycle and HCO_3^- -use are not necessarily an either/or proposition. Even though *Hydrilla* and *Egeria* have a facultative C_4 cycle the C_3 leaves acidify the abaxial leaf surface in a light- and ATP-dependent manner to improve access to inorganic carbon (van Ginkel et al., 2001; Lara et al., 2002). This CO_2 flux mechanism (CFM) is up-regulated as the leaves transition to a C_4 cycle and it facilitates CO_2

access at the cell membrane, which in turn supports the chloroplast CCM.

Although submersed C₄ species seem to be relatively uncommon, the same comment can be applied to researchers working on the physiology, biochemistry and molecular biology of photosynthesis in submersed angiosperms. As a consequence, lack of research may be a factor in the failure to identify more submersed single-cell C₄ systems, and possibly other unusual photosynthetic mechanisms, in the aquatic environment.

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Photorespiration: The Bridge to C₄ Photosynthesis

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Summary	81
I. Introduction.....	82
II. Biochemistry and Genetics of the C ₂ Cycle.....	84
A. Chloroplasts Produce 2-Phosphoglycolate by Oxygenation of RubP.....	85
B. 2-Phosphoglycolate Becomes Dephosphorylated to Glycolate	85
C. Glycolate Becomes Oxidized to Glyoxylate and H ₂ O ₂ in the Peroxisomes.....	86
D. At Least Two Peroxisomal Transaminases Convert Glyoxylate to Gly.....	87
E. Mitochondrial Reactions of Gly Yield Ser, CO ₂ , NH ₃ , and NADH	88
1. Gly decarboxylase	88
2. Ser hydroxymethyltransferase	91
F. Hydroxypyruvate Is Produced from Ser and Becomes Reduced to Glycerate.....	91
G. Glycerate Becomes Phosphorylated and 3PGA Re-enters the Calvin Cycle	92
H. Transcriptional Regulation of Photorespiratory C ₂ Cycle Genes	93
III. Related Reactions and Interactions with Other Metabolic Pathways	93
A. Reassimilation of Photorespiratory NH ₃	93
B. Regulatory Interaction with Respiration	94
C. One-Carbon Metabolism	94
D. Alternative Sources and Destinies of C ₂ Cycle Metabolites	95
IV. Measurement of Photorespiration	95
A. Post-illumination Burst of CO ₂ (PIB).....	95
B. Measurement of ¹⁴ CO ₂ Evolution.....	96
C. Extrapolation from CO ₂ Response (A/c _i) Curves.....	96
D. Estimation from Rubisco Kinetics and Gas Exchange Measurements	96
V. The Role of Photorespiration for the Evolution of C ₄ Photosynthesis.....	97
VI. Future Prospects	101
Acknowledgments	103
References	103

Summary

Photorespiration is one of the major highways of carbon metabolism in C₃ plants and hence in the biogeosphere. By mass flow, excelled only by photosynthesis, it actually constitutes the second-most important process in the land-based biosphere. The underlying biochemical pathway, the photorespiratory carbon oxidation or C₂ cycle, compensates for the oxygenation of ribulose 1,5-bisphosphate by serving as a carbon-recovery system reconverting 2-phosphoglycolate to 3-phosphoglycerate. While this ancient ancillary metabolic process enables C₃ plants to thrive in an oxygen-containing environment, it also

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sacrifices a significant part of the freshly assimilated carbon to the atmosphere. Biochemically, this sacrifice is made by the decarboxylation of the C_2 cycle intermediate Gly.

C_3 plants lose much photorespiratory CO_2 to the atmosphere. In contrast, photorespiration is very low in C_4 plants. C_3 - C_4 intermediate plants, the phylogenetic predecessors of C_4 plants, use Gly as a vehicle to transport freshly assimilated carbon from the mesophyll to the bundle sheath where it is released as photorespiratory CO_2 . Possibly, this extra CO_2 supply was a pacemaker for the subsequent substantial accumulation of chloroplasts in the bundle sheath cells of C_3 - C_4 plants. Eventually, this photorespiration-driven CO_2 pump was first superimposed and then replaced by the C_4 cycle, another auxiliary pathway to the Calvin cycle, which creates even more favorable photosynthetic conditions within the bundle sheath. It thus appears as if photorespiration triggered C_4 plant evolution not only indirectly by exerting selective pressure in favor of low-photorespiration carbon assimilation, but primarily by providing the first strategy on how to improve the intercellular CO_2 distribution in leaves.

This chapter will review molecular aspects of photorespiration and introduce some measurement techniques. It will then briefly describe current knowledge about C_3 - C_4 photosynthesis and discuss the workings of a photorespiration-driven CO_2 concentration mechanism as one of the first steps in the evolution of C_4 photosynthesis.

I. Introduction

Photorespiration is the light- and O_2 -dependent release of CO_2 by plants, but the term also denotes the underlying biochemical processes in plants and microorganisms. It occurs concurrently with photosynthetic CO_2 uptake. Photorespiration is therefore normally concealed and becomes apparent only under specific experimental conditions. In fact, one of the first indications of photorespiratory processes came with Decker's observation (1955) in the mid-1950s that respiratory rates of leaves immediately after the end of illumination were substantially higher than those after a cou-

ple of minutes in darkness. This phenomenon was later called the post-illumination CO_2 outburst (PIB).

Photorespiratory CO_2 evolution is not restricted to these artificial conditions. For instance, when a leaf is placed in a closed measuring chamber and illuminated, the CO_2 concentration will decrease until the rate of photosynthetic CO_2 uptake equals the combined rates of photorespiratory and respiratory CO_2 release. This final steady-state equilibrium concentration of CO_2 , the CO_2 compensation point (Γ), is strongly determined by the photosynthetic type of the respective plant. In C_3 plants, Γ is approximately $40\text{--}60\ \mu\text{l l}^{-1}$ whereas C_4 plants usually show values below $10\ \mu\text{l l}^{-1}$. Historically, many C_4 plants have been identified in Γ -based screening programs.

Photorespiratory CO_2 release and photosynthetic CO_2 assimilation are tightly linked on the biochemical level. This is because they are both initiated by the dual activities of Rubisco. The relative rates of these two reactions, carboxylation versus oxygenation of RubP, are solely determined by intrinsic catalytic properties of Rubisco and by the CO_2/O_2 concentration ratio. Carboxylation of RubP yields two molecules of glycerate 3-phosphate (3PGA), which become reduced to triosephosphate in subsequent reactions of the Calvin cycle. In contrast, oxygenation of RubP leads to the synthesis of equimolar amounts of 3PGA and glycolate 2-phosphate

Abbreviations: BSC – Bundle sheath cells; CAT – Catalase; CCM – CO_2 -concentrating mechanism; GGT – Glu:glyoxylate aminotransferase; GS – Gln synthetase; GOGAT – Gln:2-oxoglutarate amidotransferase (Glu synthase); GDC – Gly decarboxylase; GLYK – Glycerate 3-kinase; 3PGA – Glycerate 3-phosphate; GOX – Glycolate oxidase; 2PG – Glycolate 2-phosphate; PGLP – Glycolate 2-phosphate phosphatase; 3HP – Hydroxypyruvate; IRGA – Infrared gas analyzer; MC – Mesophyll cell; HPR1 – NADH-dependent hydroxypyruvate reductase; HPR2 – NADPH-dependent hydroxypyruvate reductase; 2OG – 2-Oxoglutarate; PIB – Post-illumination CO_2 burst; RubP – Ribulose 1,5-bisphosphate; Rubisco – RubP carboxylase/oxygenase; SHMT – Ser hydroxymethyltransferase; SGT (AGT) – Ser:glyoxylate aminotransferase; S – Specificity factor of Rubisco; THF – Tetrahydrofolate

(2PG). 2PG cannot enter the Calvin cycle but is converted to 3PGA in a series of enzymatic reactions that collectively comprise the C₂ oxidative photosynthetic carbon cycle, or shorter, the C₂ cycle (historical overview in Lorimer, 1981). Decarboxylation of Gly, an intermediate compound of the C₂ cycle, by the mitochondrial enzyme Gly decarboxylase (GDC) results in the release of photorespiratory CO₂ at rates which are much higher than those of other respiratory processes.

In rough accordance with earlier estimates, recent *in vivo* ¹³C labeling experiments indicate that photorespiration is about 17% of photosynthesis for a well-watered and fertilized plant under temperate conditions (Cegelski and Schaefer, 2006). This fraction can be significantly higher in warmer and drier environments. Photorespiration hence represents a major metabolic process in C₃ plants, which can occur with rates comparable to those of photosynthetic CO₂ fixation. Due to the unproductive re-release of 20–50% of the freshly assimilated CO₂, photorespiration considerably slows down the rates of net CO₂ fixation in C₃ plants. These negative effects triggered the search for mutations or inhibitors that specifically block photorespiration, but leave photosynthesis unimpaired. Subsequent to some early enthusiastic reports, however, it was realized that such attempts do not increase photosynthetic efficiency, but rather exacerbate the problems of photorespiration. This conclusion received strong support from the analysis of *Arabidopsis* mutants with defects in C₂ cycle enzymes (historical perspective in Somerville, 2001).

What is the biological function of photorespiratory processes? The answer, in essence, is quite simple: the synthesis of 2PG by RubP oxygenase as the primary reaction of the C₂ cycle appears to have no ‘function’ at all! It is an inevitable consequence of the chemistry of the RubP carboxylase reaction and reflects the evolutionary origin of plant photosynthesis in the anaerobic environment of the Precambrian ocean (Lorimer and Andrews, 1973). The C₂ cycle, as an important ancient auxiliary metabolic process, compensates for the oxygenation of RubP by serving as a carbon-recovery system reconverting 2PG carbon to 3PGA. During this process, a significant part of the freshly assimilated carbon is sacrificed to survive in an

otherwise toxic environment (Osmond, 1981). It is the C₂ cycle, at least for plants, which makes autotrophic CO₂ fixation possible in an environment containing high oxygen concentrations. This is the central and primary function of the C₂ cycle.

In addition to this central and indispensable function of the C₂ cycle, however, there exist several secondary adaptation benefits for the life of plants under certain environmental conditions. The most prominent example is the possible contribution of the C₂ cycle to the protection of the photosynthetic apparatus from photoinhibition (pros and cons discussed in Osmond et al., 1997). Overexpression of the C₂ cycle enzyme hydroxypyruvate reductase led to an improved tolerance of tobacco to warm temperatures (Oliver et al., 1995). It is also related to thermotolerance that photorespiratory metabolism, in a not understood manner, affects the biosynthesis of monoterpenes (Penuelas and Llusia, 2003). More recently, linkages have been revealed between photorespiration and oxidative stress-related repair processes within photosystem II (Takahashi et al., 2007), and light-stress protection of cyanobacteria (Hackenberg et al., 2009).

How, then, can photorespiration be ‘a bridge to C₄ photosynthesis’? It is a widely accepted notion that the negative effects of photorespiratory CO₂ losses on plant growth exerted one of the selective pressures that led to the evolution of C₄ traits. However, there exists a second relation between C₄ photosynthesis and photorespiration. This relation becomes apparent from specific features of C₃-C₄ intermediate plants, which use photorespiratory Gly as a vehicle for shuffling CO₂ from the mesophyll to the bundle sheath (Rawsthorne, 1992). Molecular phylogenetic studies place this photosynthetic type between C₃ and C₄ in the evolution of C₄ photosynthesis (Kopriva et al., 1996b; McKown et al., 2005). It thus appears that the evolution of the C₄ syndrome was triggered by and possibly even required the preceding presence of a much simpler CO₂ transport system, which was based on relatively small alterations to photorespiratory Gly metabolism. In fact, fully developed C₄ plants did not lose but in contrast retained a fully functional C₂ cycle.

This chapter will give a survey on the biochemistry and genetics of the photorespiratory C₂

cycle and a short introduction into the measurement of photorespiration. Then, specific features of photorespiratory metabolism in C_3 - C_4 intermediate plants will be discussed in their relation to the evolution of C_4 photosynthesis.

II. Biochemistry and Genetics of the C_2 Cycle

The biochemistry and cellular biology of the C_2 cycle of higher plants is very complex. It involves a core of nine individual enzymatic reactions and at least three auxiliary enzymes distributed over three different organelles and the cytosol (Fig. 1). Rubisco produces 2PG by oxygenation of RubP in the chloroplast, several C_2 cycle enzymes are localized within the peroxisomes

and the actual release of photorespiratory CO_2 from Gly occurs in the mitochondria. Gly decarboxylation also produces NH_3 , which becomes reassimilated in the chloroplasts. The flow of reactions implies a fixed stoichiometry of RubP oxygenation, Gly decarboxylation, and photorespiratory 3PGA synthesis. It should be noted, however, that *in vivo* ^{13}C and ^{15}N labeling experiments recently substantiated earlier suggestions that this stoichiometry may not be that strictly fixed (Hanson and Peterson, 1985; Cegelski and Schaefer, 2006).

Knowledge about the transmembrane transport of the core intermediates of the highly compartmentalized C_2 cycle is very much incomplete (Reumann and Weber, 2006; Weber and Fischer, 2007). Transport of glycolate, hydroxypyruvate (3HP), and several other compounds related to

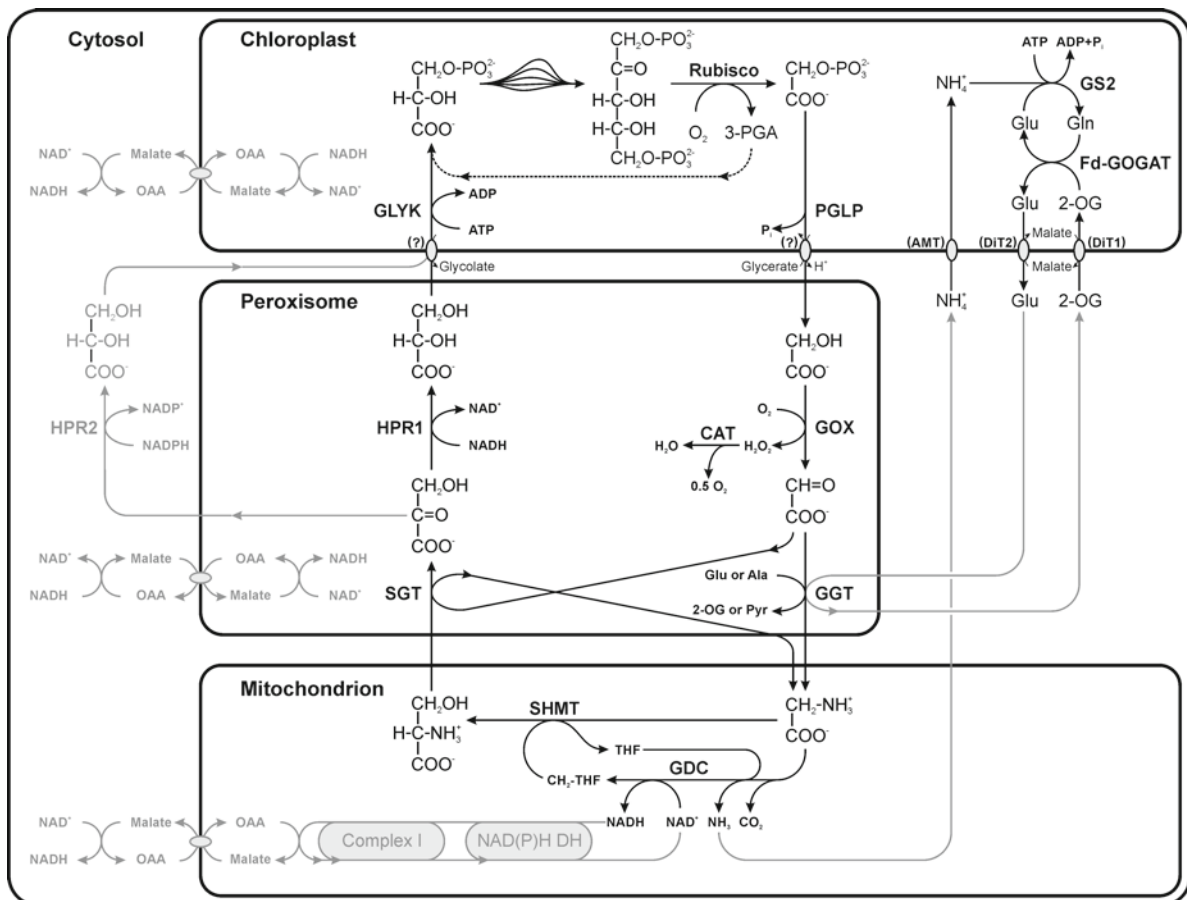


Fig. 1. Flow-chart of reactions constituting the core of the C_2 cycle and several auxiliary reactions including those of the photorespiratory nitrogen cycle.

photorespiratory metabolism across the peroxisomal membrane occurs via porin-like channels (Reumann, 2000). A chloroplastic glycolate/glycerate transporter has been described (Howitz and McCarty, 1991), but was not yet identified on the molecular level. Even less is known about the passage of Gly and Ser through the inner mitochondrial membrane. While the presence of Gly/Ser antiporters in the inner mitochondrial membrane was suggested already more than 20 years ago (Walker et al., 1982) and significant progress exists in the identification of human Gly transporters (e.g. Betz et al., 2006), the respective transporters in plants are not yet known.

A. Chloroplasts Produce 2-Phosphoglycolate by Oxygenation of RubP

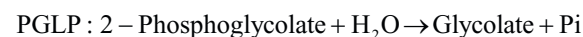
Rubisco initiates the C₂ cycle by the synthesis of 2PG (Bowes et al., 1971; historical perspective in Portis and Parry, 2007). The multimeric enzyme is present in the chloroplast stroma in the very high concentrations of 200–250 mg ml⁻¹ and constitutes about 30–50% of the soluble protein in leaves of C₃ plants. With an estimated amount per capita of about 10 kg, the enzyme is the most abundant protein on Earth. In plants as in all chlorophyll a-containing organisms, Rubisco comprises two types of polypeptide subunits, a plastome-encoded ~55 kDa large subunit carrying the catalytic site and a nuclear-encoded ~15 kDa small subunit. The subunits are organized as hexadecamers (L₈S₈, so-called form I Rubisco) to form a 560 kDa holoenzyme, but other forms exist in prokaryotes and are thought to have evolved from a primordial archaeal Rubisco-like protein (Tabita et al., 2008; Saito et al., 2009). These ancestor enzymes have no Rubisco activity but are enolases involved in methionine salvage pathways (Ashida et al., 2005).

Very crucial for Rubisco catalysis is the preceding activation via carbamylation of an active site lysyl residue. Subsequent stabilization of the lysyl carbamate by binding of Mg²⁺ then provides the cofactor necessary for both carboxylation and oxygenation of RubP (Cleland et al., 1998). Before this activation can occur, bound RubP or other sugar phosphates need to be removed from the non-carbamylated site by Rubisco activase (Portis et al., 2008).

The relative rates of carboxylation and oxygenation are determined by the kinetic properties of Rubisco and by the CO₂/O₂ concentration ratio. To conveniently describe this situation, the $V_{\max}$ and K_m values are often combined in the so-called specificity factor $S (V_c \cdot K_o / V_o \cdot K_c)$, where V and K represent $V_{\max}$ and K_m of the respective reactions of Rubisco. This parameter, in addition to the absolute k_{cat} values for the carboxylation and oxygenation reaction, respectively, is an important diagnostic factor for the overall efficiency of the enzyme and shows significant natural variability. Computational simulations suggest that substantial increases in crop carbon gain could result if Rubiscos with higher specificity factors were expressed in C₃ plants. Consequently, there is much interest in identifying ‘better’ natural or mutant Rubisco variants and integrating them into plants. Such experiments have not yet been very successful and some skepticism is also expressed (Tcherkez et al., 2006). Latest strategies attempt to produce such hypermorphs by directed evolution and genetic selection in engineered *E. coli* (Mueller-Cajar and Whitney, 2008).

B. 2-Phosphoglycolate Becomes Dephosphorylated to Glycolate

2PG is hydrolyzed by a chloroplastic phosphatase (PGLP) with high specificity for 2PG. The enzymatic properties of the enzyme from different eukaryotic organisms are very similar suggesting a common evolutionary origin. During catalysis, PGLP becomes phosphorylated. It requires Cl⁻ and Mg²⁺ ions for full activity and is reversibly inhibited by Ca²⁺ ions.



Two orthologs are present in the genomes of both Arabidopsis and rice, the photorespiratory PGLP1 (all relevant C₂ cycle enzymes are listed in Table 1) and the cytosolic PGLP2. PGLP1-deficient mutants cannot grow under ambient conditions and are viable only at 0.5–1% CO₂ (Schwarte and Bauwe, 2007). Similar to all other C₂ cycle enzymes, PGLP activity and *PGLP1* transcript levels increase rapidly during illumination of etiolated seedlings. PGLP2 does not contribute to photorespiratory metabolism but could be involved in the degradation of minor

Table 1. Enzymes of the photorespiratory C₂ cycle and their encoding genes in Arabidopsis. Related genes with unidentified functions or functions outside of the core C₂ cycle are not listed. Where the knockout results in a low CO₂-sensitive phenotype, which indicates a major function in photorespiration, the respective gene is printed in bold. References: PGLP (Schwarte and Bauwe, 2007); GOX nomenclature (Reumann et al., 2004); SGT (Liepman and Olsen, 2001); GGT (Igarashi et al., 2003); GLDP (Engel et al., 2007); LPD (Lutziger and Oliver, 2000); SHMT (Voll et al., 2006); HPR (Mano et al., 1997); GLYK (Boldt et al., 2005).

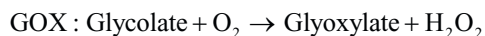
	Arabidopsis	TAIR Code	Localization
2PG phosphatase	AtPGLP1	At5g36790	Chloroplasts
Glycolate oxidase	AtGOX1	At3g14420	Peroxisomes
	AtGOX2	At3g14415	Peroxisomes
	AtGOX3	At4g18360	Peroxisomes
	AtHAOX1	At3g14130	Peroxisomes
	AtHAOX2	At3g14150	Peroxisomes
Ser:glyoxylate aminotransferase	AtAGT1	At2g13360	Peroxisomes
Glu:glyoxylate aminotransferase	AtGGT1	At1g23310	Peroxisomes
	AtGGT2	At1g70580	Peroxisomes
Gly decarboxylase P-protein	AtGLDP1	At4g33010	Mitochondria
	AtGLDP2	At2g26080	Mitochondria
Gly decarboxylase H-protein	AtGLDH1	At2g35370	Mitochondria
	AtGLDH2	At2g35120	Mitochondria
	AtGLDH3	At1g32470	Mitochondria
Gly decarboxylase T-protein	AtGLDT1	At1g11860	Mitochondria
Gly decarboxylase L-protein	AtmLPD1	At3g17240	Mitochondria
	AtmLPD2	At1g48030	Mitochondria
Ser hydroxymethyltransferase	AtSHM1	At4g37930	Mitochondria
	AtSHM2	At5g26780	Mitochondria
Hydroxypyruvate reductases	AtHPR1	At1g68010	Peroxisomes
	AtHPR2	At1g79870	Cytosol
Glycerate kinase	AtGLYK	At1g80380	Chloroplasts

amounts of 2PG as they are produced in all cells during DNA repair processes.

C. Glycolate Becomes Oxidized to Glyoxylate and H₂O₂ in the Peroxisomes

Peroxisomes are closely associated with both chloroplasts and mitochondria which appears to be related to the cooperation of these three organelle types in photorespiration. This physical association with other organelles reflects that peroxisomes, the ‘organelles at the crossroad’, metabolically link different cell compartments (Igamberdiev and Lea, 2002).

Glycolate oxidase (GOX) is the first peroxisomal enzyme of the C₂ cycle. The flavinmononucleotide (FMN)-dependent enzyme transfers electrons from glycolate to molecular O₂ to produce glyoxylate and H₂O₂. In the first part of this two-step reaction, glycolate is oxidized by the flavin and in the second part the reduced FMN becomes re-oxidized by oxygen to produce H₂O₂ in an irreversible reaction.



GOX is usually isolated as a tetra- or octamer composed of identical subunits of ~43 kDa and

its primary structure had initially been determined by peptide sequencing (Cederlund et al., 1988). The enzyme was crystallized many years ago and represents one of the few peroxisomal proteins for which high resolution crystal structures were obtained.

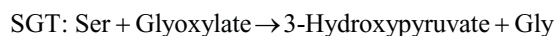
In an evolutionary context, it is interesting to note that GOX only occurs in the charophycean algae (which contain peroxisomes), the most likely predecessors of land plants, and the other members of the Streptophyta. The sister group of chlorophycean algae uses mitochondrial or plastidial glycolate dehydrogenases instead (e.g. Stabenau and Winkler, 2005). In Arabidopsis, GOX is encoded by five redundant genes, which explains the lack of mutants with a high-CO₂-requiring phenotype. Notably, four of these genes are arranged as two pairs in close vicinity on chromosome 3. The two GOX genes which are most likely involved in photorespiration, *AtGOX1* and *AtGOX2*, form a tandem, while *AtHAOX1* and *AtHAOX2* are separated by just one gene. The distance between the two gene pairs is as low as ~130 kb indicating one ancestral locus for all four genes. The localization of *AtGOX3* on another chromosome might be due to a segmental duplication similar to the events that led to the formation of the majority of Arabidopsis genes. Very similar to Arabidopsis, five probably functional *GOX* genes exist in the rice genome, but none of these genes is located in close vicinity to its homologs. In contrast, only one GOX gene is present in the C₄ plant maize. Knockout of this gene resulted in a high-CO₂ requiring phenotype, which demonstrates the need of an intact photorespiratory metabolism not only in C₃ but also in C₄ plants (Zelitch et al., 2009). In tobacco, RNAi-induced GOX deficiency resulted in a distinctly higher susceptibility to photoinhibition (Yamaguchi and Nishimura, 2000).

The large amount of H₂O₂ produced during photorespiration is degraded by the peroxisomal enzyme catalase (CAT). This reaction, although not covered in this chapter, is a very important side reaction of the photorespiratory C₂ cycle, and CAT-deficient mutants show severe leaf bleaching under normal environmental conditions (Blackwell et al., 1988; Queval et al., 2007). Photorespiratory H₂O₂ production and CAT activity gain further importance because H₂O₂ is a key player in several cellular processes including systemic

acquired acclimation to excess light and other stress and programmed cell death responses.

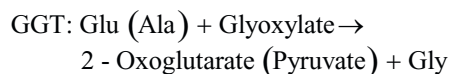
D. At Least Two Peroxisomal Transaminases Convert Glyoxylate to Gly

Rates of non-enzymatic decarboxylation of glyoxylate are normally very low, and glyoxylate becomes more or less completely converted to Gly by at least two aminotransferases. In addition to their function in the C₂ cycle, these enzymes also regulate leaf amino acid content (Igarashi et al., 2006). Because of their multiple substrate specificities, there is still some confusion with respect to the identity and naming of these enzymes.



The homo-dimeric Ser:glyoxylate aminotransferase (SGT) uses Ser as the amino donor and is encoded by single genes in Arabidopsis (*AGT1*, Liepman and Olsen, 2001) and in rice. The enzyme catalyzes transamination reactions in the combinations Ala:glyoxylate, Ser:glyoxylate, and Ser:pyruvate. Kinetic data suggest that the quasi-irreversible Ser:glyoxylate transamination is the preferred in vivo reaction (Nakamura and Tolbert, 1983).

Mutation of *AGT1* in the conditionally lethal *Arabidopsis sat* mutant leads to a loss of SGT activity, but AGT and GGT activities were maintained at nearly wild-type levels (Somerville and Ogren, 1982; Liepman and Olsen, 2001). The mechanism of a reported contribution of SGT to pathogen defense in some species is not yet understood (Taler et al., 2004).



The re-import of amino groups from Glu into the C₂ cycle is catalyzed by Glu:glyoxylate aminotransferase (GGT). In Arabidopsis, GGT is encoded by a pair of nearly identical paralogous genes, *GGT1* and *GGT2* (Liepman and Olsen, 2003; Igarashi et al., 2003). The respective recombinant enzymes displayed biochemical characteristics very similar to one another and to the Arabidopsis protein purified from leaves. Confirming earlier

reports, they catalyze amino group exchanges in the combinations Glu:glyoxylate, Ala:glyoxylate, Glu:pyruvate, and Ala:2-oxoglutarate (2OG), but not with Ser as the amino-group donor.

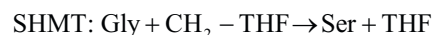
GGT1 represents the major GGT form in leaves. This follows from the analysis of an Arabidopsis *ggt1* knockout-mutant, which showed by about 80% reduced activities with the donor:acceptor combinations Glu:glyoxylate and Ala:glyoxylate. In contrast, activities with the combinations Ala:2OG and pyruvate:Glu (the reverse reaction) were reduced only by some 30–40%. Glu levels slightly increased and the levels of both Gly and Ser decreased when the mutant was grown in normal air conditions (Igarashi et al., 2003).

Schemes of the C_2 cycle usually neglect that Ala might well be an additional amino donor for photorespiratory Gly formation. Early observations in that direction now receive increasing support, e.g. by the capability of GGT to use Glu and Ala as the amino donor at equal rates (Igarashi et al., 2003). Such high activities with Ala are remarkable because the equilibrium of the reaction with glyoxylate lays toward the complete conversion of Ala to pyruvate. Moreover, the pool of Ala in the photosynthesizing cell is very large and Ala is rapidly labeled in the carboxyl group during $^{14}\text{CO}_2$ fixation (Nishimura and Akazawa, 1975). Similarly, both Ser and Ala can equally serve as the amino donor for SGT (Liepman and Olsen, 2001). This possibility of an open flux of nitrogen by import and export of amino acids into and from the photorespiratory nitrogen cycle is also reflected in the ^{15}N -labeling patterns of Glu, Gly, Ser, and Ala (Masclaux-Daubresse et al., 2006). All these data strongly indicate the position of Ala as another important amino group donor within or to the C_2 cycle.

E. Mitochondrial Reactions of Gly Yield Ser, CO_2 , NH_3 , and NADH

In illuminated C_3 plants grown in ambient air, photorespiratory Gly is synthesized at very high rates. While some Gly can be directed into other pathways, most of it becomes rapidly converted to Ser in the mitochondrial matrix. This NAD^+ - and tetrahydrofolate (THF)-dependent process requires two enzymes, the multienzyme complex Gly decarboxylase (GDC) and Ser

hydroxymethyltransferase (SHMT). Notably, both GDC and SHMT are highly susceptible to oxidation in vivo (Taylor et al., 2004) and possible targets for regulation by thioredoxin (Marti et al., 2009). As side products of the GDC reaction, photorespiratory CO_2 and NH_3 are released and NADH is generated.



1. Gly decarboxylase

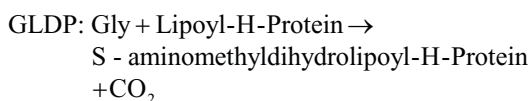
Gly decarboxylation was first discovered in non-plant organisms and the enzymology and function of GDC in plants has been frequently reviewed (e.g. Douce et al., 2001). In addition to its crucial role in photorespiration, GDC is also indispensable for general one-carbon metabolism with its multitude of biosynthetic reactions. This dual function of GDC is mirrored in the finding that deletion of GDC is fatal to plants and corresponding null mutants cannot be recovered even under non-photorespiratory conditions (Engel et al., 2007).

GDC is an atypical multienzyme complex comprising the four not very tightly associated protein components P-, H-, T-, and L-protein. All four proteins are nuclear-encoded and their combined presence within the mitochondrial matrix is necessary for catalytic activity of the complex. Although the individual proteins were purified earlier from non-plant organisms, the GDC complex has been isolated for the first time from pea leaf mitochondria, where it occurs in very high concentrations of up to 200 mg ml^{-1} . The GDC subunits assemble spontaneously within the mitochondrial matrix with an approximate subunit ratio of two P-protein dimers, 27 H-protein monomers, and nine T-protein monomers per one L-protein dimer. In analogy to the E2 dihydrolipoyl acyltransferase core of α -ketoacid dehydrogenases, H-protein supposedly could provide a central association core in this process. In comparison with other multienzyme complexes, however, the interaction between the individual GDC protein components is very fragile and

stable only at high concentration. The structure of the holoenzyme has not yet been resolved; however, some structure-function relationships between the individual subunits are known from crystallographic data and from nuclear magnetic resonance studies.

A. P-Protein

The actual binding and decarboxylation of Gly is catalyzed by the pyridoxal 5-phosphate-containing P-protein (GLDP), which is a homodimer of two ~105 kDa polypeptides. P-protein needs two substrates, Gly and H-protein, and catalyzes a reversible Gly:Lipoyl-H-protein oxidoreductase reaction.



First, a Schiff base between the carbonyl group of pyridoxal-5-phosphate (PLP) and a lysyl residue of P-protein is exchanged for a Schiff base between PLP and the amino group of Gly. Then, α -elimination results in the release of CO₂, not bicarbonate (Sarojini and Oliver, 1983), and the remaining aminomethylene group becomes conveyed to the oxidized lipoyl group of H-protein. P-protein and H-protein alone can catalyze a reversible Gly-bicarbonate exchange reaction where exogenous CO₂ can replace the carboxyl carbon of Gly. Although resulting in very low catalytic efficiency, even H-protein can be replaced by lipoate in both the Gly decarboxylation and the Gly synthesis reaction.

P-protein is encoded by two genes both in Arabidopsis and in rice. The individual knockout of either of these two genes in Arabidopsis does not significantly alter metabolism and photosynthetic performance indicating functional redundancy. In contrast, as already mentioned, a double-mutant with two inactivated *GLDP* genes does not develop beyond the cotyledon stage in air enriched with 0.9% CO₂ and the seedlings do not survive for longer than about 3–4 weeks under these non-photorespiratory conditions (Engel et al., 2007). This feature distinguishes the GDC-lacking double-mutant from all other known photorespiratory mutants. An earlier reported GDC-deficient Arabidopsis

mutant, *gld1* (Somerville and Ogren, 1982) is defective in mitochondrial lipoate synthesis and contains only minor amounts of functional H-protein (Ewald et al., 2007).

B. H-Protein

The ~14 kDa H-protein (GLDH, also known as aminomethyl carrier protein) carries covalently bound lipoic acid as its prosthetic group and represents the dominant lipoylated protein in photosynthesizing plant cells (Gueguen et al., 2000). Lipoylation of H-protein occurs via a two-step mechanism and nearly fully depends on the intra-mitochondrial biosynthesis of octanoyl-ACP (where ACP stands for acyl-carrier protein) by octanoyl-ACP synthase. First, lipoyltransferase conveys the octanoyl residue from octanoyl-ACP to the H-apoprotein. Next, S-adenosylmethionine-dependent lipoate synthase attaches the two sulphur atoms from S-adenosylmethionine on-site (reviewed in Rébeillé et al., 2007).

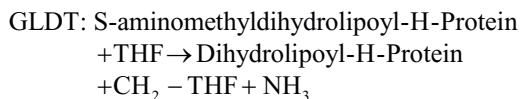
H-protein has no catalytic activity itself, but interacts as a mobile substrate via its lipoamide arm one after the other with P-, T-, and L-protein. In light of this central function for catalysis and the suspected role in the assembly of the holoenzyme, it was named the ‘structural and mechanistic heart’ of the GDC complex (Douce et al., 2001). One of the major functions of H-protein is the stabilization of the methylamine moiety remaining after decarboxylation of Gly by P-protein thus preventing its spontaneous degradation. Once aminomethylated by P-protein, the lipoate arm becomes locked within a cleft at the surface of the H-protein and released only by interaction with T-protein. This differs from structural models for other α -ketoacid dehydrogenase complexes where the lipoyl domain of the E2 subunit, which is structurally and functionally analogous to the H-protein, can freely rotate in the complex (e.g. Guilhaudis et al., 1999).

Plant H-proteins are often encoded by small multigene families and the individual H-proteins supposedly fulfil different functions in plant metabolism (Kopriva and Bauwe, 1995; Rajinikanth et al., 2007). In Arabidopsis, H-protein is encoded by a small gene family comprising three members. Two of the encoded proteins are very similar to each other but share only limited (about 60%) identity with the third homologue protein.

Notably, the lipamidyl residue of H-protein is very sensitive to modification by 4-hydroxy-2-nonenal, which is a cytotoxic product of lipid peroxidation under environmental stress. This has been taken as an indication that GDC and hence the proper functioning of the C₂ cycle are among the first casualties of oxidative damage to leaves (Taylor et al., 2004). A corresponding 'lipoate lyase' able to remove damaged lipoic acid, regenerating the H-apoprotein and allowing reattachment of an unmodified lipoate remains to be discovered.

C. T-Protein

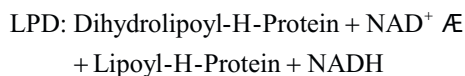
The ~41 kDa monomeric T-protein (GLDT) acts as an aminomethyl transferase and needs both THF and aminomethylated H-protein as substrates. In the absence of THF, formaldehyde is produced instead of CH₂-THF. T-protein is the only GDC subunit that is most likely encoded by single-copy genes both in Arabidopsis and in rice. This singular occurrence could indicate a central role of T-protein in the regulation of GDC biosynthesis and might explain the yet unsuccessful search for knockout mutants for this gene.



The folate binding site is formed via the interaction with H-protein in a 1:1 molar ratio and comprises several lysyl residues that interact with the glutamyl α -carboxyl groups of THF. The current model implies that the polyglutamyl tail of the folate substrate is inserted into a formed cavity leaving the pteridine ring near the entrance of the cavity in the context of the catalytic reaction (Okamura-Ikeda et al., 2003). As already mentioned, interaction results in a change of the conformation of the H-protein, leading to the release of the aminomethylated lipoate arm from the protecting cleft followed by nucleophilic attack on the methylene carbon by the N-5 atom of the THF's pterin ring (Guilhaudis et al., 2000). Products of this reaction are photorespiratory NH₃, CH₂-THF, and H-protein with a fully reduced dihydrolipoyl group.

D. L-Protein

The re-oxidation of the H-protein's dihydrolipoyl group closes the H-protein reaction cycle. This final reaction is catalyzed by L-protein (LPD, dihydrolipoamide dehydrogenase), which is a homo-dimer of ~50 kDa polypeptides and needs both FAD and NAD⁺ for catalysis. Except the catalytic interaction with the lipoyl arm, there is no apparent molecular recognition and interaction between L-protein and the reduced H-protein (Douce et al., 2001). During oxidation of the dihydrolipoyl-H-protein, FAD is reduced to FADH₂ which, in turn, becomes re-oxidized by NAD⁺ resulting in the synthesis of one NADH per decarboxylated Gly.



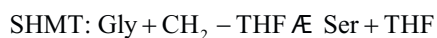
The massive carbon flux through the GDC reaction requires high rates of NADH re-oxidation, which are substantially higher than the predicted rates of mitochondrial electron transport and oxidative phosphorylation (e.g. Noguchi and Yoshida, 2008). As much as 25–50% of the produced reducing equivalents is thought to be exported to the cytosol via a malate/oxaloacetate shuttle and contributes to the peroxisomal reduction of 3HP to glycerate. The responsible transporter (DCT, At5g19760), as the first dicarboxylate/tricarboxylate carrier in plants, has been cloned and functionally characterized (reviewed in Picault et al., 2004). The mitochondrial malate shuttle system is seemingly complemented by a glycerol 3-phosphate shuttle operating in the opposite direction to ensure proper malate/OAA ratios in the cytosol (Shen et al., 2006). It is not yet clear how much of the remaining GDC-generated NADH actually drives oxidative phosphorylation to provide ATP to the cytosol, but this fraction may vary depending on many circumstances. Most of it is probably recycled to NAD⁺ via a non-phosphorylating by-pass comprising one of the mitochondrial non-proton pumping NAD(P)H dehydrogenases (NDA1, At1g07180) and alternative oxidase which thus closely interact with photorespiration. The mitochondrial uncoupling protein UCP1, which bypasses ATP synthesis by facilitating the re-entry of H⁺ into the mitochondrial matrix

(Sweetlove et al., 2006), also helps in maintaining adequate redox poise of the mitochondrial electron transport chain.

L-protein is a component not only of GDC but also, as the so-called E3 subunit, a component of the three 2-oxoacid dehydrogenase complexes oxidizing pyruvate, 2OG, and the branched-chain 2-oxoacids (Mooney et al., 2002). The mitochondrial L-protein is encoded by two genes in *Arabidopsis*, *mtLPD1* and *mtLPD2*. The knockout of *mtLPD2* did not result in an apparent phenotype and the two enzymes hence appear as entirely interchangeable among the different multienzyme complexes (Lutziger and Oliver, 2001).

2. Ser hydroxymethyltransferase

SHMT consists of four pyridoxal 5-phosphate-containing ~53 kDa subunits and catalyzes the CH₂-THF-dependent conversion of Gly to Ser. SHMT, in addition to T-protein represents the second of two major folate-dependent mitochondrial proteins. The two enzymes bind a large fraction of the mitochondrial folate pool thereby also preventing oxidative degradation of this very labile and oxidizable compound.



As a second result of the SHMT reaction, CH₂-THF is recycled to THF for its re-use in the GDC reaction. The more than twofold higher activity of GDC relative to SHMT ensures a high CH₂-THF/THF ratio in the mitochondrial matrix, which drives the SHMT reaction in the thermodynamically not favoured direction towards the synthesis of Ser (Douce et al., 2001). In a second catalytic activity, SHMT can produce considerable amounts of 5-formyl-THF. This compound is a strong inhibitor of SHMT and becomes recycled to THF in a series of recently identified reactions including 10-formyl-THF deformylase. Deletion of this enzyme results in a remarkable increase of leaf Gly levels, indicating the essential function of this pathway for photorespiration (Goyer et al., 2005; Collakova et al., 2008).

Intriguingly, AtSHMT1 appears to require the presence of a second enzyme, ferredoxin-dependent glutamate synthase (Fd-GOGAT). Fd-GOGAT is responsible for NH₃ assimilation

in chloroplasts, but also targeted to the mitochondria, where it has been shown to physically interact with SHMT1. This interaction appears to be necessary for full catalytic activity of SHMT1 in vivo, but the underlying mechanism remains speculative (Jamai et al., 2009).

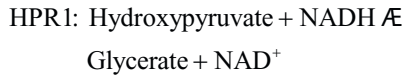
SHMT, other than GDC, is not exclusively restricted to the mitochondria. Specific isoforms are present in the cytosol and in plastids, where they catalyze the Ser-to-Gly reaction to provide CH₂-THF for a large number of biosynthetic reactions in both photosynthesizing and non-photosynthesizing tissues. The resulting Gly is then re-converted to Ser by the mitochondrial GDC-SHMT system. Accordingly, the *Arabidopsis* genome contains at least five and perhaps even seven SHMT genes, *AtSHM1* to 7. Only *AtSHM1* and *AtSHM2* encode mitochondrial proteins. Genetic mapping and knockout studies have identified AtSHM1 as the photorespiratory SHMT, whereas functional deletion of AtSHM2 does not lead to any apparent phenotypic alterations and cannot complement the *shm1* mutation (Voll et al., 2006). The rice genome probably contains five SHMT genes. Only *OsSHM1*, an ortholog of *AtSHM1*, encodes a protein with a mitochondrial targeting peptide.

Disruption of *AtSHM1* not only impairs photorespiratory metabolism, but also leads to a constitutive expression of salicylic acid-inducible genes and genes involved in H₂O₂ detoxification. Notably, *shm1* mutants were also more susceptible than control plants to infection with biotrophic and necrotrophic pathogens (Moreno et al., 2005). This once more indicates a contribution of photorespiratory processes to dissipatory mechanisms that minimize the level of reactive oxygen species. Excessive accumulation of reactive oxygen species impairs cell death containment and could counteract the effectiveness of the plant defenses to restrict pathogen infection.

F. Hydroxypyruvate Is Produced from Ser and Becomes Reduced to Glycerate

After export from the mitochondria, Ser becomes converted to 3HP by the aforementioned peroxisomal enzyme SGT (AGT1), which transfers the

amino group of Ser to glyoxylate, yielding 3HP and Gly. In the penultimate step of the C_2 cycle, most 3HP becomes reduced to D-glycerate by the peroxisomal NADH:3HP reductase (HPR1) using NADH imported from the cytosol via malate shuttles.



HPR1 consists of two identical ~42 kDa polypeptides. Together with catalase, glycolate oxidase, and NAD^+ :malate dehydrogenase, HPR1 is one of the four major proteins in peroxisomes. In vivo, the enzyme functions primarily as a NADH:3HP reductase (Tolbert et al., 1970). Although with much lower affinity, however, HPR1 also accepts glyoxylate as substrate and was therefore originally described as NADH-specific glyoxylate reductase.

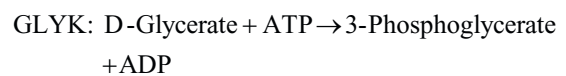
Intriguingly, two *HPR1* genes are present in soybean with one of the corresponding enzymes being a primary target for the P34 syringolide elicitor. After binding of the elicitor, a hypersensitive response is induced by inhibiting one or more yet unknown HPR1 functions in soybean (Okinaka et al., 2002). In many other plants, however, HPR1 is encoded by single genes. Functional deletion of HPR1 in *Arabidopsis* results in an only minor impairment of plant growth in normal air, very much resembling the low- CO_2 response of an earlier reported HPR-deficient barley mutant (Murray et al., 1989) and that of a recently described double-knockout mutant without peroxisomal malate dehydrogenase (Cousins et al., 2008). The missing low- CO_2 response is very atypical for photorespiratory mutants and indicates the operation of an HPR1-independent route of 3HP-to-glycerate conversion. While a candidate enzyme for this alternative reaction had been suggested (Givan and Kleczkowski, 1992), it was only recently that the responsible enzyme was properly identified as a cytosolic NADPH-dependent 3HP reductase, HPR2, and its metabolic function analyzed by a reverse genetics approach (Timm et al., 2008).

The current model of cooperation between HPR1 and HPR2 is based on the well established fact that NADH cannot permeate through the peroxisomal membrane. Instead, reducing

equivalents are imported into peroxisomes in the form of malate provided from chloroplasts and mitochondria via malate/oxaloacetate shuttles, which can be limiting in specific environmental conditions (Yu and Huang, 1986; Reumann and Weber, 2006). Hence, likely determined by the rate of NADH supply to the peroxisomes, a variable fraction of 3HP exits the peroxisomes and becomes reduced in the cytosol. Flux analyses indeed indicate that the cytosolic route is operating not only under the condition of abnormally high photorespiration, but also in moderate environments (Timm et al., 2008). This new model implies that the C_2 cycle, by the cooperation of peroxisomal and cytosolic oxidoreductase reactions, gains additional flexibility in the adaptation to different environmental and possibly developmental conditions. Notably, some plants produce peroxisomal and cytosolic isoforms of HPR1 by alternative splicing of a single pre-mRNA, and the transcript level of the cytosolic form is greatly enhanced under photorespiratory conditions (Mano et al., 1999).

G. Glycerate Becomes Phosphorylated and 3PGA Re-enters the Calvin Cycle

Glycerate enters the chloroplast via a yet unidentified glycolate/glycerate transporter (Howitz and McCarty, 1991). As the ultimate step of the C_2 cycle, glycerate 3-kinase (GLYK) completes the C_2 cycle by returning three fourth of the initially 'misdirected' carbon atoms back to the Calvin cycle in the form of 3PGA.



The molecular structure and the encoding gene of the ~41 kDa monomeric enzyme were identified only recently (Boldt et al., 2005). GLYK is encoded by a single gene in *Arabidopsis*. T-DNA insertion knockout mutants show no GLYK activity and are not viable in normal air; however, they grow under elevated CO_2 providing direct evidence for the obligatory nature of the ultimate step of the C_2 cycle under photorespiratory conditions. The enzyme is phylogenetically unrelated to known glycerate kinases from bacteria and animals, which produce 2-phosphoglycerate

(Bartsch et al., 2008), but orthologous proteins are present in other plants, fungi, and some cyanobacteria.

H. Transcriptional Regulation of Photorespiratory C₂ Cycle Genes

The C₂ cycle is a subset of the reactions of photosynthetic carbon assimilation. It is therefore not surprising that C₂ cycle enzymes and their transcripts are present in heterotrophic tissue in only low amounts and that their biosynthesis is induced by light or responsive to cytokinin. Light induction is probably mediated via phytochrome A and cryptochromes. Transcriptional regulation of C₂ cycle enzymes has been examined by the analysis of individual genes or enzymes, organellar proteomes (e.g. mitochondria, Bardel et al., 2002), and expressed sequence tag (EST) arrays. In these latter studies, a light-dependent increase of nearly all genes encoding C₂ cycle enzymes in Arabidopsis was observed after illumination with blue, red, and far-red light. The induction by far-red or blue light was observed not only in wild-type plants but also in *phyA* and cryptochrome null (*cry1/cry2*) mutants. On the other hand, *phyB* mutants did not show induction by red light (Ma et al., 2001). Related *in silico* analyses of Arabidopsis genes encoding peroxisomal proteins revealed distinct co-expression patterns for photorespiration-related genes (Reumann and Weber, 2006). This latter finding may prove very helpful for future work because it opens a new strategy to identify yet unknown components of the photorespiratory pathway, such as transporters.

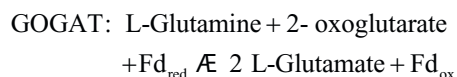
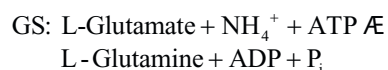
III. Related Reactions and Interactions with Other Metabolic Pathways

A. Reassimilation of Photorespiratory NH₃

Photorespiratory CO₂ evolution is intrinsically associated with the generation of equimolar amounts of NH₃, which are recaptured via the 'photorespiratory nitrogen cycle' (Keys et al., 1978; Keys, 2006). This pathway is also responsible for the entry of NH₃ derived from other sources into general metabolism, and a close interaction of

photorespiration and nitrate assimilation has been proposed (Rachmilevitch et al., 2004). NH₃ recovery occurs with very high efficiency, and only ~0.01% of photorespiratory NH₃ is lost (Mattsson et al., 1997).

Biochemically, this process occurs in the chloroplastic glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. The chloroplastic isoform of GS, GS2, combines Glu and NH₃ to form Gln, and the NH₃ acceptor molecule Glu is re-synthesized by the ferredoxin-dependent isoform of GOGAT.



Arabidopsis like all higher plants studied so far contains a single gene (*GLN2*) for GS2 and multiple genes for cytosolic GS1. Mutation of *GLN2* results in a conditional lethal phenotype under photorespiratory conditions (Blackwell et al., 1988). One out of two genes encoding Fd-GOGAT isoforms in Arabidopsis, *GLU1*, is dominantly expressed in leaves and was mapped to the same location as an earlier Fd-GOGAT mutant. Leaves of this *gls* mutant contain less than 5% of the wild-type levels of Fd-GOGAT activity (Somerville and Ogren, 1982). A second, NADH-dependent isoform accounts for only about 5% of total leaf GOGAT (*GLT1*) activity and is involved in the non-photorespiratory ammonium assimilatory pathway in roots (Coschigano et al., 1998; Suzuki and Knaff, 2005). *GLN2* and *GLU1* hence play the major roles in photorespiration and primary nitrogen assimilation in leaves.

At least in Arabidopsis, GS2 is dual targeted to chloroplasts and mitochondria indicating that some photorespiratory NH₃ could be directly fixed within the mitochondria (Taira et al., 2004). In course of these reactions, Gln produced in the mitochondria is supposedly used for the synthesis of carbamoylphosphate which combines with ornithine to yield citrulline. Citrulline is supposedly exported from the mitochondria via a hypothetical and not yet identified ornithine-citrulline transporter. Considering that NH₃ refixation is a

two-step process requiring glutamate synthase, which is exclusively located in plastids, this hypothesis was recently extended by the suggestion of two alternative NH_3 shuttles between the mitochondria and the chloroplast, an ornithine-citrulline shuttle and a Gln-Glu shuttle (Linka and Weber, 2005).

It is not known whether NH_3 leaves the mitochondria and enters the chloroplasts by diffusion or via specific transporters. Ammonium transport across the plasma membrane is facilitated by ammonium transporters and, in animal systems, specific aquaporins. Such transport systems were not yet identified in mitochondrial or plastidial membranes. Much more is known about the uptake of 2OG into chloroplasts and the export of Glu for peroxisomal transamination reactions. This process is mediated by a two-translocator system. Both translocators, DiT1 for 2OG and DiT2 for Glu, facilitate transport of the respective compounds by counter-exchange with malate, resulting in no net malate transport. Antisense α -DiT1 plants and *dit2* mutant Arabidopsis and barley plants show a heavily disturbed phenotype (e.g. Weber and Fischer, 2007).

B. Regulatory Interaction with Respiration

Regulatory interactions between photorespiration and respiration in the light are not very well understood, but received increasing attention in recent years. Many observations point to an important role for TCA cycle function in the regulation of photosynthesis and photorespiration well beyond the suggested functions of mitochondria in providing ATP for sucrose synthesis and carbon skeletons necessary for nitrate reduction in the cytosol (e.g. Nunes-Nesi et al., 2007; Noguchi and Yoshida, 2008).

Day respiration, i.e. the processes by which non-photorespiratory CO_2 is produced by illuminated leaves, is only about 20–50% of the night respiration rate (Atkin et al., 2000). Some recent data suggest an even higher inhibition of TCA cycle decarboxylations in combination with an opposed partial upregulation under conditions of elevated photorespiration to meet the increased demands of NH_3 reassimilation (Tcherkez et al., 2008). Mechanistically, the downregulation of the TCA cycle activity in the light is not fully under-

stood; however, it is inhibited by its products, NADH and ATP, and activated by its substrates, NAD and ADP. The relative levels of these compound affect the supply of other substrates to the cycle and determine the activity of several internal enzymes including, for example, isocitrate dehydrogenase, which is inhibited by high NADH/NAD (Igamberdiev and Gardeström, 2003). Mitochondrial pyruvate dehydrogenase is inhibited by NADH and controlled by regulatory phosphorylation. The inactivating kinase is activated by NH_4^+ , which is present in large amount in illuminated leaf mitochondria (Tovar-Mendez et al., 2003). In the dark, NH_4^+ and NADH levels drop and a phosphatase reactivates PDH, which corresponds well to the lower light suppression of TCA cycle reactions observed in GDC-deficient transgenic plants (Bykova et al., 2005).

C. One-Carbon Metabolism

With GDC and the mitochondrial SHMT, the C_2 cycle shares two major enzymatic reactions with cellular one-carbon (C_1) metabolism. CH_2 -THF as the central C_1 carrier compound, directly or after conversion to methyl-, methenyl- and formyl-THF or the synthesis of S-adenosylmethionine, provides C_1 units to very many biosynthetic pathways not only in plants but also in all other prokaryotic and eukaryotic organisms (e.g. Hanson and Roje, 2001).

Although plant mitochondria contain fivefold more folate compounds than chloroplasts, this high CH_2 -THF/THF pool does not equilibrate with the cytosolic or chloroplastic pools and cannot be a major direct source of one-carbon units for biosynthetic reactions outside the mitochondria (e.g. Orsomando et al., 2005). Instead, all the non-mitochondrial subcellular compartments seem to rely on their own SHMT-catalyzed synthesis of C_1 units, which occurs in cooperation with GDC via a Ser-Gly cycle (Mouillon et al., 1999). That is, GDC and SHMT interconnect the metabolism of one-, two-, and three-carbon compounds in most if not all organisms, and it appears likely that both enzymes were recruited for their novel role in the C_2 cycle from C_1 metabolism.

It is a yet unresolved question whether C_1 metabolism, in comparison with the C_2 cycle, has different regulatory requirements to GDC and SHMT. Different enzymatic properties are

apparently not required for T-protein, the only GDC component that is encoded by single-copy genes in Arabidopsis, rice and other plants. In contrast to T-protein, however, all other GDC components are encoded by multiple genes. It is remarkable that the number of GDC subunit genes is the same in Arabidopsis and in rice. Any specific functions or, alternatively, the degree of functional redundancy of the multi-copy paralogous genes and their products are yet to be examined. Some data suggest that specific forms of H-protein are involved in C₁ metabolism in developing xylem rather than in photorespiration of photosynthesizing tissue (Wang et al., 2004). Likewise, the observation that alternative H-proteins, originating by light-regulated alternative splicing of a single mRNA in some species, are present in leaves and roots in different ratios may also indicate different requirements for enzymatic regulation in the C₂ cycle vs. C₁ metabolism of heterotrophic tissue (Kopriva et al., 1996a).

D. Alternative Sources and Destinies of C₂ Cycle Metabolites

The aforementioned findings (e.g. Cegelski and Schaefer, 2005) of a not very strictly fixed stoichiometry indicate that the C₂ cycle could be a more flexible metabolic pathway than it is usually thought. Accordingly, some earlier experiments, performed mainly in the 1960–1970s, suggest the presence of alternative routes of glycolate synthesis (e.g. Eickenbusch and Beck, 1973). Although by far most of the 2PG in photosynthesizing cells originates from RubP oxygenation, it is also continuously generated in small amounts by other processes, for instance those related to DNA repair. While contributions of such processes to 2PG synthesis may be negligible, bypassing reactions to the core C₂ cycle or export to other pathways could be of higher significance. Due to the complex biochemistry of these compounds, the presence of such reactions is most likely for glycolate and 3HP.

Unicellular algae lacking peroxisomes, in contrast to higher plants and many multicellular algae, have no GOX activity and oxidize glycolate by a mitochondrial glycolate dehydrogenase (Stabenau and Winkler, 2005). An equivalent enzyme has recently been identified in Arabidopsis mitochondria, indicating the conservation of the algal photorespiratory pathway (Bari et al., 2004).

In unicellular algae and in higher plants, glycolate can also be oxidized directly within the chloroplasts by a glycolate:plastoquinone oxidoreductase (also known as glycolate dehydrogenase). This enzyme is associated with the thylakoid membranes and supposedly acts as an electron donor in cyclic photophosphorylation providing additional ATP without NADPH accumulation. The generated glyoxylate is probably recycled to glycolate by a chloroplastic NADPH-specific glyoxylate reductase (Goyal and Tolbert, 1996).

IV. Measurement of Photorespiration

The exact determination of photorespiration rates is difficult for two reasons. Firstly, photorespiratory CO₂ evolution is concealed by CO₂ uptake and re-fixation in the Calvin cycle as well as by CO₂ release from mitochondrial ‘dark’ respiration continuing in the light. Secondly, photorespiratory O₂ uptake occurs concurrently with photosynthetic O₂ evolution and with O₂ consumption during mitochondrial respiration and other metabolic processes including the direct reduction of O₂ at photosystem I, the so-called Mehler reaction.

There are several methods available for the more or less accurate determination of photorespiration rates. Each of these methods has some technical or theoretical problems, which compromise the results, and sizeable differences can be obtained using different methods. Three of the more common methods will be shortly described below but more are available. For instance, photorespiration can also be assessed by the combination of gas exchange measurements with the determination of electron transport rates by fluorescence, mass spectrometry (Haupt-Herting et al., 2001), and nuclear magnetic resonance techniques (Cegelski and Schaefer, 2006). More detailed treatises on how to measure photorespiration can be found in former reviews (Sharkey, 1988; Laisk and Oja, 1998; von Caemmerer, 2000; Long and Bernacchi, 2003).

A. Post-illumination Burst of CO₂ (PIB)

One possible way to rapidly terminate photosynthesis is switching off the light. Thereafter, C₂ cycle metabolism continues for much longer than

CO₂ assimilation, and an approximate measure of the efflux of CO₂ can be obtained using an open infrared gas analyzer system (IRGA). Two peaks are commonly observed during the PIB, but only the first one is associated with photorespiration while the second peak rises during equilibration of mitochondrial respiration. With well-designed equipment allowing rapid-response measurements, CO₂ uptake usually approaches 0 after 5 s and the rate of CO₂ evolution peaks approximately 13 s from darkening (Laisk and Oja, 1998). Due to the masking of the initial slope by continuing CO₂ assimilation that is supported by decreasing but for a while still existing pools of RubP and its precursors, however, this method fails to give quantitative measures of photorespiration.

B. Measurement of ¹⁴CO₂ Evolution

A much more reliable technique, developed by Olav Keerberg and colleagues (Pärnik and Keerberg, 2007), distinguishes between the two opposite fluxes of photosynthetic CO₂ fixation and (photo)respiratory CO₂ evolution, but requires a more complex measuring system. In short, metabolically active leaf carbon pools are labeled with ¹⁴CO₂, and ¹⁴CO₂ evolution is then monitored in the presence of very high (30 ml l⁻¹) concentrations ¹²CO₂. Refixation of ¹⁴CO₂ evolved inside the leaf is close to zero under this condition. Photorespiration and respiration are distinguished on the basis of data obtained from measurements of ¹⁴CO₂ evolution under normal (210 ml l⁻¹) and low (15 ml l⁻¹) concentrations of oxygen, while different time periods during the initial ¹⁴CO₂ labeling allow differentiation between CO₂ release from primary versus stored photosynthates. This approach not only yields accurate estimates of photorespiration rates, but can also quantitatively assess internal re-fixation of photorespiratory CO₂.

C. Extrapolation from CO₂ Response (A/c_i) Curves

This method does not require equipment for the on-line analysis of ¹⁴CO₂, but is somewhat less accurate. Apparent CO₂ exchange rates (A) are measured in an open IRGA system at several CO₂ concentrations. Intracellular CO₂ concentrations (c_i), which are indirectly determined from the simultaneous measurement of transpiration,

are used to eliminate effects of the boundary layer and stomata aperture. The preferred CO₂ concentration range spans from below the CO₂ compensation point Γ (i.e., the atmospheric CO₂ concentration where total CO₂ uptake equals total CO₂ efflux) up to 200–300 $\mu\text{l l}^{-1}$ CO₂. The CO₂ response curve is nearly linear in this range. Extrapolation to zero CO₂ yields the rate of CO₂ release from the leaf, i.e., the sum of photorespiration and respiration in the light.

CO₂ exchange rates, of course, are minimal under such conditions resulting in experimental errors. Moreover, at CO₂ concentrations below twofold Γ the CO₂ response curve is commonly curvilinear, bending toward lower values of apparent photorespiration. This effect is due to smaller RubP pools and can be avoided in very rapid measurements (Laisk and Oja, 1998). Therefore, although convenient for comparisons between species, this method also gives only rough estimates of photorespiration rates and is restricted to the conditions of unphysiologically low CO₂/O₂ ratios.

D. Estimation from Rubisco Kinetics and Gas Exchange Measurements

An important feature of Rubisco is the competition of CO₂ and O₂ for the same catalytic site. This and other features have been integrated into mathematical models of photosynthesis and combined with the analysis of whole leaf gas-exchange parameters. The most broadly used 'Farquhar model' (Farquhar et al., 1980) allows the determination of the in vivo RubP oxygenation rate from gas exchange measurements thus separating contributions from respiration in the light to total CO₂ efflux. This approach requires the determination of the chloroplastic CO₂ concentration where the rate of RubP carboxylation equals the rate of photorespiratory CO₂ release. This CO₂ concentration is defined as the photosynthetic CO₂ compensation point in the absence of respiration in the light, Γ^* , and can be determined from the interception point of CO₂ curves measured at different light intensities. The same data set gives an estimate for R_d, the respiration in the light. As an experimentally sometimes more convenient alternative, Γ^* can also be obtained from the (in C₃ plants) linear O₂ dependence of Γ between 1.5% and 21% O₂ (e.g. Laisk and Oja, 1998).

The mathematical modeling approach, in short, is as follows (von Caemmerer, 2000). The ratio of RubP oxygenation to carboxylation, v_o/v_c , is determined solely by the kinetic constants of Rubisco as shown in Eq. 6.1 where V_o , V_c , K_o , and K_c are the in vivo maximal rates and the Michaelis-Menten constants for oxygenation and carboxylation, respectively. O and C represent the chloroplastic O₂ and CO₂ concentrations. S is the so-called specificity factor of Rubisco.

$$\Phi = \frac{v_o}{v_c} = \left(\frac{V_o K_c}{K_o V_c} \right) \cdot \frac{O}{C} = \frac{1}{S} \cdot \frac{O}{C} \quad (1)$$

Net flux of CO₂ will be zero if exactly two oxygenations of RubP occur for every carboxylation. This happens at the chloroplastic CO₂ concentration Γ^* .

$$\frac{v_o}{v_c} = 2 = \frac{1}{S} \cdot \frac{O}{\Gamma^*} \quad (2)$$

Re-arranging Eq. 6.2 yields an expression for the specificity factor.

$$S = \frac{O}{2\Gamma^*} = \frac{\Delta O}{2\Delta\Gamma} \quad (3)$$

By using Eq. 6.3 in combination with Eq. 6.1, one can now calculate the oxygenation to carboxylation ratio in vivo at any reasonably given chloroplastic CO₂ and O₂ concentration. The rate of photorespiratory CO₂ production can then be determined with the help of two more experimental parameters to be determined by gas exchange measurements, the net CO₂ assimilation rate A and the rate of respiration in the light Rd (Eq. 6.4).

$$A = v_c - 0.5 v_o - R_d \quad (4)$$

Using Eq. 6.4 and keeping in mind that the stoichiometry of the C₂ cycle requires two molecules 2PG to produce one molecule of CO₂, primary rates of photorespiratory CO₂ evolution can be calculated according to Eq. 6.5.

$$0.5 v_o = \frac{A + R_d}{\frac{2}{\Phi} - 1} \quad (5)$$

V. The Role of Photorespiration for the Evolution of C₄ Photosynthesis

As already indicated, photorespiration reflects the evolutionary origin of oxygenic photosynthesis about 2.5 billion years ago in the anaerobic environment of the Precambrian ocean (Canfield, 2005). Due to the lack of free oxygen, RubP oxygenation could not occur and photorespiratory glycolate recycling was unnecessary. Much more recently, about 1.1–0.54 Ga ago (e.g. Kennedy et al., 2006), the level of oxygen in the atmosphere rose to relatively high levels, resulting in increasingly significant competition of O₂ with CO₂ for the active site of Rubisco.

This had severe consequences for the further evolution of photosynthetic bacteria, algae, and later land plants including those of the C₄ photosynthetic type. While details of the evolution of C₂ cycle are not well known, the pathway is not restricted to the Chlorobionta, but already present in cyanobacteria. In these ancestors of plastids, it overlaps and cooperates with the bacterial glycerate pathway (Eisenhut et al., 2006). Cyanobacteria improve their photosynthetic efficiency very much by active uptake of CO₂ and bicarbonate via CO₂-concentrating mechanisms (CCM; Kaplan et al., 2007). Despite this, the artificial obstruction of cyanobacterial glycolate metabolism leads to toxic Gly accumulation (Eisenhut et al., 2007). This suggests that photorespiratory glycolate metabolism is not just present but essential for cyanobacteria, where it employs multiple routes, one of which might have been conveyed endosymbiotically to plants (Eisenhut et al., 2008).

Land plants evolved from ancestral freshwater algae from the Charophyceae group that were closely related to the unicellular flagellate *Mesostigma viride* (Petersen et al., 2006; Simon et al., 2006) and to the possibly even more ancient unicellular green alga *Ostreococcus tauri* (Palenik et al., 2007). While the respective analyses still need to be extended, first data suggest that major changes in photorespiratory metabolism took place already very early during the evolution of unicellular flagellates, i.e., before the transition of plants to land approximately 480–700 million years ago. Once on land, plants had easier access to CO₂ than bacteria and algae, but also faced periods of limiting water supply or elevated temperatures and hence decreased CO₂/O₂ ratios. The resulting problems for photosynthetic

efficiency were further exacerbated by the significant decline of atmospheric CO₂ levels during the last 500 million years in combination with at least one intermediate rise of atmospheric oxygen to about 35% (Beerling and Berner, 2000; Pearson and Palmer, 2000). The most effective option to deal with the elevated potential for RubP oxygenation was the addition of a CCM to the Calvin cycle, the C₄ cycle. C₄ plants usually show only small or even no apparent photorespiratory CO₂ release. This is because of the C₄ cycle-mediated CO₂ enrichment within the bundle sheath, which out-competes oxygen and efficiently reduces 2PG synthesis by Rubisco. Hence, smaller amounts of photorespiratory CO₂ are produced and more or less completely recaptured by the entry enzyme into the CO₂ concentration cycle of C₄ plants, phosphoenolpyruvate carboxylase.

Details of the individual steps leading from C₃ to C₄ photosynthesis in the last 15–30 million years are still far from being well understood. C₄ photosynthesis differs from the original C₃ photosynthesis by quite a number of molecular and morphological features, which implies the likelihood of transitional, C₃-C₄ intermediate, species. *Mollugo verticillata* (Aizoaceae) was the first plant species reported which has a number of features, for example leaf anatomy, photorespiration, and primary photosynthetic products, that can neither be easily associated with C₃ nor with C₄ photosynthesis (Kennedy and Laetsch, 1974). Since then, C₃-C₄ intermediate and C₄-like species have been identified in about 30 species of 11 genera representing nine different families (Table 2). These plants represent a broad variety of transitions between C₃ and C₄. Phylogenetic analyses demonstrate that C₃-C₄ intermediate plants represent transient forms between C₃ and C₄ *Flaveria* species (Powell, 1978; Kopriva et al., 1996b; McKown et al., 2005) and have likewise suggested that they may be derived from C₃ plants in *Moricandia* (Bauwe, 1983).

Photosynthetic rates of C₃ and C₃-C₄ intermediate species are comparable in a range of environmental conditions, but the responses of gas-exchange parameters that provide a measure of photorespiratory activity differ widely between these two groups. For instance, leaves of C₃-C₄ intermediate species show a higher CO₂ affinity than related C₃ species (e.g. Ku and Edwards,

1978; Bauwe and Apel, 1979). The higher CO₂ affinity of leaves from C₃-C₄ intermediate species is mirrored in lower values for Γ . At atmospheric O₂ concentration, the respective Γ values are generally between 10 and 30 $\mu\text{l l}^{-1}$ and hence much lower than the typical range for Γ of C₃ plants. Historically, it is interesting to note that several C₃-C₄ intermediate plants, such as *Moricandia arvensis* (L.) DC and *Panicum milioides* Nees ex Trin., have been identified in the aforementioned Γ -based screening programs set up to identify further C₄ plants.

At low photon flux densities, close to the light compensation point, Γ of a C₃-C₄ intermediate species can be almost as high as that of a C₃ species; however, in contrast to C₃ plants where Γ is essentially unaffected by light intensity, Γ of C₃-C₄ intermediate species is strongly light dependent and declines steeply as the light intensity increases (e.g. Brown and Morgan, 1980). This response was an early indication that the mechanism which lowers Γ (i.e., reduces apparent photorespiration) in C₃-C₄ intermediate species is dependent on the rate of photosynthesis.

Rather than the linear response of Γ generally observed with C₃ plants (compare Eq. 6.2), C₃-C₃ intermediate plants show a biphasic response of Γ to increasing O₂ concentration (e.g. von Caemmerer, 1989), which is another very indicative feature of C₃-C₄ intermediate photosynthesis. As the O₂ concentration is raised to 10–15%, relatively low increases of Γ occur, followed by a steeper and linear response above 20%. Typically, the slope of the Γ/O_2 response curve (γ) at higher O₂ concentrations is lower than corresponding values for C₃ plants. In C₃ plant photosynthesis models, γ is proportional to the specificity factor *S* of Rubisco; however, no corresponding differences exist in the kinetic properties of Rubisco of C₃ and C₃-C₄ intermediate plants (Bauwe, 1984). In combination with the presence of Rubisco in both mesophyll cells (MC) and bundle sheath cells (BSC), and in accordance with the higher CO₂ affinity of leaves, lower values for γ indicate that a significant fraction of total Rubisco operates at an elevated CO₂/O₂ concentration ratio in C₃-C₄ intermediate species.

C₃-C₄ intermediate species are usually native to warm environments like Mexico and Florida (*Flaveria*) or Mediterranean countries (*Moricandia*).

Table 2. C₃-C₄ intermediate land plants.

Family	Species	References
Aizoaceae	<i>Mollugo verticillata</i>	(Kennedy and Laetsch, 1974)
	<i>M. nudicaulis</i>	(Raghavendra et al., 1978)
Poaceae	<i>Panicum</i> (= <i>Steinchisma</i>) <i>decipiens</i>	(cf. Sage et al., 1999)
	<i>P. milioides</i>	(cf. Sage et al., 1999)
	<i>P. schenckii</i>	(cf. Sage et al., 1999)
Boraginaceae	<i>Heliotropium convulvolaceum</i>	(Vogan et al., 2007)
	<i>H. greggii</i>	(Vogan et al., 2007)
	<i>H. racemosum</i>	(Vogan et al., 2007)
Poaceae	<i>Neurachne minor</i>	(cf. Sage et al., 1999)
Brassicaceae	<i>Moricandia arvensis</i>	(cf. Sage et al., 1999)
	<i>M. nitens</i>	(cf. Sage et al., 1999)
	<i>M. sinaica</i>	(cf. Sage et al., 1999)
	<i>M. spinosa</i>	(cf. Sage et al., 1999)
	<i>M. suffruticosa</i>	(cf. Sage et al., 1999)
	<i>Diplotaxis tenuifolia</i>	(cf. Sage et al., 1999)
Asteraceae	<i>Flaveria angustifolia</i>	(cf. Sage et al., 1999)
	<i>F. anomala</i>	(cf. Sage et al., 1999)
	<i>F. chloraefolia</i>	(Holaday et al., 1984)
	<i>F. floridana</i>	(Holaday et al., 1984)
	<i>F. linearis</i>	(cf. Sage et al., 1999)
	<i>F. pubescens</i>	(cf. Sage et al., 1999)
	<i>F. ramosissima</i>	(cf. Sage et al., 1999)
Asteraceae	<i>Parthenium hysterophorus</i>	(cf. Sage et al., 1999)
	<i>Bougainvillea</i> cv. <i>Mary Palmer</i>	(Sabale and Bhosale, 1984)
Amaranthaceae	<i>Alternanthera ficoidea</i>	(cf. Sage et al., 1999)
	<i>A. tenella</i>	(cf. Sage et al., 1999)
Chenopodiaceae	<i>Salsola arbusculiformis</i>	(Voznesenskaya et al., 2001)
Cleomaceae	<i>Cleome paradoxa</i>	(Voznesenskaya et al., 2007)

While most of the described gas exchange data have been obtained under laboratory conditions, some data are available from field measurements. For *F. floridana*, in its natural habit, it was demonstrated that Γ was still low at leaf temperatures between 35°C and 40°C, but dramatically increased in a sympatric C₃ species, and photosynthetic rates were 2.4–4 times higher in the C₃-C₄ intermediate species at the same conditions (Monson and Jaeger, 1991). These data show that the improved gas-exchange characteristics discussed above indeed translate into improved fitness and so provide the basis for the evolution of more C₄-like traits.

With few possible exceptions, significant levels of C₄ cycle activity were not observed in most C₃-C₄ intermediate species. While there is usually some enhanced incorporation of ¹⁴CO₂-carbon into C₄ compounds, this carbon is not conveyed to the C₃ cycle. Hence (the sometimes called ‘type I’) C₃-C₄ intermediate species typically do not have a functional C₄ cycle. This is different in the so-called C₄-like (‘type II’ C₃-C₄ intermediate) *Flaveria* species *F. brownii*, *F. palmeri* or *F. vaginata*, where a limited C₄ cycle operates in parallel with direct CO₂ fixation by Rubisco (Bassüner et al., 1984; Edwards and Ku, 1987; Moore et al., 1989).

Despite the absence of a C_4 cycle, however, leaves of C_3 - C_4 intermediate plants have a distinctive anatomy which is clearly different from C_3 plant and similar to C_4 plants. In comparison with C_3 plants, e.g. the vascular bundles show increased vein density in leaves of C_3 - C_4 intermediate plants and are surrounded by BSC containing a remarkably high number of organelles reminiscent of the Kranz anatomy of C_4 plants. In addition, mitochondria and peroxisomes are found in close association with chloroplasts. A recent re-examination of anatomical features for a broad range of *Flaveria* species demonstrated that key anatomical features of C_4 plants are required for C_3 - C_4 intermediacy and Kranz anatomy is fully developed before complete C_4 biochemistry is achieved (McKown and Dengler, 2007).

It has been early suggested that these specific ultrastructural properties could facilitate reassimilation of photorespiratory CO_2 via the Calvin cycle (Brown, 1980), and C_3 - C_4 intermediate plants are indeed capable of a much more efficient internal recycling of photorespiratory CO_2 than C_3 plants (Holbrook et al., 1985). Quantitative estimates showed that between 70% and 90% of photorespiratory CO_2 is recaptured before it escapes from the leaf of a C_3 - C_4 intermediate plant, which is much higher than the about 30–50% refixation typically observed with C_3 plant leaves (Bauwe et al., 1987).

Following up reports on some atypical features of Gly catabolism, it was hypothesized that a relatively large portion of the GDC activity may take place in the BSC resulting in higher CO_2 recycling and low photorespiration rates in leaves of C_3 - C_4 intermediate species. Because of the similarity of this model to the metabolic situation in C_4 plants, where GDC is confined to the BSC, it was also suggested that C_3 - C_4 intermediate species may have partially developed this biochemical feature of C_4 photosynthesis (e.g. Edwards and Ku, 1987).

This hypothesis was validated by extensive immunolocalisation studies. Where it has been subsequently examined, at least the P-protein of GDC is not uniformly distributed in the leaves of C_3 - C_4 intermediate species, but confined to the BSC. In the leaves of C_3 - C_4 intermediate *Panicum* and *Flaveria* species, e.g. all four GDC subunits are missing in the MC, whereas significant levels of H-, T-, and L-protein are present in the

mesophyll of C_3 - C_4 intermediate *Moricandia* species (e.g. Hylton et al., 1988; Morgan et al., 1993; Devi et al., 1995; Ueno et al., 2003; Voznesenskaya et al., 2007). Because the presence of all GDC subunits is necessary for GDC activity, the deletion of only one subunit will unavoidably cause GDC deficiency in the mesophyll. It shall therefore be noted that an *absolute* confinement of the respective GDC subunits to the bundle sheath neither has been proven nor is likely, because of the un-replaceable function of GDC in C_1 metabolism in all cells (Engel et al., 2007). C_1 metabolism, however, requires much less GDC activity in comparison with the high fluxes through photorespiratory metabolism.

Based on these localization studies and following on from earlier models, a model for photorespiratory metabolism in leaves of C_3 - C_4 intermediate plants has been proposed which can satisfactorily explain the high degree of light-dependent recapture and the low apparent rate of photorespiration (Rawsthorne, 1992). Both MC and BSC of C_3 - C_4 intermediate species contain a fully functional Calvin cycle leading to oxygenation of RubP and hence photorespiratory Gly production in both cell types. Due to the lack of GDC in the mesophyll, Gly accumulates to a considerable steady-state level driving its passive transport into the GDC-containing bundle sheath where decarboxylation results in photorespiratory CO_2 release. This requires high activities of both GDC and SHMT in the bundle sheath, and an about fivefold elevated SHMT activity was indeed detected in the bundle sheath of C_3 - C_4 intermediate plants (Rawsthorne et al., 1988). It should be mentioned that SHMT is also required for the rapid recycling of CH_2 -THF to THF. In contrast to Gly and Ser, neither of these two compounds can equilibrate between different cells. The distinctly elevated levels of Ser in the leaves of C_3 - C_4 intermediate plants suggest that a considerable fraction of this amino acid diffuses back into the mesophyll to ensure continued operation of the mesophyll-located Calvin cycle.

The physiological consequences of this ‘Gly shuttle’ are usually discussed with respect to the high refixation of photorespiratory CO_2 achieved by C_3 - C_4 intermediate plants. In contrast, the inherent potential for elevating bundle sheath CO_2 levels is less often considered (e.g. von Caemmerer, 1989; Bauwe and Kolukisaoglu, 2003).

This latter aspect, however, is possibly of much higher importance for the subsequent evolution of C₄ plants than the pure refixation aspect. In leaves of C₃-C₄ intermediate plants, the C₂ cycle uses Gly as vehicle to move a substantial fraction of freshly assimilated carbon from the mesophyll (the photorespiratory CO₂ ‘pump’s’ inlet) to the bundle sheath (the ‘pump’s’ outlet) where it is released as photorespiratory CO₂. This must have led to an elevated CO₂ concentration in the bundle sheath, as it also follows from model calculations (von Caemmerer, 1989). C₃ plants typically invest very little in chloroplasts in the bundle sheath, which appears economical because of the probably low CO₂ concentration in this location (Fig. 2). The operation of the C₂ pathway, for the time being stabilized by improved refixation, would provide more favorable photosynthetic conditions in BSC, making the accumulation of chloroplasts an advantageous consecutive step. Correspondingly, relocation of the photorespiratory Gly-to-Ser conversion could have been a universal starting point for the subsequent evolution of C₄ photosynthetic features. Further support for this hypothesis will certainly require the identification of C₃-like plants that have GDC already confined to BSC, but still contain only few chloroplasts in these cells. Promising genera to search for such plants could be the well analyzed genus *Flaveria* (McKown and Dengler, 2007) and the more recent additions to the list of C₃-C₄-containing genera, *Cleome* (Marshall et al., 2007) and *Heliotropium* (Vogan et al., 2007).

All known C₃-C₄ intermediate species already partition a significant number of chloroplasts to the BSC and hence possess, roughly speaking, two fractions of Rubisco operating under different CO₂/O₂ ratios. Most likely, the BSC fraction of Rubisco operates under (at least ‘somewhat’) more favorable conditions than the MC fraction. This hypothesis has been tested by quantitative ¹⁴CO₂ labeling experiments with leaves of several *Flaveria* species designed to allow the determination of in-vivo RubP carboxylation/oxygenation ratios (B. Bassünér, T. Pärnik, O. Keerberg and H. Bauwe, unpublished). These data indicate that the C₃-C₄ intermediate species *F. pubescens* has an about twofold increased carboxylation/oxygenation ratio of RubP relative to the C₃ plant *F. cronquistii*. As mentioned above, Rubiscos of these two species show identical in vitro properties

and the enzyme of the C₃-C₄ intermediate plant should hence operate under an approximately doubled mean CO₂ concentration in comparison with that of the C₃ plant.

VI. Future Prospects

Photorespiration is a major and indispensable, although costly, part of the network of C₃ plant primary metabolism. It’s often disputed function is very clear: recycling of the (in C₃ plants) more or (in C₄ plants) less massive amounts of 2PG produced by Rubisco. In light of the fact that many essentials of the C₂ cycle were already discovered in the 1960–1980s, it is surprising that a significant share of the components of the pathway, such as metabolite transporters, probably metabolic enzymes, and perhaps regulatory proteins are not yet known. Likewise, our understanding of interactions with other major metabolic pathways is still very limited, and most of the accumulated knowledge is restricted to the C₃ type of land plant photosynthesis.

Photorespiration is not only a multifaceted and high-throughput biological process of its own importance. It is also a determinant factor of C₃ crop biomass production. In nature, plant growth is probably not very often limited by photosynthetic carbon fixation, but by the availability of nutrients and water, attack of pathogens, and other stresses. In an agricultural environment where those limitations are managed, productivity is co-determined by multiple factors as well, but it fundamentally depends on the photorespiration-affected efficiency of crop plants to convert atmospheric CO₂ into biomass by the process of photosynthesis. Therefore, the negative impacts of photorespiratory CO₂ losses on agricultural productivity have challenged researchers for many decades. It was only very recently that an artificial redirection of photorespiratory C fluxes indeed led to improved growth rates and biomass production of a C₃ plant (Kebeish et al., 2007). In contrast to earlier attempts to modify photorespiratory metabolism, which all failed to improve plant growth, this new result eventually proved that photorespiratory metabolism can be optimized by molecular plant breeding.

Photorespiration occurs in aquatic microorganisms, too, but land plants are in a different

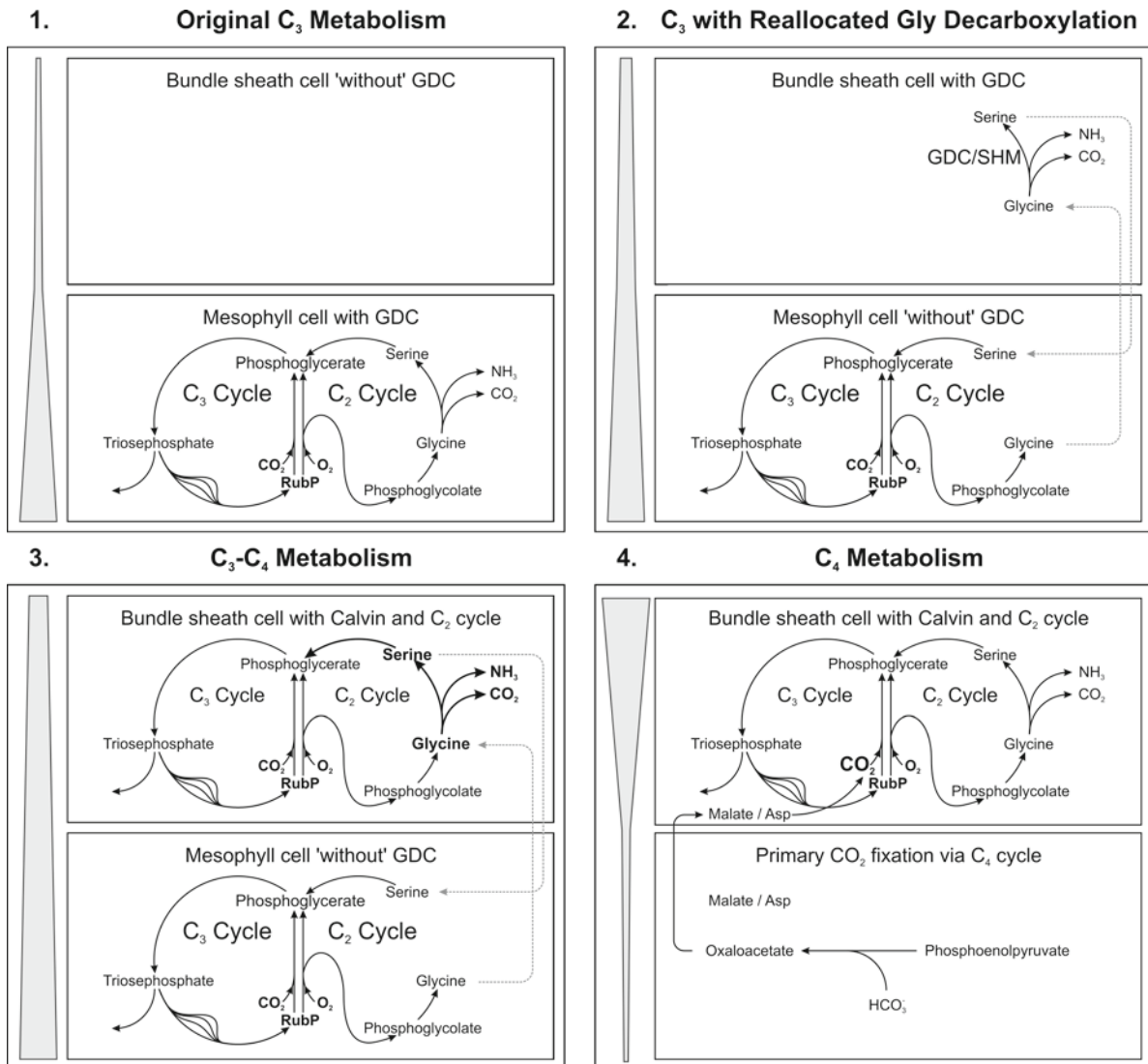


Fig. 2. A plausible sequence of major steps towards C_4 photosynthesis. By reallocation of GDC from the mesophyll (1.) to the bundle sheath, Gly starts to act as a vehicle for transferring a significant fraction of freshly assimilated carbon to the mesophyll (2.) This makes metabolic investment in more bundle sheath chloroplasts beneficial (3.) In addition to the extra CO_2 from Gly decarboxylation, enhanced Calvin cycle activity in the bundle sheath is also pushed by the additional 3PGA, while RubP synthesis in the mesophyll may be discriminated. Once this key anatomical component of C_4 photosynthesis exists, the C_4 cycle gradually first superimposes (in the C_4 -like species) and finally replaces (in C_4 species) the photorespiratory CO_2 pump. Thereafter, smaller investments in mesophyll chloroplast numbers in the mesophyll become feasible (4.). The left-handed grey areas indicate approximate CO_2 concentration gradients between mesophyll and bundle sheath in the four panels.

situation. While they have easier access to CO_2 than bacteria and algae, stomata close under conditions of limiting water supply, which reduces transpiration and enhances photorespiration. Elevated temperatures increase photorespiratory CO_2 losses to even higher levels. These two stressors,

limiting water supply and high temperatures, led to the evolution of C_4 photosynthesis. Photorespiration is hence closely related to the evolution of C_4 plants including highly productive crops. These plants, however, do not merely 'avoid' photorespiratory CO_2 losses. Paradoxically, their

evolution started with a photorespiration-driven primary CCM, which used the large daily amounts of glycine produced during photorespiration instead of a C₄ acid as a transport vehicle for CO₂. It thus appears as if photorespiration contributed to the evolution of C₄ photosynthesis in two ways, by exerting selective pressure in favor of more efficient photosynthesis and by providing the first plan on how to improve the intercellular CO₂ gradients in leaves.

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Nitrogen and Sulfur Metabolism in C₄ Plants

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Summary	109
I. Introduction.....	110
II. Nitrogen Assimilation.....	110
A. Plant Nitrate Assimilation	110
B. Regulation of Nitrate Assimilation	111
C. Nitrate Assimilation in C ₄ Plants.....	113
III. Sulfate Assimilation	114
A. Plant Sulfate Assimilation.....	114
B. Regulation of Sulfate Assimilation	115
C. Sulfate Assimilation in C ₄ Plants	116
IV. Glutathione Synthesis and Reduction	117
A. Regulation of GSH Synthesis.....	117
B. Localization of GSH and GSH Synthesis	118
C. GSH Synthesis in C ₄ Plants	119
V. Physiological Significance of the Distribution of Nitrate and Sulfate Assimilation	120
A. Open Questions on Nitrate Assimilation in C ₄ Plants	120
B. Significance of BSC Localization of Sulfate Assimilation	121
C. Consequences of BSC Localization of Sulfate Assimilation.....	122
VI. Conclusions.....	122
Acknowledgments	122
References	123

Summary

C₄ photosynthetic mechanism is based on a spatial separation of CO₂ assimilating enzymes. The assimilation of two mineral nutrients, nitrogen and sulfur, is also localized in a cell-specific manner in most C₄ species. N assimilation seems to be confined to mesophyll whereas sulfate reduction has been previously reported to be bundle sheath specific. The latter view has been challenged by finding an ubiquitous presence of enzymes of sulfate assimilation in the dicot C₄ species of *Flaveria*. Although inter- and intracellular distribution of enzymes of N assimilation in C₄ plants differ from C₃ plants and C₄ plants have a better N use efficiency, very little is known about the physiological consequences of this distribution. Analogically, no evolutionary advantage for the BSC localization of sulfate assimilation has been identified. On the other hand, the organization and general regulation of the pathways is the same in C₃ and C₄ plants. In this chapter the two essential pathways of plant primary metabolism, nitrate and sulfate assimilation, as well as the synthesis of glutathione, the major sulfur containing metabolite involved in stress defense, will be described. The general regulation of the pathways as well as specific features connected with C₄ photosynthesis will be discussed. The major open questions of N and S metabolism in C₄ plants will be addressed.

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I. Introduction

A characteristic feature of C_4 plants is a cell-specific localization of many enzymes of primary metabolism in bundle sheath cells (BSC) or mesophyll cells (MC) (for details see Chapter 12, this volume). Clearly, the enzymes involved in the primary CO_2 fixation and malate and/or aspartate synthesis, such as cytosolic carbonic anhydrase, phosphoenolpyruvate carboxylase, pyruvate phosphate dikinase, and NADP-malate dehydrogenase, are localized predominantly in the MC, whereas NAD(P)-malic enzyme, Rubisco, Rubisco activase, and some enzymes of the Calvin cycle are found exclusively in BSC (reviewed in Sheen, 1999; Edwards et al., 2001). In most C_4 species analyzed, including maize and *Sorghum*, BSC chloroplasts lack photosystem II and therefore exhibit very little oxygen evolution (Hatch and Osmond, 1976). Consequently, noncyclic electron flow and the capacity for NADPH formation are restricted in BSC chloroplasts. In addition, glycine decarboxylase, a key enzyme of photorespiration, is localized exclusively in BSC of C_4 and C_3 - C_4 intermediate plants (Hylton et al., 1988; see Chapter 6, this volume). The C_3 - C_4 intermediate plants were originally identified by having a CO_2 compensation point intermediate between C_3 and C_4 species. They can be considered as evolutionary intermediates in the path from C_3 to C_4 photosynthesis (Monson and Moore, 1989; Kopriva et al., 1996). The intermediate species possess a Kranz-like anatomy and their apparent photorespiration rate is reduced, due to the confinement of glycine decarboxylase to the BSC and efficient refixation of photorespired CO_2 in these cells (Hylton et al., 1988; Rawsthorne, 1992; cf. Chapter 6, this volume). Some C_3 - C_4 plants are to some extent able to fix CO_2 into malate and aspartate as C_4 species (Bassünér et al., 1984; Monson et al., 1986), but

the compartmentalization of the photosynthetic enzymes is not complete (Bauwe, 1984).

Interestingly, apart from enzymes involved in the C_4 carbon cycle enzymes participating in the assimilation of nitrogen and sulfur are also localized in cell-specific manner in C_4 plants. In this chapter, the two pathways will be described in detail with the focus on their subcellular distribution and the consequence of such distribution for the regulation of the pathways and for the performance of C_4 plants.

II. Nitrogen Assimilation

Nitrogen (N) is the most abundant mineral nutrient in plant tissues but also the element most frequently limiting plants growth (Vance, 2001). The major N sources are inorganic nitrate and ammonium, however, plants developed several other strategies to meet their demand for N. The best known alternative source of N for plant nutrition are the symbiotic N_2 fixing root nodules (Day et al., 2001). Also mycorrhizal fungi have been shown to contribute to plant N acquisition (Chalot and Brun, 1998), as well as N_2 fixing bacteria in the phyllosphere (Papen et al., 2002). In addition, uptake of organic N compounds, such as amino acids, may cover substantial part of N acquisition in certain habitats (Persson et al., 2003). C_4 plants are capable of mycorrhiza symbiosis; however, no C_4 species form nodules with symbiotic bacteria.

A. Plant Nitrate Assimilation

The most common form of N plants acquire from the soil is nitrate. Nitrate is transported into plant cells by nitrate transporters (Fig. 1). Three uptake systems are responsible for uptake of nitrate into the roots: a constitutive high affinity uptake system, an inducible high affinity system and a low affinity system (Miller et al., 2007). However, further transporters are necessary to facilitate xylem loading and distribution of nitrate throughout the plant as well as its storage in the vacuoles. Two major classes of nitrate transporters exist in plants, the NRT1/PRT family responsible for low affinity nitrate uptake as well as amino acid and peptide transport, which contains 53 genes in Arabidopsis, and NRT2 family of high affinity

Abbreviations: APR – Adenosine 5'-phosphosulfate reductase; APS – Adenosine 5'-phosphosulfate; ATPS – ATP sulfurylase; BSC – Bundle sheath cells; Γ ECy-Glutamylcysteine; Γ ECSy-Glutamylcysteine synthetase; GOGAT – Glutamate synthase; GR – Glutathione reductase; GS – Glutamine synthetase; GSH – Glutathione; GSHS – Glutathione synthetase; GSSG – Oxidized glutathione, glutathione disulfide; MC – Mesophyll cells; NR – Nitrate reductase; OAS – O-Acetylserine; ROS – Reactive oxygen species;

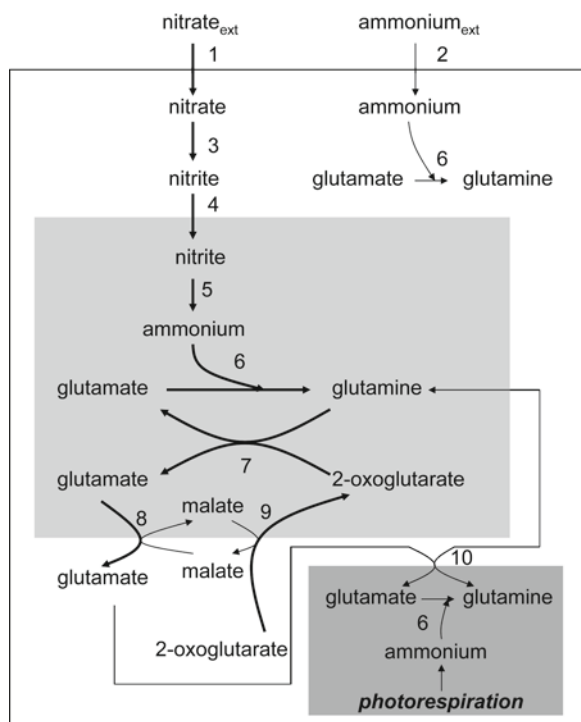


Fig. 1. Schematic representation of plant nitrate assimilation. *Dark shaded rectangle* represents mitochondria, *light shaded* one denotes plastid. Enzymes are symbolized by numbers: 1 – nitrate transporter, 2 – ammonium transporter, 3 – nitrate reductase, 4 – nitrite transporter, 5 – nitrite reductase, 6 – glutamine synthetase, 7 – glutamate synthase, 8 – plastidic glutamate–malate translocator, 9 – plastidic 2-oxoglutarate–malate translocator, 10 – mitochondrial glutamate–glutamine translocator. The major pathway of nitrate assimilation is printed **bold**.

nitrate transporters composed of seven members in this species. Genes of both classes have been found throughout the plant kingdom, including C₄ plants (Santi et al., 2003).

Nitrate is reduced to ammonium in two spatially separated steps. In the cytosol, nitrate reductase (NR) transfers electrons from NADH to nitrate to form nitrite. Nitrite is transported into plastids and reduced to ammonium by ferredoxin dependent nitrite reductase. Ammonium is assimilated into organic compounds by glutamine synthetase (GS) which uses glutamate as the ammonium acceptor. GS is coupled with glutamate synthase (GOGAT) which transfers the amino group of glutamine to 2-oxoglutarate to form two molecules of glutamate, in a GS/GOGAT cycle. The cycle thus uses one molecule of 2-oxoglutarate to assimilate one NH_4 molecule and export one molecule of the

amino acid glutamate (Fig. 1, Mifflin and Habash, 2002; Stitt et al., 2002; Weber and Flugge, 2002). Glutamate is the source of reduced N for the synthesis of other amino acids, with the first step in this route being usually transamination with oxaloacetate to form aspartate catalyzed by aspartate aminotransferase.

Ammonium in plants originates not only from nitrate reduction. It is present as a nutrient in the soil and can be transported into plants by ammonium transporters (von Wiren et al., 2000). In fact, ammonium is the preferred source of inorganic N for many plant species and optimal growth is often achieved only when both nitrate and ammonium are present (Bloom et al., 1993). Ammonium is also produced by photorespiration, in the mitochondrial glycine decarboxylation reaction (cf. Chapter 6, this volume; Linka and Weber, 2005). An efficient assimilation and re-assimilation of ammonium thus must occur in all compartments to prevent its accumulation to toxic levels. Indeed, plastidic and cytosolic isoforms of GS exist in all plant species (Inokuchi et al., 2002) and the plastidic GS is dual targeted also to mitochondria (Taira et al., 2004). GOGAT is present in plants in multiple forms as well, the major ferredoxin dependent enzyme is found predominantly in leaves, whereas in non-photosynthetic cells NADH-GOGAT is the prevalent isoform. Both forms of GOGAT are, however, localized to plastids (Tobin and Yamaya, 2001). Another enzyme, the glutamate dehydrogenase, which in vitro synthesizes glutamate from ammonium and 2-oxoglutarate, has been long implicated to participate in ammonium assimilation. Recent findings however suggest that the major function of this enzyme is the provision of carbohydrate skeletons for carbon metabolism from amino acids during protein degradation (Miflin and Habash, 2002).

B. Regulation of Nitrate Assimilation

Nitrate and ammonium assimilation is strongly regulated by the N demand of the plant and N supply and is closely connected to carbon metabolism. The components of the pathway undergo a coordinated regulation which often occurs on multiple levels. So, nitrate uptake is induced in the presence of nitrate and feedback inhibited by amino acids and ammonium. It is regulated by

light and CO₂ concentration via the availability of carbohydrates (Rufty et al., 1989; Stitt et al., 2002). Accordingly, nitrate uptake was demonstrated to be under diurnal regulation with maximum activity during day and minimum activity at night (Lejay et al., 1999). The decrease of uptake at night can be reversed by feeding sucrose. The coordination between N demand and N supply in control of nitrate uptake was proposed to be achieved by internal cycling of amino acids and/or cytokinins between the roots and the shoots (Gessler et al., 2004). Ammonium transport seems to be regulated in a similar manner to nitrate transport: it undergoes a day night rhythm and the reduction of uptake in night can be prevented by sucrose treatment and is inhibited by glutamine (Lejay et al., 2003; Loque and von Wiren, 2004). However, in contrast to nitrate transport ammonium uptake seems to be regulated primarily by local and not by systemic signals (Loque and von Wiren, 2004).

NR is a well studied enzyme which undergoes a complex regulation. NR expression is controlled by circadian clock and it is also induced by light and by sugars in the dark (Cheng et al., 1992; Campbell, 1999). The level of NR transcript is modulated by CO₂ availability; it decreases when CO₂ assimilation is diminished, e.g. due to water stress (Foyer et al., 1998) and increases upon exposure of plants to elevated CO₂ (Fonseca et al., 1997). The rapid response of NR activity to environmental stimuli is, however, caused mostly by a rapid post-translational regulation. Upon sudden decrease in photosynthesis NR is rapidly and reversibly phosphorylated (Kaiser and Huber, 2001). The phosphorylation of NR enables its binding to 14-3-3 proteins, which seems to be the actual mechanism of inactivation and might be a signal for NR degradation (Lillo et al., 2004). The rapid response seems to be necessary to prevent accumulation of toxic levels of nitrite. NR is present in leaves and roots with different distribution of the activity among these organs in different plant species. Even a relatively low activity, however, is important for correct C/N balance as revealed by experiments with tobacco lacking root NR (Kruse et al., 2002).

A central role in controlling nitrate and ammonium assimilation is occupied by GS. In contrast to NR and nitrite reductase, which are usually encoded by one to two genes, GS and GOGAT

are encoded by small multigene families of three to six members. This accounts for a great versatility of regulation with some genes expressed in tissue specific manner and at certain developmental stages while some being universal (Mifflin and Habash, 2002). The organel-targeted GS2 is especially important for reassimilation of the photorespiratory NH₃ and is, therefore, highly expressed in green tissues and inducible by light (Oliveira and Coruzzi, 1999). Cytosolic GS1 often fulfils specialized roles, such as in nodules to assimilate the ammonium produced by nitrogen fixation in the bacteroids (Stanford et al., 1993) or in tissues involved in transport of reduced N (Mifflin and Habash, 2002). GS is regulated transcriptionally but undergoes also post-transcriptional and post-translational regulation. Both GS forms are subjected to phosphorylation and bind 14-3-3 proteins. Cytosolic GS is increased during senescence to facilitate the remobilisation of N (Habash et al., 2001). GS is also often associated with improved yield of crop plants by quantitative genetics (Hirel et al., 2001). Indeed, overexpression of pine cytosolic GS in poplar resulted in significantly enhanced growth (Gallardo et al., 1999) whereas disruptions of specific GS1 genes in maize led to reduced kernel size and/or number (Martin et al., 2006).

Despite the importance of N assimilation surprisingly little is known about the molecular mechanisms of the regulation of N acquisition and metabolism. Nitrate transporter NRT1;1 seems to play an important role in N sensing (Remans et al., 2006). Nitrate has clearly a role as a signal since microarray analysis of Arabidopsis mutants in nitrate reductase revealed that expression of 595 genes including several transcription factors responded to nitrate treatment (Wang et al., 2004). Different amino acids act as feedback inhibitors of N assimilation, whereas glutamine seems to be most important in regulation of nitrate and ammonium uptake, glutamate, cysteine, and asparagine inhibit nitrate reduction (Stitt et al., 2002; Gessler et al., 2004). Sugars are clearly involved in the regulation of N assimilation, both as inducers and repressors, when their content is low, however the mechanisms of their action are not understood (Stitt et al., 2002). A special role in regulation of N assimilation, specifically in signalling N-status of the plant, is played by the phytohormones cytokinins. Cytokinins are produced in the root

at sufficient N supply and after transport into the shoots induce expression of genes of N assimilation (Wagner and Beck, 1993; Mok and Mok, 2001). They are, however, also transported from the shoot to the root and affect nitrate uptake (Collier et al., 2003).

C. Nitrate Assimilation in C₄ Plants

Most of our knowledge on regulation of N metabolism has been derived from C₃ plants and no differences in organization and regulation of nitrate assimilation between C₃ and C₄ plants were described. However, C₄ plants differ significantly in the compartmentalization of N metabolism and also in N use efficiency. It has long been known that in terms of growth rate C₄ grasses respond better to applied N than C₃ grasses (Hallock et al., 1965). The C₄ species clearly exhibited a higher N use efficiency, expressed as biomass per unit N in plant (Brown, 1978). The greater N use efficiency seems to be connected with a lower content of Rubisco in the leaves due to the CO₂ concentrating mechanism. The N use efficiency in C₄ plants will be discussed in detail by Ghannoum et al. in Chapter 8 of this volume.

The intracellular localization of N assimilation in maize follows the pattern of C₃ plants, i.e. cytosolic localization of NR and the presence of nitrite reductase in chloroplast (Ritenour et al., 1966). Because of the observed differences in distribution of carbon metabolism in C₄ plants and in structures of MS and BSC chloroplasts the question of intercellular localization of nitrate assimilation has frequently been addressed in the early years of C₄ photosynthesis research. By enzyme activity assays Mellor and Tregunna (1971) found that NR is prevalently present in MC of three C₄ species with different types of BSC chloroplasts: maize, *Sorghum sudanense*, and *Gomphrena globosa*. NADH dependent glutamate dehydrogenase, which at that time was believed to be the only ammonium assimilating enzyme, was localized in BSC. Nitrite reductase activity has been found in MC of maize and *S. sudanense*, but in BSC of *G. globosa* which seems to imply that it is associated with the presence of chloroplasts with grana (Mellor and Tregunna, 1971). Also in *Eleusine coracana* NR and nitrite reductase occurred predominantly in MC (Rathnam and Das, 1974). In a more systematic approach

analysing five C₄ species of all three metabolic subtypes, i.e. NADP-malic enzyme, NAD-malic enzyme, and phosphoenolpyruvate carboxykinase, NR, nitrite reductase, GS, and GOGAT were coordinately localized in MC, except *Panicum maximum*, where nitrite reductase was present both in MC and BSC (Rathnam and Edwards, 1976). On the other hand, in another study with maize, GS and GOGAT have been found in both cell types but predominantly in BSC (Harel et al., 1977). Similarly, in *Digitaria sanguinalis* NR and nitrite reductase were found exclusively in MC, GS was equally distributed between MC and BSC, and GOGAT was predominantly localized to BSC (Moore and Black, 1979).

Since all the localization studies were based on distribution of enzyme activities in differentially homogenized leaves, it was difficult to conclude on exclusivity of the intercellular distribution of the N assimilation pathway and its components. Importantly, therefore, a clear evidence for an exclusive localization of maize NR in the cytosol of MC was obtained by immunogold labelling (Vaughn and Campbell, 1988). Similarly, the ambiguities in distribution of ammonium assimilation were clarified by Becker et al. (1993) who showed that both cytosolic and plastidic GS are present in both cell types, whereas ferredoxin dependent GOGAT is almost completely confined to BSC chloroplasts.

An interesting feature of C₄ plants is a relatively high cytosolic GS activity. Whereas in most C₃ species cytosolic GS1 accounts for less than 30% of total leaf GS activity, in most C₄ plants analyzed to date GS1 activity is higher or equal to the plastidic GS2 (McNally et al., 1983). Analyzing *Panicum* species of different type of photosynthesis Hirel et al. (1983) showed that the presence of GS1 activity in leaf correlated with C₄ photosynthesis, with the C₃–C₄ intermediate *P. milliodes* possessing foliar GS1 activity intermediate between the C₃ and C₄ species. The ratio in accumulation of the two GS isoforms also differs in between the two cell types of C₄ plants. GS1 seems to be more abundant in MC, whereas both isoform are present at the same level in BSC (Becker et al., 2000). Clearly, due to substantially reduced and BSC confined photorespiration much less ammonia is produced in C₄ leaves than in C₃ leaves and therefore the demand for its rapid refixation by GS is lower. The MC localization of

nitrate reduction implies an increased need for transport of reduced N between MC and BSC, which might explain the need for higher cytosolic GS. The evolutionary advantage of the spatial distribution of nitrate assimilation in C4 plants is, however, not known. Interestingly, assimilation of another essential mineral nutrient, sulfate, is also distributed in cell-specific manner in C4 plant species.

III. Sulfate Assimilation

Sulfur is essential for all living organisms as a constituent of the amino acids cysteine and methionine, many coenzymes such as iron sulfur centers, thiamine, lipoic acid, etc., and various other compounds of primary and secondary metabolism. In most of these compounds sulfur is present in the reduced (−2) form of organic sulfide. The most common form of sulfur in nature is, however, oxidized as inorganic sulfate (+6). Plants, algae, and many microorganisms are able to directly utilize sulfate, reduce it and incorporate into bioorganic molecules, which are the form of sulfur accessible to animals and other organisms. The pathway of sulfate assimilation is thus an essential component of plant primary metabolism.

A. Plant Sulfate Assimilation

The pathway of sulfate assimilation in plants has been completely resolved only relatively recently (Suter et al., 2000) and has lately been subjected to several comprehensive reviews (Leustek et al., 2000; Saito, 2004; Rausch and Wachter, 2005; Kopriva, 2006). Sulfate uptake into plant cells is facilitated by sulfate transporters (Fig. 2). Also sulfate transporters form a large multigene family with 12–15 members differing in affinity to sulfate and tissue distribution (Buchner et al., 2004). Because it is a very stable and inert compound, before reduction sulfate has to be activated to adenosine 5'-phosphosulfate (APS) by adenylation catalyzed by ATP sulfurylase (ATPS). APS is reduced to sulfite by APS reductase (APR). Sulfite is further reduced to sulfide by ferredoxin dependent sulfite reductase. Sulfide is then incorporated into the amino acid skeleton of *O*-acetylserine (OAS), which is synthesized by

acetylation of serine by serine acetyltransferase, to form cysteine in a reaction catalyzed by *O*-acetylserine (thiol)lyase (Fig. 2; Leustek et al., 2000; Kopriva, 2006). Cysteine is the source of reduced sulfur for synthesis of methionine, iron sulfur clusters, and other compounds.

Sulfate assimilation is confined to plastids, however, some reactions occur also in other compartments. The cysteine synthesis, e.g., proceeds in all three compartments capable of protein synthesis, i.e. plastids, cytosol, and mitochondria (Wirtz et al., 2004). ATPS activity was detected both in plastids and in the cytosol (Lunn et al., 1990; Rotte and Leustek, 2000). On the other hand the enzymes involved in reductive steps of the pathway, APR and sulfite reductase, are localized exclusively in plastids (Brunold and Suter, 1989; Prior et al., 1999; Koprivova et al., 2001). Total foliar ATPS and APR activity decrease with

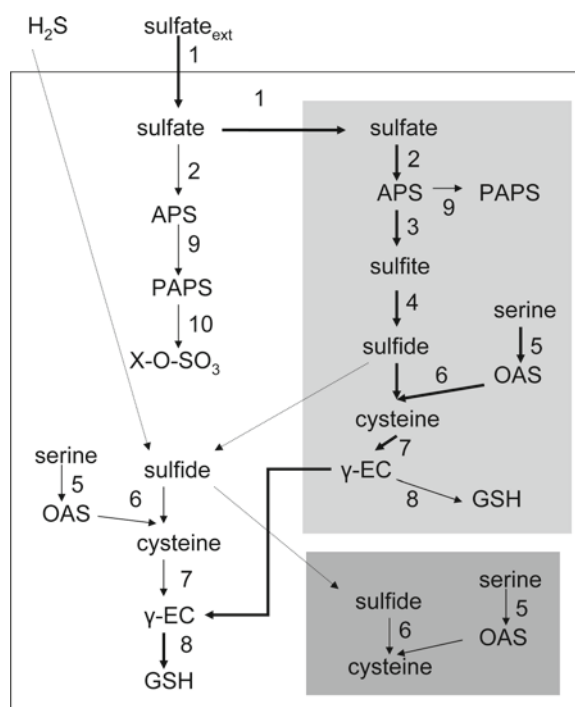


Fig. 2. Schematic representation of plant sulfate assimilation. Dark shaded rectangle represents mitochondria, light shaded one denotes plastid. Enzymes are symbolized by numbers: 1 – sulfate transporter, 2 – ATP sulfurylase, 3 – APS reductase, 4 – sulfite reductase, 5 – serine acetyltransferase, 6 – *O*-acetylserine (thiol)lyase, 7 – γ -glutamylcysteine synthetase, 8 – glutathione synthetase, 9 – APS kinase, 10 – sulfotransferase. The major pathway of sulfate assimilation and glutathione synthesis (in Arabidopsis) is printed bold.

the leaf age in *Arabidopsis* (von Arb and Brunold, 1986; Rotte and Leustek, 2000), however, the cytosolic and plastidic ATPS are regulated differently. Whereas the plastidic activity declines with time, the cytosolic is increasing with leaf age. This indicates different roles of ATPS in the two compartments: a provision of APS for sulfate reduction for biosynthetic processes required for growth in plastids and involvement in synthesis of secondary compounds in the cytosol (Rotte and Leustek, 2000).

The reduction of sulfate occurs predominantly in leaves, and reduced sulfur compounds are distributed to sink tissues via phloem (Herscbach and Rennenberg, 2001). It appears, however, that most tissues are capable of sulfate reduction, including roots and developing seeds (Brunold and Suter, 1989; Tabe and Droux, 2002). Indeed, available microarray data in the Genevestigator database reveals the presence of mRNA for the genes of sulfate assimilation, such as APR or sulfite reductase, in all *Arabidopsis* organs, including flowers and siliques (Zimmermann et al., 2004). These findings were corroborated using promoter::GUS fusions which clearly showed activity of APR and ATPS promoters in all tissues of *Arabidopsis* (A Koprivova, C Matthewman, S Kopriva, 2008, unpublished). However, whether the sulfate reduction rate in these cells is sufficient to cover their needs for reduced sulfur instead of relying on long distance transport of organic sulfur compounds, such as glutathione or S-methylmethionine, remains to be seen (Herscbach and Rennenberg, 2001). However, there is a group of plants that lacks the ability to reduce sulfate in a great portion of their cells, the monocot C₄ plants (reviewed in Kopriva and Koprivova, 2005).

B. Regulation of Sulfate Assimilation

The sulfate assimilation pathway is extensively regulated in a demand-driven manner to prevent accumulation of toxic intermediate and to provide optimal rate of cysteine production (Lappartient and Touraine, 1996; Leustek et al., 2000; Kopriva, 2006). Thus, when demand for reduced sulfur is increased due to enhanced protein synthesis or increased turnover of the sulfur containing tripeptide glutathione (see below) the flux through the pathway is increased (Lappartient and Touraine, 1996). On the other hand, when plants are exposed

to reduced sulfur in the atmosphere or rhizosphere or when there is not sufficient supply of the amino acid acceptor due to carbon or nitrogen deficiency, the pathway is downregulated (Koprivova et al., 2000; Westerman et al., 2001; Kopriva et al., 2002; Vauclare et al., 2002). Although regulation of all components of the sulfate assimilation has been described, control flux analysis revealed that APR and sulfate uptake possess the highest control over the pathway (Vauclare et al., 2002). In line with these observations, APR activity and mRNA accumulation undergoes a diurnal rhythm with a maximum during light and minimum at night (Kocsy et al., 1997; Kopriva et al., 1999). The high activity coincides with the highest flux through the pathway (Kopriva et al., 1999).

Despite a comprehensive knowledge on the physiological responses of sulfate assimilation to various environmental stimuli, very little is known about the molecular mechanisms of regulation and the signals involved. Several compounds have been proposed as molecular signals in regulation of the pathway. OAS accumulates during sulfur deficiency and when supplied exogenously induces mRNA accumulation of many genes of sulfate uptake and assimilation (Neuenschwander et al., 1991; Koprivova et al., 2000; Hopkins et al., 2005). Indeed, system biology approaches revealed a correlation of OAS content with transcript accumulation of many genes during a sulfur starvation response and large set of genes regulated in the same way by sulfate starvation and OAS treatment (Hirai et al., 2003, 2005). However, not all genes induced by sulfur deficiency are regulated by OAS and the timing of OAS accumulation seems to fall behind the induction of sulfate uptake by sulfur starvation in potato, so that OAS is probably not the only signal in the sulfur starvation response (Hirai et al., 2003, 2005; Hopkins et al., 2005). Glutathione (GSH) may also represent the signal of sulfur status of the plant, as depletion of GSH by treatment with an inhibitor of its synthesis, buthionine sulfoximine (BSO), leads to upregulation of APR, whereas GSH itself inhibits sulfate uptake and reduction (Lappartient and Touraine, 1996; Vauclare et al., 2002; Hartmann et al., 2004). ATPS and APR activity are reduced also in plants treated with cysteine, however, the feedback inhibition is alleviated when simultaneously BSO blocks the synthesis of GSH from the additional cysteine

(Lappartient and Touraine, 1996; Vauclare et al., 2002). In addition, several phytohormones have been shown to modulate expression or activity of various components of sulfate assimilation, such as jasmonate (Harada et al., 2000; Jost et al., 2005), cytokinins (Ohkama et al., 2002), or abscisic acid (Barroso et al., 1999). Only very recently, however, the first transcription factor and *cis* element responsible for regulating genes for sulfate transporter by sulfur starvation have been identified (Maruyama-Nakashita et al., 2005, 2006).

C. Sulfate Assimilation in C_4 Plants

In a search for further metabolic processes spatially distributed in C_4 plants it was soon discovered that in maize 75–100% of total leaf ATPS activity is confined to BSC (Gerwick and Black, 1979; Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1984). These findings were extended to 17 other C_4 species of all three C_4 subtypes, where 95–100% of total leaf ATPS was localized in BSC chloroplasts (Gerwick et al., 1980). Also APR was found almost exclusively in BSC of maize (Schmutz and Brunold, 1984; Burgener et al., 1998), while the activities of sulfite reductase and OAS(thiol)lyase could be measured at comparable levels in MC and BSC (Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1985). In attempts to decipher the mechanism of the spatial distribution of these enzymes, northern analysis of BSC and MC specific RNA revealed that mRNA levels for ATPS, APR, and sulfite reductase were detected in BSC only, whereas the mRNA for OAS(thiol)lyase was found in both MC and BSC (Kopriva et al., 2001). The cell-specific localization of enzymes of sulfate assimilation in maize seems, therefore, to be regulated on the transcriptional level, at least under standard growth conditions. However, the situation might be different under stress. Maize is especially sensitive to chilling which induces a strong oxidative stress characterized by production of reactive oxygen species (ROS). In maize plants subjected to chilling APR activity and mRNA level were greatly increased in BSC, and mRNA but not enzyme activity was also detectable in MC. This indicates an additional post-transcriptional mechanism to ensure the BSC specific localization of sulfate assimilation in maize (Kopriva et al., 2001).

The exclusive localization of ATPS and APR in BSC of maize means that an efficient transport of reduced sulfur compounds from BSC to MC must exist. MC are capable of cysteine synthesis, therefore, the transport form of reduced sulfur could be sulfide, cysteine, methionine or glutathione. Feeding of isolated bundle sheath strands from maize with [^{35} S]sulfate resulted in secretion of labelled cysteine into the nutrient solution (Burgener et al., 1998). It seems therefore, that cysteine (or its oxidized form cystine) is the most probable transport metabolite for reduced sulfur (Fig. 2). Interestingly, [^{35} S]sulfate feeding experiments also imply that glutathione synthesis was predominantly localized in MC (Burgener et al., 1998; and see below).

The biological significance of the BSC specific localization of sulfate assimilation in C_4 plants is not obvious and, similarly, it is not clear whether this localization is a pre-requisite or a consequence of C_4 photosynthesis. To answer this question we addressed the distribution of ATPS and APR in *Flaveria* species with different types of photosynthesis (Koprivova et al., 2001). The dicot genus *Flaveria* (*Flaveriinae*–*Asteraceae*) is an excellent model to study the evolution of C_4 photosynthesis because, beside C_3 and C_4 species, it comprises a relatively large number of C_3 – C_4 intermediates (Ku et al., 1991). A continuous gradation in the physiology and biochemistry of C_4 photosynthesis can be found among *Flaveria* species (Monson and Moore, 1989). Indeed, we showed previously that the C_3 – C_4 intermediate *Flaveria* species are true evolutionary intermediates in the path from C_3 to C_4 photosynthesis, based on phylogenetic analysis of the H-protein subunit of glycine decarboxylase (Kopriva et al., 1996).

The aim of the study with *Flaveria* was to compare the intercellular distribution of sulfate assimilation in C_3 , C_3 – C_4 , and C_4 species. We expected a BSC localization of APR and ATPS in C_4 *Flaveria* species and a ubiquitous distribution in C_3 species. The distribution of the two enzymes in the intermediate ones would be an excellent indication for the evolutionary sequence of the processes leading to the BSC localization of sulfate assimilation. Surprisingly however, northern analysis of cell-specific RNA and in situ hybridization revealed that in the C_4 species *F. trinervia* mRNA for ATPS and APR were present at comparable levels in both MC and BSC. Immunogold

electron microscopy confirmed the presence of APR protein in chloroplasts of both cell types (Koprivova et al., 2001). Consequently, the localization of assimilatory sulfate reduction in BSC cannot be a general C₄ trait.

How can these findings be explained taking into account the results of Gerwick et al. (1980) who showed the exclusive BSC localization of ATPS in 17 C₄ species? Whereas the 17 species analyzed before were monocots, *F. trinervia* and *F. australasica* were the first C₄ dicots where the subcellular localization of the pathway was addressed. It has to be concluded that sulfate assimilation is exclusively localized in BSC only in C₄ monocots and this distribution is thus neither a pre-requisite nor a consequence of C₄ photosynthesis (Koprivova et al., 2001; Kopriva and Koprivova, 2005). However, the BSC localization of ATPS in maize and other C₄ plants analyzed by Gerwick et al. (1980) is not a general trait of monocots either, since in wheat, a C₃ monocot, ATPS and APR are present in all cell types (Schmutz and Brunold, 1984). Whether C₄ *Flaveria* species are exceptions or the rule for C₄ dicots remains to be established, however, it is evident that the previously generally accepted link between C₄ photosynthesis and BSC localization of sulfate assimilation is no longer valid.

The investigations of sulfate assimilation in *Flaveria* resulted in an additional interesting finding. The APR activity and levels of thiols were significantly higher in leaves of C₄-like and C₄ species than in those of C₃ and C₃-C₄ species (Koprivova et al., 2001). APR, cysteine and GSH content correlated with the degree of development of C₄ photosynthesis expressed by CO₂ compensation points (Kopriva and Koprivova, 2005). The actual foliar concentration of GSH is highly dependent on environmental conditions and, therefore, varies significantly among different plant species but also within single species. Since the *Flaveria* species were grown at identical controlled conditions, it seems that the clear tendency to towards higher APR activity and GSH content with increasing C₄ photosynthesis might be a result of the adaptation to different habitats. C₄ photosynthesis is especially advantageous in dry and warm conditions. The higher GSH contents in C₄ *Flaveria* might thus be a mechanism to cope with increased oxidative stress caused by such environmental conditions. According to the

demand-driven regulation, APR activity would have to be elevated to supply sufficient cysteine for the GSH synthesis.

IV. Glutathione Synthesis and Reduction

Among sulfur containing metabolites the tripeptide glutathione (γ -glutamyl-cysteinyl-glycine) plays the most versatile role being essential for plant stress defense, redox regulation and signaling, control of cell cycle, and as a storage and transport form of reduced sulfur (May et al., 1998a; Noctor et al., 1998a; Foyer and Rennenberg, 2000). GSH is involved in plant defense against ROS as a reductant of dehydroascorbate in the glutathione-ascorbate cycle (Noctor and Foyer, 1998). It is indispensable for protection against heavy metals as a substrate for synthesis of phytochelatin, which chelate the metals and enable their sequestration into the vacuoles (Cobbett and Goldsbrough, 2002). Conjugation of xenobiotics with GSH is the first step in their detoxification and a molecular basis of resistance to some herbicides (Dixon et al., 1998). Not surprisingly, GSH accumulates to high levels reaching concentration of several mM (Meyer and Fricker, 2000; Hartmann et al., 2003). Due to its role in stress defense and specifically in the response to chilling, GSH synthesis and its regulation was often addressed in C₄ plants (Rueggsegger and Brunold, 1993; Doulis et al., 1997; Kocsy et al., 2001; Gómez et al., 2004; Kopriva and Koprivova, 2005).

A. Regulation of GSH Synthesis

GSH is synthesized from its constituent amino acids in two ATP dependent steps. Firstly a γ -glutamylcysteine synthetase (γ -ECS) synthesizes γ -glutamylcysteine (γ -EC) from glutamate and cysteine. Subsequently, glycine is added to the γ -EC by glutathione synthetase (GSHS). GSH synthesis is an essential process, since disruption of γ ECS gene by T-DNA insertion is embryolethal (Cairns et al., 2006). The rate of GSH synthesis is primarily controlled by the availability of the constituent amino acids, with the regulation of γ -ECS playing an additional substantial role (Kopriva and Rennenberg, 2004). Most of our knowledge on this regulation is derived from

studies with poplar overexpressing bacterial genes for γ -ECS and GSHS (e.g. Strohm et al., 1995; Noctor et al., 1996, 1998b). γ -ECS and GSHS are induced at conditions of high demand for GSH, such as after exposure to heavy metals or herbicide safeners (Rueggsegger and Brunold, 1992; Farago and Brunold, 1994; Schaefer et al., 1998). γ -ECS is more important for the control of GSH synthesis because GSH content was increased only in poplars overexpressing γ -ECS and not GSHS (Strohm et al., 1995). The enzyme seems to undergo a complex regulation on different levels. Heavy metals induce mRNA accumulation of γ -ECS (and GSHS) (Schaefer et al., 1998; Xiang and Oliver, 1998), but a post-transcriptional regulation has also been described (May et al., 1998b). On the other hand, the enzyme is feedback inhibited by GSH, which causes a reversible conformational change due to a reduction of an internal disulfide bond (Jez et al., 2004; Hothorn et al., 2006). The γ -ECS regulation thus seems to follow the same demand-driven manner as described for the components of sulfate assimilation. Indeed, since at most physiological situations cysteine limits GSH synthesis γ -ECS is often regulated in the same way as APR or other enzymes of sulfate assimilation (Rueggsegger et al., 1990; Brunner et al., 1995). At some conditions however, e.g. during night or at non-photorespiratory conditions, synthesis of GSH is controlled by availability of glycine (Noctor et al., 1999).

During its function in the glutathione–ascorbate cycle GSH is oxidized. The oxidized form of glutathione, GSSG, is reduced by the action of glutathione reductase (GR) which utilizes the electrons from NADPH. GR maintains the reducing environment in cells so that GSSG normally forms no more than 5% of total glutathione. Increased ratio of GSSG to total GSH is thus indicative of oxidative stress (Mullineaux and Rausch, 2005).

B. Localization of GSH and GSH Synthesis

GSH is present in all cellular compartments; however, the quantitative distribution is not resolved yet. Currently, two approaches are being used to quantify GSH on the subcellular level. The confocal laser scanning microscopy method makes use of the fluorescence of the GSH conjugate

with monochlorobimane (Meyer et al., 2001). However, since the conjugation is catalyzed enzymatically by GSH transferase, the method can directly measure only cytosolic GSH, whereas the organellar pools can be estimated by HPLC after cell disruption and additional chemical labeling of the remaining GSH by monobromobimane (Hartmann et al., 2003). The second method is based on immunohistochemistry with antibodies against GSH (Zechmann et al., 2005). This approach shows the highest density of labeling in mitochondria and the lowest in plastids. Unfortunately, none of the methods have been used for localization of GSH in C_4 plants.

GSH synthesis has been shown to take place in the cytosol and plastids (Hell and Bergmann, 1990; Noctor et al., 1998a). However, recently it was revealed that the two steps of GSH biosynthesis may be spatially separated, at least in some plant species. In *Arabidopsis* and *Brassica juncea* γ -ECS seems to be exclusively localized in the plastids whereas GSHS is present in both plastids and cytosol (Wachter et al., 2005). The γ -EC may thus not only be a precursor of GSH but also play important roles in transport of reduced sulfur from plastids to the cytosol and possibly in signaling of the redox status of the chloroplast. This conclusion is supported by the identification of the *regulator of APX2 (rax1)* mutant in *Arabidopsis* (Ball et al., 2004). This mutant constitutively expresses cytosolic ascorbate peroxidase, which is normally inducible by photooxidative stress, and contains only approximately 50% of normal foliar GSH levels. In *rax1* thus the chloroplast–cytosol signaling is clearly disturbed. The *rax1* phenotype is caused by an R(229)-K substitution in γ -ECS protein (Ball et al., 2004). Several other *Arabidopsis* mutants are associated with γ -ECS and low GSH levels, the *cadmium sensitive cad2* (Cobbett et al., 1998), *root meristemless rml1* (Vernoux et al., 2000), and *phytoalexin-deficient pad2* (Parisy et al., 2007), but none of them displays the same effect on the chloroplast–cytosol signaling.

On the other hand, γ -ECS and GSHS were detected by immunolocalization in both chloroplasts and cytosol of maize (Gómez et al., 2004). Also, expression of the bacterial gene for γ -ECS both in plastids and in the cytosol led to an increase in foliar GSH content in poplar (Noctor et al., 1996, 1998b). Interestingly, the increase

in GSH content in poplar plants overexpressing γ -ECS in the cytosol seems to be confined to this compartment which implies a limited exchange of GSH between cytosol and plastids (Hartmann et al., 2003). GR is localized in cytosol, plastids, and mitochondria (Edwards et al., 1990). Interestingly, a single gene encodes both plastidic and mitochondrial isoform of GR due to a presence of a dual-specificity targeting peptide (Chew et al., 2003).

GSH is present and can be synthesized in all plant organs. Obviously, in many occasions cell specific differences in GSH levels have been found. In maize roots, GSH levels form a clear gradient from the highest at root tip to the lowest in the mature portion of the root (Kopriva et al., 2001). When investigated at the cellular level by confocal laser scanning microscopy approximately twofold higher cytosolic GSH concentration was measured in atrichoblasts than in trichoblasts (Meyer and Fricker, 2000). In addition high GSH levels were detected in root cap while markedly lower GSH was found in quiescent centers (Sanchez-Fernandez et al., 1997). On the other hand, in poplar leaves analyzed by the same approach surprisingly uniform concentration of cytosolic GSH has been detected (Hartmann et al., 2003). Also in leaves, however, certain cell types distinctly differ, as very high GSH concentration has been found in leaf trichoms (Gutierrez-Alcala et al., 2000). Consequently, γ ECS, GSHS, and genes for enzymes of cysteine synthesis were highly expressed in these cells. These cell specific differences in GSH levels are probably caused by differential rates of GSH synthesis and indicate that GSH transport between cells may not be efficient enough to balance such differences.

C. GSH Synthesis in C₄ Plants

Due to its role in detoxification of ROS GSH is particularly important in low temperature sensitive C₄ plants, such as maize, since chilling induces oxidative stress via the photochemical production of H₂O₂. Consequently, at low temperatures GSH content and reduction state are higher in chilling tolerant genotypes of tomato, *Sorghum*, wheat, and maize than in the sensitive ones (Walker and McKersie, 1993; Kocsy et al., 1996, 2000a; Badiani et al., 1993; see Chapter 10, this volume). Brunner et al. (1995) demonstrated that in maize chilling

caused the content of GSH to increase and, in accordance with the demand-driven model of regulation, also enzyme activities of APR, γ -ECS, and GSHS. Similarly, at 12°C the activities of APR and GR and GSH content were higher in a chilling tolerant maize genotype than in a sensitive one (Kocsy et al., 1997). Accordingly, treatment with 1 mM BSO, which decreased GSH content to very low levels, resulted in reduction of fresh weight and in visible leaf injury of chilling-tolerant maize at 5°C but not at ambient temperature (Kocsy et al., 2000b). Addition of GSH or γ -EC together with BSO protected the plants from the chilling injury by increasing GSH content and GR activity. Similarly, when GSH content in the chilling sensitive maize had been increased via treatment with herbicide safeners, the chilling induced injury was significantly reduced (Kocsy et al., 2001). A simultaneous addition of BSO counteracted the safener-induced protection. These experiments thus clearly showed that, at least in maize, sensitivity to chilling is a trait connected with GSH content and/or reduction state (see Chapter 10, this volume).

Despite its essential function in stress defense GSH is not equally distributed between MC and BSC in maize. GSHS activity is greater in MC than in BSC resulting in a predominant GSH synthesis rate in the MC (Burgener et al., 1998) and higher GSH levels in this cell type (Doulis et al., 1997; Burgener et al., 1998; Kopriva et al., 2001). Surprisingly, GR was found exclusively in MC of maize (Doulis et al., 1997; Pastori et al., 2000). The MC specific localization of GR might be explained by a limited capacity for NADPH production in BSC. GR mRNA, however, was found in both cell types revealing involvement of a post-transcriptional regulation of its cell-specific distribution (Pastori et al., 2000). In contrast to GR, the enzymes of GSH synthesis were localized in both MC and BSC of maize by immunohistochemistry (Gómez et al., 2004). In line with previous findings foliar GSH content increased in cold treated plants, however, chilling caused induction of γ -ECS mRNA in BSC but not in MC (Gómez et al., 2004). It seems therefore, that both cell types possess the capacity to synthesize GSH, but the enzymes in BSC are more affected by stress. The apparent contrast of these results with previous data (Doulis et al., 1997; Burgener et al., 1998) is likely to be caused by cell-type specific

post-translational modification of the enzymes resulting in changes in GSH synthesis rates and thus distribution of GSH. As γ -ECS is redox regulated, variation in redox environment in MC and BSC can be responsible for such differences in activity. The preferential stress response in BSC might be explained by the fact that cysteine, the limiting factor in GSH synthesis, is synthesized only in BSC and can be used for GSH synthesis without the necessity for any transport steps (Burgener et al., 1998; Kopriva et al., 2001). On the other hand, the oxidized form of glutathione can probably be reduced only in MC (Pastori et al., 2000). Consequently, GSSG formed during the stress in BSC has to be transported to MC for reduction and thus the GSH pool in MC increases while the BSC pool becomes depleted. This is likely to result in increased demand for GSH synthesis in BSC but not in MC and in activation γ -ECS and sulfate assimilation.

V. Physiological Significance of the Distribution of Nitrate and Sulfate Assimilation

Nitrate and sulfate assimilation are clearly localized in MC and BSC, respectively, in many C_4 plants. For sulfate assimilation it is obvious that this localization is not linked to C_4 photosynthesis, as C_4 *Flaveria* species possess the pathway in both cell types. The physiological significance of this localization and evolutionary advantage is, however, not evident as yet.

A. Open Questions on Nitrate Assimilation in C_4 Plants

The cell specific distribution of N assimilation in C_4 plants is well established, however, many questions are still open. Obviously, our findings on sulfate assimilation in *Flaveria* (Koprivova et al., 2001) incite questioning the universality of results on N assimilation derived from maize and a few other C_4 species. Although similarly to Gerwick et al. (1980) C_4 species of all three types were analyzed for localization of NR and GS with the same result, no dicot species were included (Rathnam and Edwards, 1976; McNally et al., 1983). Only in the very first analysis by Mellor and Tregunna (1971) a C_4 dicot (*G. globosa*) was

analyzed and, indeed, the results pointed to possible alternative distribution of the enzymes in this species compared, e.g. to maize. The findings have to be interpreted with caution, obviously, due to the methodology based on enzyme activities in MC and BSC specific extracts obtained by differential tissue extraction, which surely have been cross-contaminated. Nevertheless, unlike in other species analyzed, nitrite reductase activity in *G. globosa* was higher in BSC than in MC. In analogy with sulfate assimilation, this would imply that the MC distribution of nitrate assimilation may not be universal and not be connected with C_4 photosynthetic mechanism.

A second question concerns the increased N use efficiency of C_4 plants. Reduced accumulation of Rubisco, the major sink for reduced N in C_3 plants, is thought to be the major reason for better N use efficiency, as less N is required for the same or a better CO_2 fixation rate (Brown, 1978). Whether the cell-specific distribution of NR and/or the high content of cytosolic GS contribute to the improved N use efficiency is not clear.

The physiological consequences of the alterations in localization of N assimilation are also not known. Major differences in regulation of N uptake and assimilation between C_3 and C_4 plants have not been reported. Clearly, more transport steps are necessary to provide all cells with sufficient reduced N and the MC with nitrate (Fig. 3). On the other hand, the MC localization of NR and nitrite reductase might prevent competition for reduction equivalents between nitrate and CO_2 assimilation (Moore and Black, 1979). Alternatively, the low NADPH production capacity in BSC chloroplasts due to the lack of grana and photosystem II was cited as a reason for MC localization of NR (Mellor and Tregunna, 1971). This may be true for maize and some other NADP-malic enzyme-type C_4 plants, but plants of the other types do possess photosystem II in BSC and still reduce nitrate in MC only (Rathnam and Edwards, 1976; Ketchner and Sayre, 1992). Most likely explanation takes into account that nitrite is an alternative acceptor of photosynthetic electrons and its reduction is coupled to oxygen evolution (Mifflin, 1972). Localization of this reaction in MC chloroplast thus prevents O_2 evolution in BSC, which would counteract the CO_2 concentrating mechanism of C_4 plants and support the oxygenase reaction of Rubisco (Moore and Black, 1979). Limitation

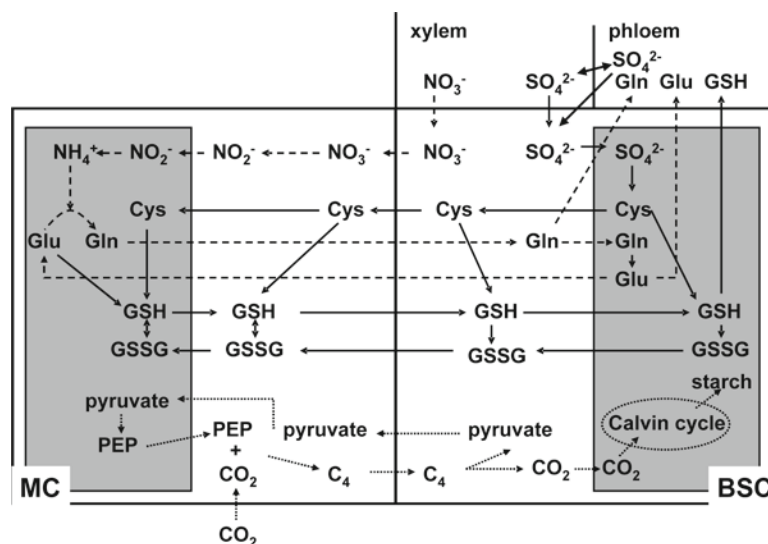


Fig. 3. Schematic representation of distribution of major steps in assimilation of carbon, nitrogen, and sulfur between mesophyll (MC) and bundle sheath (BSC) of maize. Transport steps of C compounds are marked by dotted arrows, dashed and full arrows symbolize transport of N and S compounds, respectively (Reprinted from Kopriva and Koprivova, 2005).

of NR to MC is essential to prevent accumulation of nitrite and its toxicity in BSC. This might be the biggest evolutionary advantage of the cell-specific distribution of N assimilation in C₄ plants. This explanation would be strengthened if the MC localization of NR and nitrite reductase would be confirmed also for the dicot C₄ species.

B. Significance of BSC Localization of Sulfate Assimilation

Although not necessarily linked with C₄ photosynthetic mechanism, sulfate is reduced only in BSC of maize. A link with low rate of NADPH production as discussed for nitrate assimilation and GSH reduction (Mellor and Tregunna, 1971; Doulis et al., 1997) can be excluded since then the sulfate reduction would have to be localized in MC. Burgener et al. (1998) speculated that low concentration of oxygen in BSC would prevent oxidation of intermediates of sulfate assimilation, sulfite and sulfide, and thus increase the efficiency of the pathway. However, if such oxidation would indeed significantly reduce the rate of sulfate assimilation, the pathway would not be functional in chloroplasts of C₃ plants. Consequently, sulfate would be reduced in mitochondria, what actually is the case in *Euglena gracilis* (Brunold and Schiff, 1976), or elaborate structures

would have evolved possibly including bacterial symbiosis, such as for N₂ fixation in legumes.

Another attractive explanation is the co-localization with photorespiration, namely with GDC, the major source of serine in plants since activated serine is the acceptor of sulfide and a direct precursor of cysteine. However, serine synthesized in BSC must be transported into MC for protein synthesis anyway and thus a shortage of this amino acid in MC seems unlikely. Moreover, in C₄ *Flaveria* species GDC is BSC specific but sulfate assimilation is not (Hylton et al., 1988; Koprivova et al., 2001). In addition, the MC specific ferredoxin FdI (Matsumura et al., 1999) was an efficient electron donor for sulfite reductase (Yonekura-Sakakibara et al., 2000), so cell specific differences in electron flux can also be excluded.

To find out the significance of the BSC specific distribution of sulfate assimilation, one should ask for the advantage maize may have gained this way. Unfortunately, there does not seem to be any obvious one. In contrast to nitrogen nutrition, a difference in sulfur use efficiency between C₃ and C₄ plants was not observed. As with other metabolic processes, maize probably invests less into synthesis of the proteins of sulfate assimilation pathway, but on the other hand it must possess an efficient transport system for cysteine and GSH. Maize does not require significantly more or

less sulfate than other plant species and it is not known for especially good or poor sulfur use efficiency. It is not particularly resistant or sensitive to heavy metals, which trigger a high demand for reduced sulfur (Nussbaum et al., 1988). On the other hand, sulfate assimilation and GSH synthesis are associated with tolerance to chilling and detoxification of herbicides, so the BSC localization might even be a limitation for the capacity to provide sufficient GSH to cope with such stress. It seems, therefore, that the significance of compartmentalization of sulfate assimilation in maize will further remain an open question.

C. Consequences of BSC Localization of Sulfate Assimilation

To understand the cell-specific localization of sulfate assimilation one also has to ask about its consequences. Maize was a frequent subject of investigations of assimilatory sulfate reduction in the pre-Arabidopsis era. The results on regulation of sulfate assimilation obtained with maize fitted well to the general hypothesis of demand driven control (Lappartient and Touraine, 1996). Coordinate increase in mRNA levels for sulfate transporters, ATPS, and APR was observed in maize roots and leaves upon sulfate starvation (Bolchi et al., 1999; Hopkins et al., 2004) and the ATPS mRNA level was repressed in presence of reduced sulfur compounds (Bolchi et al., 1999). Accordingly, ATPS and APR activities were increased upon treatments of maize with cadmium or chilling (Brunner et al., 1995; Nussbaum et al., 1988; Rueggsegger and Brunold, 1992). In all these reports the regulation of sulfate assimilation in maize was not distinguishable from other plants, such as *Lemna minor*, poplar, potato, and *Arabidopsis* (Kopriva, 2006). Differences appeared, however, when the mechanisms of regulation were addressed (Bolchi et al., 1999). To identify the signal responsible for feedback inhibition of sulfate assimilation by thiols, plants can be treated with cysteine, glutathione, and cysteine together with BSO which prevents its conversion to GSH. Such analysis performed with *Brassica napus*, *Arabidopsis*, and poplar unambiguously identified GSH as the molecular regulator (Lappartient and Touraine, 1996; Vauclare et al., 2002; Hartmann et al. 2004) whereas in maize cysteine acted directly without the necessity of conversion

to GSH (Bolchi et al., 1999). The molecular mechanisms of this feedback inhibition are not known, but it is reasonable to expect that the responsible sensor/transcription factor in maize is specific for cysteine whereas the corresponding protein(s) in other plants are GSH specific. This variation might well be a consequence of the BSC localization of sulfate assimilation. As GSH can be synthesized both in MC and BSC (Gómez et al., 2004) but only cysteine is transported out of BSC protoplasts (Burgener et al., 1998) it is likely that the GSH pools in MC and BSC are not rapidly interchangeable. On the other hand, cysteine pools in the two cell types have to be linked to enable efficient protein and GSH synthesis in MC and rapid modulation of cysteine biosynthesis in BSC upon even subtle changes in demand for reduced sulfur in the whole leaf. Therefore, cysteine may be much better suited as a signal of the sulfur status at the site of sulfate assimilation than GSH.

VI. Conclusions

Nitrate and sulfate assimilation in C_4 plants are another processes differentially distributed between MC and BSC. MC specific localization of nitrate assimilation has been demonstrated in only a few species and despite being generally accepted, it has to be proven that this indeed is a general C_4 trait. This is even more imperative after it was revealed that the BSC association of sulfate assimilation is not a general feature of C_4 photosynthesis but is probably limited to C_4 monocots. For both pathways the evolutionary advantage of such compartmentalization is not understood and should be addressed in future studies. Given the high interest in improvements of nutrient use efficiency of crops the N metabolism in C_4 plants should certainly be further explored to find whether the C_4 specific alterations contribute to the improved N use efficiency of C_4 plants. Clearly, there are still more open questions than answers in N and S metabolism of C_4 plants.

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Chapter 8

Nitrogen and Water Use Efficiency of C₄ Plants

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Summary	129
I. Introduction.....	130
II. Nitrogen Use Efficiency.....	131
A. Nitrogen Use Efficiency of C ₄ Grasses.....	131
B. CO ₂ Assimilation Rate, Leaf N and Leaf Mass per Area	132
C. C ₄ Species and the Global Plant Trait Network	133
D. Leaf N Budget	134
E. Rubisco and Nitrogen Use Efficiency of C ₄ Species	136
III. Water Use Efficiency	138
A. Water Use Efficiency of C ₄ Grasses	138
B. Water Use Efficiency and Carbon Isotope Discrimination in C ₄ Species.....	139
C. Effects of Environmental Conditions on Water Use Efficiency of C ₄ Grasses	141
IV. Conclusions.....	143
References	143

Summary

Species with the C₄ photosynthetic pathway have evolved biochemical CO₂ concentrating mechanisms that allow Rubisco to function in a high CO₂ environment. This increases both their nitrogen and water use efficiency compared to C₃ species. A comparison between Australian C₄ grasses and global data (Glopanet) reveals that C₄ species have greater rates of CO₂ assimilation than C₃ species for a given leaf nitrogen when both parameters are expressed either on a mass or an area basis. The comparison also revealed that although the range in leaf N content per unit area is less in C₄ compared to C₃ species, the range in leaf nitrogen concentration per unit dry mass is similar for both C₄ and C₃ species. While C₃ and C₄ species invest a similar fraction of leaf N into photosynthetic components, C₄ species allocate less to Rubisco protein and more to other soluble proteins and thylakoid components. Hence, the driving force that increases CO₂ assimilation rate per unit leaf nitrogen in C₄ species is greater catalytic turnover

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rate of Rubisco in vivo. This is exemplified by the fact that differences in photosynthetic nitrogen use efficiency amongst C_4 species of the NAD-ME and NADP-ME decarboxylation types is linked to variation in Rubisco kinetic properties amongst these species. Improved leaf and plant water use efficiency in C_4 species is due to both higher photosynthetic rates per unit leaf area and lower stomatal conductance. By contrast, leaf and plant water use efficiency is increased in C_4 plants under elevated CO_2 because of reduced stomatal conductance. The geographic distribution of the different C_4 subtypes is strongly correlated with rainfall. One might expect that these distribution differences are linked to differences in water use efficiency. The convergence found in water use efficiency and leaf gas exchange characteristics under most growth conditions, between NAD-ME and NADP-ME grasses, is therefore a curious reminder that geographic distribution may not be related fully to the physiology of photosynthesis.

I. Introduction

Rubisco, the primary CO_2 fixing enzyme of photosynthesis, is a poor catalyst at current atmospheric conditions (Andrews and Lorimer, 1987; Tcherkez et al., 2006). Many species, including unicellular algae (Badger and Price, 1992), crassulacean acid metabolism plants and C_4 plants (Leegood et al., 1997), have evolved mechanisms to concentrate CO_2 at the site of carboxylation and enhance Rubisco catalysis. Species with the C_4 photosynthetic pathway have evolved a biochemical CO_2 concentrating mechanism which in most species involves the close collaboration of two photosynthetic cell types, the mesophyll and bundle sheath cells. CO_2 is initially fixed by phosphoenolpyruvate carboxylase (PEPC) in the mesophyll cells into C_4 acids which then diffuse to the bundle sheath where they are decarboxylated to supply CO_2 for Rubisco. This allows Rubisco to operate close to its maximal activity. The C_4 photosynthetic pathway has evolved many times in both dicot and monocot genera (Sage, 2004). Three major biochemical subgroups of C_4 plants have been characterised, utilising different C_4 -acid decarboxylases (Hatch et al., 1975). NADP malic enzyme (NADP-ME) types decarboxylate C_4 acids in bundle sheath

chloroplasts, NAD-ME types decarboxylate in the mitochondria and phosphoenolpyruvate carboxykinase (PCK) types decarboxylate primarily in the cytosol. These biochemical variations are accompanied by a suite of distinct anatomical and ultrastructural modifications (Hatch, 1987).

To compensate for poor Rubisco kinetic properties, C_3 plants have high Rubisco contents in their leaves, which impose a considerable nitrogen requirement. In C_4 plants, the CO_2 concentrating mechanism enhances CO_2 assimilation rate (A) under normal air and high light, which increases both photosynthetic nitrogen use efficiency (PNUE, defined as A per unit leaf N) and leaf water use efficiency (W, defined as the ratio of A to leaf transpiration rate) compared to C_3 species. This has been validated by a number of studies which compared PNUE of C_3 and C_4 species belonging to the same genera such as *Flaveria* (Monson, 1989) or *Panicum* (Bolton and Brown, 1980). This topic was reviewed by Long (1999) for both crop and natural ecosystems. In addition to increases in A , differences in plant nitrogen use efficiency (NUE, plant dry mass per leaf N) and/or plant water use efficiency (WUE, plant dry mass produced per water transpired) can be caused by a number of factors. For example, plants can acclimate to their growth environment at several levels. They can vary the amount of biomass invested in leaves, stems and roots; modulate biomass per unit leaf area by altering leaf anatomy or change relative investment of nitrogen between photosynthetic components. These traits have been investigated in detail for C_3 species (Poorter and Evans, 1998; Evans and Poorter, 2001; Wright et al., 2004), but not for C_4 species. Here we draw on experiments conducted

Abbreviations: A – CO_2 assimilation rate; g_s – Stomatal conductance; K_c – Michaelis–Menten constant for CO_2 ; k_{cat} – Catalytic turnover rate; LMA – Leaf dry mass per area; NADP-ME – NADP malic enzyme; NUE – Nitrogen use efficiency; PCK – Phosphoenolpyruvate carboxykinase; pCO_2 – CO_2 partial pressure; PEPC – Phosphoenolpyruvate carboxylase; PNUE – Photosynthetic nitrogen use efficiency; W – Photosynthetic leaf water use efficiency; WUE – Water use efficiency (whole plant); ϕ – Bundle sheath leakiness; Δ – Carbon isotope discrimination

using C_4 grasses with different decarboxylation types to examine the underlying basis of the superior nitrogen and water use efficiencies in C_4 plants.

II. Nitrogen Use Efficiency

A. Nitrogen Use Efficiency of C_4 Grasses

C_4 grasses with different biochemical subtypes have different geographic distribution according to rainfall, such as seen in Australia (Hattersley, 1992), South Africa (Ellis et al., 1980) and South America (Taub, 2000; Cabido et al., 2008). With increasing rainfall, NADP-ME grasses increase in abundance, while NAD-ME grasses become less abundant. In addition, the C_4 biochemical subtypes exhibit large differences in leaf anatomy, ultrastructure and biochemistry. For example, C_4 acid decarboxylation occurs in different cellular compartments in the different subtypes. Other differences include the lack of photosystem II activity in the bundle sheath chloroplasts of NADP-ME type species and the presence of a suberin layer in the bundle sheath cell wall of this subtype (Hatch, 1987; Hattersley, 1992). Although these differences have been known for some time, their physiological significance remains little understood.

To shed some light over the significance of this intriguing biochemical diversification of C_4 photosynthesis, Ghannoum et al. (2005) undertook a number of studies comparing NAD-ME and NADP-ME grasses. These two subtypes were chosen because they represent the two most contrasting and floristically abundant subtypes in Australia (Hattersley, 1992). Using various combinations of 27 NAD-ME and NADP-ME grasses grown in a glasshouse experiment under adequate and deficient soil N supplies, Ghannoum et al. (2005) found a surprising similarity of CO_2 assimilation rates amongst species when measured under common conditions despite the anatomical and biochemical heterogeneity of the different subtypes (Fig. 1a). However, these experiments made it clear that NAD-ME grasses contained more leaf N to achieve a given A than their NADP-ME counterparts (Fig. 1b). This resulted in NAD-ME type grasses having a lower

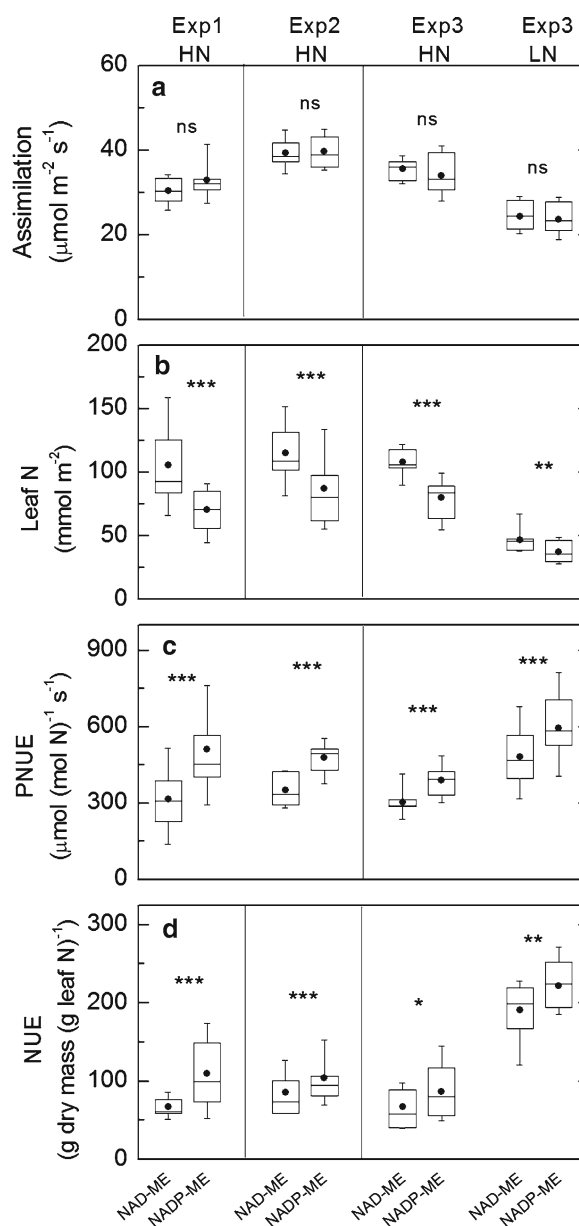


Fig. 1. Box-whisker plots of net CO_2 assimilation rates, A (a), N content per leaf area (b), photosynthetic N use efficiency, PNUE (c) and whole plant N use efficiency, NUE (d) in 13 NAD-ME and 14 NADP-ME C_4 grasses. The box and whisker represent the 25–75% and minimum–maximum distributions of the data, respectively. Means (●) and significance levels for the subtype effect are shown and they are: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; ns = not significant ($P \geq 0.05$). The experiments were run three times and a low N treatment was included in the third experiment. Each experiment used at least seven different species of each type (For details see Ghannoum et al., 2005). (www.plantphysiol.org; “Copyright American Society of Plant Biologists”).

PNUE (Fig. 1c). Although diminished relative to PNUE, the difference in whole plant NUE was also evident between the two C_4 subtypes (Fig. 1d). Along the same line Bowman (1991) found that two NADP-ME *Panicum* species accumulated more biomass per total shoot N than four NAD-ME species. When Taub and Lerdau (2000) compared the response of A to leaf N in three NAD-ME and three NADP-ME grasses, they concluded that variation between species was greater than between the two C_4 subtypes. It is likely that the small number of species used in the latter study hindered the emergence of a clear trend. Long term fertilization experiments in South Africa found that fertilization resulted in increased abundance of NAD-ME species and reduced frequency of NADP-ME type grasses. This fits with the controlled environment-based observation that NAD-ME species have higher N requirement relative to their NADP-ME counterparts (Knapp and Medina, 1999).

B. CO_2 Assimilation Rate, Leaf N and Leaf Mass per Area

The acclimation of leaves of C_4 species to different environmental conditions has not been studied as extensively as for C_3 species. Nevertheless, available data indicate that there is a strong relationship between CO_2 assimilation rate and leaf N content in both C_3 and C_4 species (Bolton and Brown, 1980; Wong et al., 1985; Sage et al., 1987; Sage and Percy, 1987; Evans, 1989; Monson, 1989).

Relationships between A and leaf N content are shown for C_4 grasses, with samples collected in the field tending to have lower leaf N contents than glasshouse-grown material (Fig. 2). NAD-ME leaves tend to have greater N for a given A than NADP-ME leaves. The correlation between A and leaf N is largely due to the fact that soluble and thylakoid proteins account for 75% of leaf N in both C_3 and C_4 species (Fig. 3). However, the underlying causes of the variations in this relationship are not always similar between the two photosynthetic types.

In C_3 species, genetically- and environmentally-driven differences in A and leaf N content may be caused by a multitude of factors, one of which is leaf thickness (Poorter and Evans, 1998). Species or plants with thicker leaves, tend to have greater A and leaf N. For example, the number of photosynthetic cells per unit leaf area increases with increasing growth irradiance (Yano and Terashima, 2004). This increases the number of chloroplasts, and hence photosynthetic capacity and N content per leaf area. However, when expressed on a mass basis, low- and high-light-grown plants have similar A and leaf N (Evans and Poorter, 2001). Consequently, leaf dry mass per area (LMA), often correlates well with A and leaf N content in C_3 leaves (Reich et al., 1997). Plasticity in stacking different numbers of cells per unit leaf area is possible in C_3 leaves because the photosynthetic building blocks are individual (mesophyll) cells.

In contrast, leaf thickness is constrained in C_4 species to a much narrower range than in C_3 species.

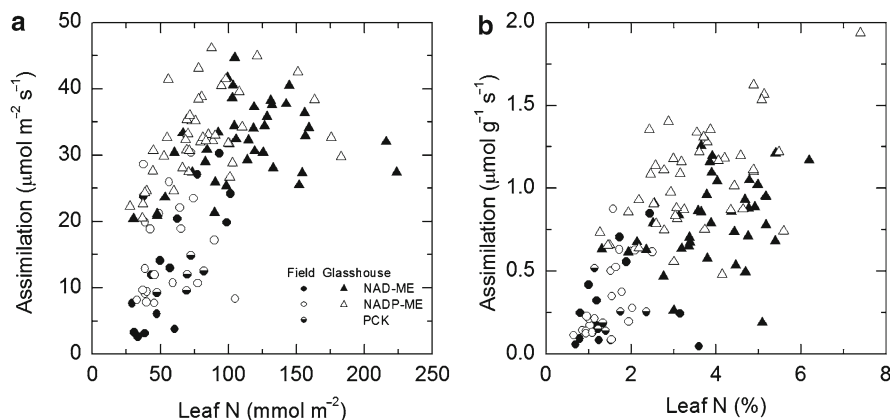


Fig. 2. Net CO_2 assimilation rate, A , as a function of leaf N expressed (a) on an area basis, or (b) on a dry mass basis for glasshouse or field grown C_4 grasses. Gas exchange measurements were made at $2,000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, ambient pCO_2 and leaf temperatures of 30°C (Data are taken from Ghannoum et al., 2001a, b, 2002, 2005).

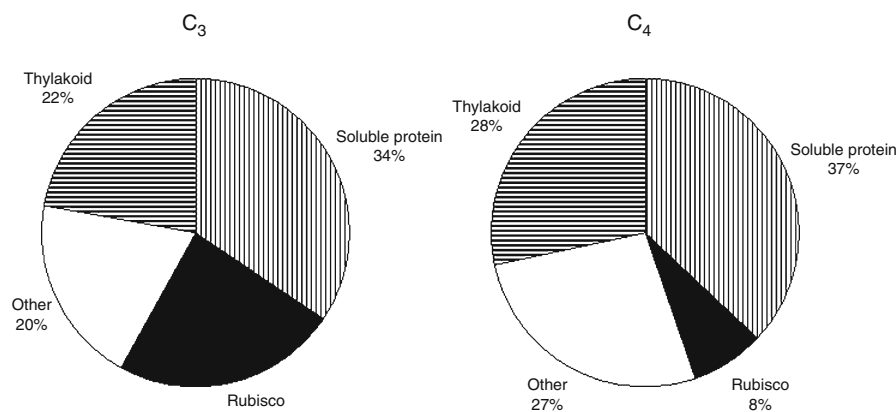


Fig. 3. Comparison of leaf N budgets between C_3 and C_4 species. The contributions of soluble proteins without Rubisco, Rubisco, thylakoid N and other N are calculated as described by Ghannoum et al. (2005). The diagram combines data from several data sets (see Table 1 for details).

The C_4 photosynthetic pathway involves the close collaboration of two photosynthetic cell types, the mesophyll and bundle sheath cells. The vast majority of C_4 plants possess the classical Kranz leaf anatomy (but for exceptions see, Freitag and Stichler, 2000; Voznesenskaya et al., 2001; Edwards et al., 2004). The need for extensive and rapid metabolite transfer between the two cell types requires that mesophyll cells abut bundle sheath cells. Leaves of C_4 plants have between two and four mesophyll cells between adjacent bundle sheaths (Hattersley and Watson, 1975; Morgan and Brown, 1979). Consequently, veins are more closely spaced in C_4 leaves compared to C_3 leaves (Morgan and Brown, 1979; Dengler et al., 1994). These features constitute anatomical traits by which C_4 plants can be identified and grouped into their various subtypes (Hattersley and Watson, 1975; Dengler et al., 1994). In general, high photosynthetic rates require large areas of mesophyll exposed to intercellular airspaces to allow for high CO_2 fluxes into mesophyll cells (Evans and von Caemmerer, 1996; von Caemmerer et al., 2007). This requirement contrasts with that of low CO_2 permeability across the mesophyll-bundle sheath interface, which is needed to minimize CO_2 leakage and achieve elevated CO_2 partial pressure (pCO_2) in the bundle sheath, ensuring the proper functioning of the CO_2 concentrating mechanism. In part, this is achieved by low bundle sheath to leaf surface area ratios (1.8 ± 0.1) that vary little amongst C_4 species

(von Caemmerer et al., 2007). As a consequence of these anatomical constraints, C_4 species have relatively thin leaves and low LMA. LMA was also found to be consistently lower in NADP-ME compared to NAD-ME grasses (Ghannoum et al., 2001a, b, 2005). There appears to be less variation in LMA under different growth irradiance or nitrogen nutrition for C_4 compared to C_3 species (Ghannoum and Conroy, 1998; Ghannoum et al., 2005; Tazoe et al., 2006). Nevertheless, there are examples in the literature where LMA was reported to change with growth irradiance in C_4 grasses (e.g. Ward and Woolhouse, 1986; Kephart et al., 1992; Ghannoum et al., 2001a).

C. C_4 Species and the Global Plant Trait Network

Wright and co-workers formed a global plant network (Glopanet) to quantify leaf economics across the world's plant species (Wright et al., 2004). They focused on key features including LMA, leaf N and CO_2 assimilation rate, A , measured under high light and ambient pCO_2 . The Glopanet dataset includes 16 C_4 out of the total of 698 species. Although C_4 plants represent only a small portion of the world's plant species (approximately 4%), they contribute about 20% to global primary productivity because of highly productive C_4 grasslands (Lloyd and Farquhar, 1994; Ehleringer et al., 1997). It is therefore important to include them in any global network analysis. In Fig. 4, we

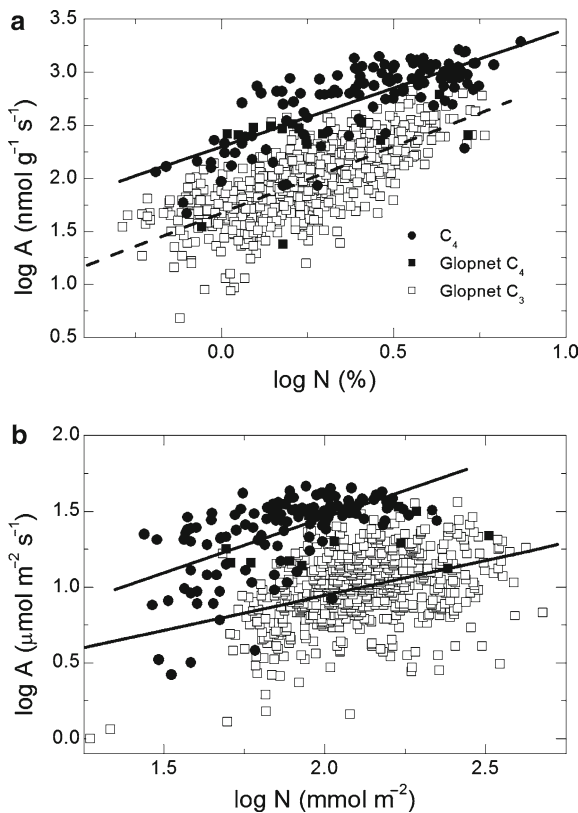


Fig. 4. (a) The relationship between $\log(A)$ versus $\log$ (leaf N) where both are expressed on a dry mass basis. (●) are C_4 grasses shown in Fig. 2, (■, □) denote C_4 and C_3 species in the Glopnet data base (Redrawn from the supplementary data of Wright et al., 2004). The regression equations are $\log(A) = 1.103 \log(N) + 2.30$ ($r^2 = 0.59$) and $\log(A) = 1.243 \log(N) + 1.68$ ($r^2 = 0.51$) for the C_4 and Glopnet C_3 data, respectively. (b) The same relationship with A and leaf N expressed on an area basis. The regression equations are $\log(A) = 0.726 \log(N) + 0.005$ ($r^2 = 0.34$) and $\log(A) = 0.46 \log(N) + 0.02$ ($r^2 = 0.14$) for the C_4 and Glopnet C_3 data, respectively.

have compared the relationship between A and leaf N for the C_4 species shown in Fig. 2 and the Glopnet data set on both a dry mass and an area basis. It is clear that the relationship for C_4 species sits apart from that for C_3 species. On a double log scale, used by Reich and co-workers (Reich et al., 1997), the different intercepts highlight that C_4 species generally have a greater PNUE. This reflects the C_4 photosynthetic mechanism and the amount of CO_2 fixed per Rubisco protein (see Section II.E). Interestingly, the range in leaf N on a unit dry mass basis is similar for both C_4 and C_3 species (Fig. 4a). However, C_4 species

do not reach such high N contents per unit leaf area as C_3 species because their maximal LMA is less (Fig. 4b).

D. Leaf N Budget

When grown under high light, C_3 leaves typically allocate 58% of leaf N to soluble protein (one third of which is Rubisco) and 22% to thylakoids (Evans, 1989; Evans and Poorter, 2001). Several studies have examined the nitrogen allocation of C_4 leaves (Makino et al., 2003; Ghannoum et al., 2005; Tazoe et al., 2006). A comparison between the leaf N budgets of C_3 and C_4 species reveals that the major difference between the two photosynthetic types is the lower investment of N into Rubisco (Fig. 3, Table 1), a fact that has been well recognised (Ku et al., 1979; Sage et al., 1987; Long, 1999; Evans and von Caemmerer, 2000). This is offset to some extent by an increase in other soluble proteins (e.g., those involved in the C_4 cycle), so that the allocation to soluble protein is 45% versus 58% for C_4 and C_3 leaves, respectively (Fig. 3). The comparison in Fig. 3 also reveals that C_4 leaves invest more N into their thylakoids than C_3 leaves (28% vs 22%, respectively). This difference can also be seen in the chlorophyll to leaf N ratio of leaves grown under similar irradiances. The mean values were 5.0 and 3.5 mmol Chl (mol N) $^{-1}$ for the C_4 and C_3 leaves, respectively (Table 1).

For some NAD-ME and NADP-ME C_4 grasses, it has been possible to examine the N distribution between soluble and thylakoid pools at the tissue level as well as the whole leaf (Fig. 5; Ghannoum et al., 2005). In the NAD-ME species, 60% of both leaf N and chlorophyll were found in the bundle sheath, whereas only 35% of both chlorophyll and N were found in the PSII-depleted bundle sheath of NADP-ME species (Fig. 5). Regardless of the C_4 type, about 28% of the nitrogen in each tissue type was associated with the thylakoids. By contrast, nitrogen allocation to soluble protein differed between tissues. The exclusive localization of Rubisco in bundle sheath cells accounted for only part of the difference. NADP-ME species allocated considerably less of the mesophyll N to soluble protein than NAD-ME species, but more detailed characterisation of these pools is needed to reveal which proteins are responsible for these differences.

Table 1. Partitioning of leaf nitrogen between soluble protein, Rubisco and thylakoids and the ratio of chlorophyll to leaf N for C₃ and C₄ species.

	Species	Percentage of leaf N in			Chl/N (mmol mol ⁻¹)	Reference
		Soluble protein	Rubisco	Thylakoids		
C ₃	<i>Datura stramonium</i>	61.8	24.4	22.4	3.29	Poorter and Evans, 1998
	<i>Echium plantagineum</i>	56.9	19.5	21.3	2.76	Poorter and Evans, 1998
	<i>Nicotiana tabacum</i>	65.6	22.8	22.1	3.33	Poorter and Evans, 1998
	<i>Physalis peruviana</i>	64.8	23.5	17.8	3.00	Poorter and Evans, 1998
	<i>Plantago major</i>	63.9	21.8	18.5	2.46	Poorter and Evans, 1998
	<i>Raphanus sativus</i>	63.0	26.4	24.4	2.81	Poorter and Evans, 1998
	<i>Spinacia oleracea</i>	47.6	15.9	26.3	3.89	Terashima and Evans, 1988
	<i>Triticum aestivum</i>	51.0	21.0	25.7	4.59	Evans, 1983; Evans and Seemann, 1984
	<i>Pisum sativum</i>	56.5	30.5	20.5	3.60	Makino and Osmond, 1991
	<i>Oryza sativa</i>	50.0	27.0	23.9	4.79	Makino et al., 2003
	<i>Chenopodium album</i>		27.0	21.0	3.85	Sage et al., 1987
	Mean ± SE	58.1 ± 2.1	23.6 ± 1.2	22.2 ± 0.8	3.5 ± 0.2	
C ₄	NAD-ME					
	<i>Amaranthus retroflexus</i>		7.0	27.0	4.52	Sage et al., 1987
	<i>Amaranthus cruentus</i>	38.1	10.5	19.1	3.33	Tazoe et al., 2006
	<i>Panicum miliaceum</i>	55.7	7.8	30.1	5.44	Ghannoum et al., 2005
	<i>Panicum coloratum</i>	60.4	9.1	29.0	4.89	Ghannoum et al., 2005
	NADP-ME					
	<i>Sorghum bicolor</i>	36.5	5.3	26.1	4.99	Ghannoum et al., 2005
	<i>Cenchrus ciliaris</i>	44.7	5.3	32.9	5.33	Ghannoum et al., 2005
	<i>Zea mays</i>	33.0	8.5	34.0	6.75	Makino et al., 2003
	Mean ± SE	44.7 ± 4.5	7.6 ± 0.7	28.3 ± 1.9	5.0 ± 0.4	

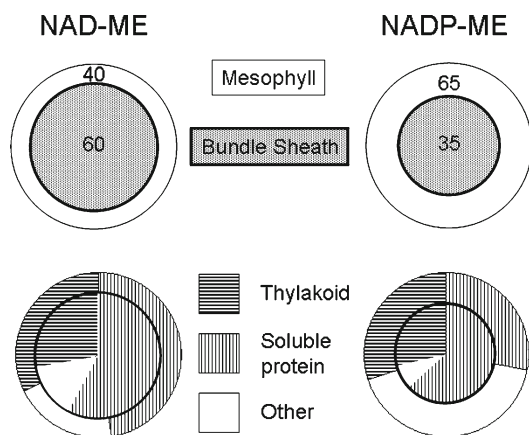


Fig. 5. Distribution of N between bundle sheath and mesophyll cells and the proportion allocated to soluble protein, thylakoid N and other N in each tissue for NAD-ME and NADP-ME grasses. Values shown are percentages (Data is taken from Ghannoum et al., 2005).

The similar fraction of tissue N allocated to the thylakoid pool conceals considerable variation in functional activity. Bundle sheath cells of the two NAD-ME species contained 60% of leaf

chlorophyll, but 17–46% of leaf functional PSII and PSI centers. For the two NADP-ME species, PSI activity per unit chlorophyll was similar for both tissues, but PSII activity was almost negligible in the bundle sheath (Ghannoum et al., 2005). The deficiency of PSII activity in the bundle sheath of sorghum and other NADP-ME types is one of the best documented examples of heterogeneity in composition in mesophyll and bundle sheath chloroplasts of C₄ species, and is thought to prevent accumulation of high O₂ partial pressures in the gas tight bundle sheath compartment (Woo et al., 1970; Mayne et al., 1974; Edwards et al., 1976).

In C₄ leaves, the close association of mesophyll and bundle sheath cells means that the bundle sheath is shaded by the mesophyll cell layers (Evans et al., 2007). The lower cytochrome *f* contents per unit chlorophyll in bundle sheath, relative to mesophyll chloroplasts are consistent with a low-light phenotype (Ghannoum et al., 2005). Consequently, it would appear that C₄ species invest more N into pigment protein complexes in their bundle sheath chloroplasts to compensate

for the shading from surrounding mesophyll cells. There are few studies that have examined the effects of N nutrition and light environment on the plasticity of thylakoid organisation in mesophyll and bundle sheath tissues of C_4 species (Drozak and Romanowska, 2006).

E. Rubisco and Nitrogen Use Efficiency of C_4 Species

Similar to photosynthesis in C_3 species, a strong correlation is observed in C_4 species between Rubisco activity (or content) measured in vitro and CO_2 assimilation rate measured at high irradiance (Usuda et al., 1984; Hunt et al., 1985; Sage et al., 1987; von Caemmerer et al., 1997). Because Rubisco operates close to CO_2 saturation in the bundle sheath, the relationship between maximal Rubisco activity and A is close to 1:1 below 30°C. It is well recognised that Rubisco kinetic properties differ between species (Jordan and Ogren, 1981; Badger and Andrews, 1987). For example, the catalytic turnover rate (k_{cat}) has often been shown to be greater in C_4 relative to C_3 species, and this is associated with a greater Michaelis–Menten constant for CO_2 (K_c) in the

C_4 type (Yeoh et al., 1980, 1981; Seemann et al., 1984; Wessinger et al., 1989; Sage, 2002). Operating under high pCO_2 , C_4 Rubisco can afford to trade higher k_{cat} at the expense of lower affinity for CO_2 (i.e., greater K_c). To compensate for the lower A per Rubisco sites, C_3 plants allocate three to four times more N to Rubisco than C_4 plants (Fig. 6, Table 2). However, it should be noted here that the full benefits of greater k_{cat} in C_4 species can be reaped only at high temperatures. The advantage of a CO_2 concentrating mechanism which allows Rubisco to operate at maximal activity is diminished at low temperature as both k_{cat} and K_c decrease with decreasing temperature. Moreover, growth at low temperatures has been shown to cause some C_4 species to allocate more of their leaf N to Rubisco (Pearcy et al., 1974; Berry and Bjorkman, 1980; Bjorkman et al., 1980; Long, 1999; Sage, 2002; Dwyer et al., 2007; Sage and Kubien, 2007). This was reviewed in detail by Long (1999) and Chapter 10, this volume.

In addition to differences between C_3 and C_4 species, Seemann et al. (1984) noted that Rubisco k_{cat} is greater in NADP-ME species compared to NAD-ME species. Ghannoum et al. (2005), who

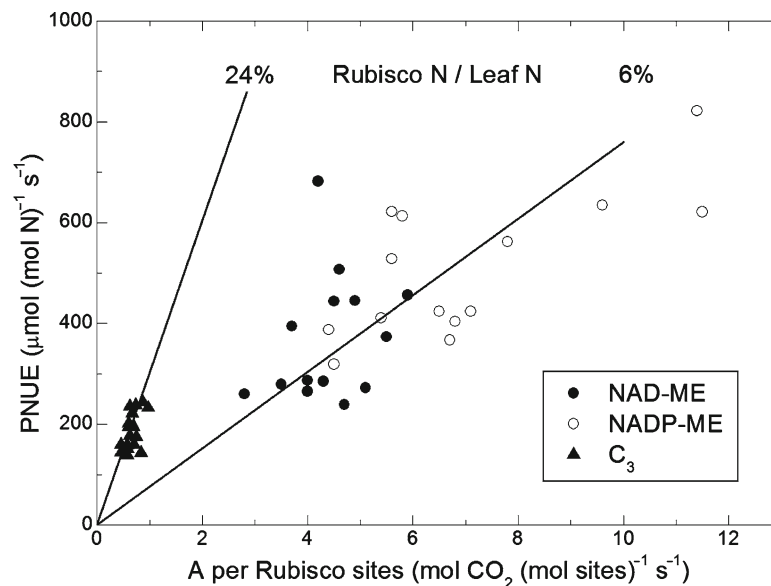


Fig. 6. Photosynthetic nitrogen use efficiency (PNUE) as a function of the ratio of net CO_2 assimilation rate to Rubisco catalytic sites for C_4 grasses measured by Ghannoum et al. (2005) and several C_3 species (Makino et al., 1988, 1997; Evans et al., 1994; Poorter and Evans, 1998; Westbeek et al., 1999). For the C_4 grasses, measurements were made at a leaf temperature of 30°C, high light and ambient pCO_2 . For the C_3 species, measurements were made at 25°C, high light and ambient pCO_2 . The lines represent the average proportion of leaf N in Rubisco for the C_4 and C_3 species. Details of the species used are given in Table 2.

Table 2. Photosynthetic nitrogen use efficiency (PNUE), CO₂ assimilation rate (*A*) per Rubisco site, nitrogen content per unit leaf area (N), leaf dry mass per unit leaf area (LMA) and Rubisco content for a number of C₄ grasses as well as a number of C₃ species

	Species	PNUE	<i>A</i> per Rubisco	N	LMA	Rubisco	
		($\mu\text{mol mol}^{-1} \text{ N s}^{-1}$)	sites ($\text{mol mol}^{-1} \text{ s}^{-1}$)	(mmol m^{-2})	(g m^{-2})	($\mu\text{mol sites m}^{-2}$)	
C ₄							
NAD-ME	<i>Astrebala lappacea</i>	395	3.7	103.2	37.0	11.1	Ghannoum et al., 2005
	<i>Astrebala lappacea</i>	682	4.2	30.1	32.3	4.9	
	<i>Astrebala pectinata</i>	272	5.1	126.3	52.6	6.8	
	<i>Astrebala pectinata</i>	239	4.7	89.6	45.5	4.6	
	<i>Eragrostis superba</i>	287	4.0	118.5	33.3	8.5	
	<i>Eragrostis superba</i>	444	4.5	47.7	31.3	4.7	
	<i>Panicum coloratum</i>	260	2.8	119.4	32.3	11.0	
	<i>Panicum coloratum</i>	445	4.9	53.2	38.5	4.8	
	<i>Panicum decompositum</i>	279	3.5	128.4	38.5	10.4	
	<i>Panicum decompositum</i>	507	4.6	60.4	33.3	6.9	
	<i>Panicum miliaceum</i>	265	4.0	142.5	32.3	9.9	
	<i>Panicum miliaceum</i>	456	5.9	47.4	26.3	3.6	
	<i>Panicum virgatum</i>	285	4.3	132.0	47.6	8.8	
	<i>Panicum virgatum</i>	374	5.5	73.3	43.5	5.0	
NADP-ME	<i>Bothriochloa biloba</i>	424	7.1	66.1	35.7	4.0	Poorter and Evans, 1998
	<i>Bothriochloa biloba</i>	621	11.5	44.5	32.3	2.4	
	<i>Bothriochloa bladhii</i>	404	6.8	81.2	37.0	4.8	
	<i>Bothriochloa bladhii</i>	411	5.4	59.9	38.5	4.6	
	<i>Cenchrus ciliaris</i>	424	6.5	97.8	27.0	6.4	
	<i>Cenchrus ciliaris</i>	822	11.4	27.5	30.3	2.0	
	<i>Dichanthium sericeum</i>	388	4.4	83.4	38.5	7.4	
	<i>Dichanthium sericeum</i>	635	9.6	38.4	37.0	2.5	
	<i>Paspalum dilatatum</i>	319	4.5	99.6	28.6	7.2	
	<i>Paspalum dilatatum</i>	622	5.6	39.9	22.7	4.5	
	<i>Paspalum notatum</i>	528	5.6	87.4	34.5	8.2	
	<i>Paspalum notatum</i>	613	5.8	36.9	34.5	3.9	
	<i>Pennisetum clandestinum</i>	367	6.7	107.9	20.4	6.0	
	<i>Pennisetum clandestinum</i>	562	7.8	36.6	18.5	2.6	
C ₃	<i>Datura stramonium</i>	195	0.60	153.2	37.1	49.9	Poorter and Evans, 1998
	<i>Echium plantagineum</i>	175	0.75	155.5	44.2	36.3	
	<i>Nicotiana tabacum</i>	143	0.55	112.3	33.8	28.9	
	<i>Nicotiana tabacum</i>	160	0.68	144	55.7	33.7	
	<i>Oryza sativa</i>	202	0.60	143	-	48.0	
	<i>Oryza sativa</i>	236	0.62	136.4	-	43.6	
	<i>Physalis peruvianum</i>	144	0.46	138.3	34.6	43.0	
	<i>Plantago major</i>	177	0.62	122.8	38.6	35.3	
	<i>Poa alpina</i>	143	0.83	140.6	39.1	23.8	
	<i>Poa annua</i>	244	0.87	91.3	24.2	26.2	
	<i>Poa compressa</i>	233	0.97	119.2	31.9	27.8	
	<i>Poa costiniana</i>	138	0.58	221.2	97.1	51.1	

(continued)

Table 2. (continued)

Species	PNUE	<i>A</i> per Rubisco sites (mol mol ⁻¹ N s ⁻¹)	N (mmol m ⁻²)	LMA (g m ⁻²)	Rubisco (μmol sites m ⁻²)	
	(μmol mol ⁻¹ N s ⁻¹)					
<i>Poa fawcettiae</i>	158	0.58	202.3	80.0	55.9	Westbeek et al., 1999
<i>Poa pratensis</i>	195	0.70	126.6	38.9	35.5	Westbeek et al., 1999
<i>Poa trivialis</i>	151	0.58	107.7	27.1	27.5	Westbeek et al., 1999
<i>Raphanus sativus</i>	222	0.67	136.2	34.1	45.3	Poorter and Evans, 1998
<i>Triticum aestivum</i>	238	0.74	114.3	-	43.6	Makino et al., 1988

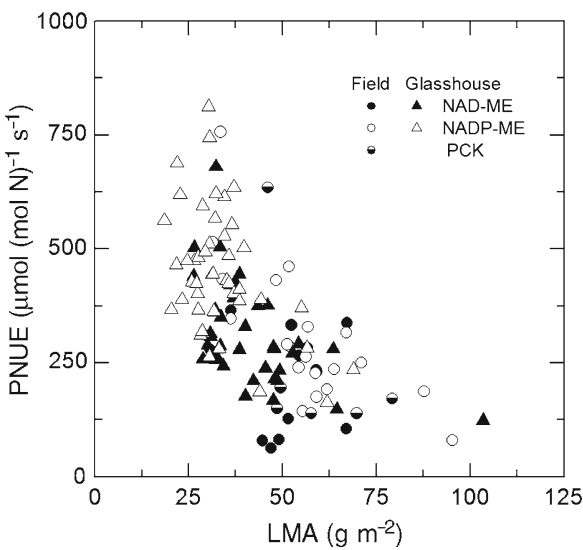


Fig. 7. Photosynthetic nitrogen use efficiency (PNUE) as a function of leaf mass per area (LMA) for glasshouse or field grown C_4 grasses described in Fig. 2

compared measurements of *A* per Rubisco content with in vitro measurements of k_{cat} , showed that the greater PNUE of NADP-ME relative to NAD-ME grasses can be explained almost entirely by differences in the in vitro k_{cat} of these species. These findings provided a direct association between PNUE and Rubisco kinetic properties in C_4 grasses with different biochemical subtypes (Fig. 6). An interesting spin-off of this association is the apparent relationship between PNUE and LMA in C_4 grasses (Fig. 7). For C_3 species, lower LMA due to thinner leaves was associated with a Rubisco with greater k_{cat} or a larger proportion of leaf N invested in photosynthesis (Poorter and Evans, 1998). In our work with C_4 grasses, low LMA was

weakly associated with greater allocation of N to Rubisco for C_4 leaves (data not shown). However, since LMA was consistently lower in NADP-ME than NAD-ME species, most of the variation of PNUE with LMA was associated with variation in k_{cat} (Fig. 7).

III. Water Use Efficiency

A. Water Use Efficiency of C_4 Grasses

Whole plant water use efficiency (WUE), the ratio of biomass produced to water used, is an important parameter in determining crop productivity and can be a key determinant of plant fitness and distribution (Long, 1999). WUE depends on the average intrinsic photosynthetic leaf water use efficiency (*W*) as well as on whole-plant biomass partitioning and respiration (Farquhar et al., 1989; Brugnoli and Farquhar, 2000). Leaf water use efficiency is given by

$$W = \frac{A}{E} = \frac{C_a(1 - C_i/C_a)}{1.6(e_i - e_a)} \quad (1)$$

where *A* is the rate of CO_2 assimilation, *E* is the rate of transpiration, C_i and C_a are the leaf intercellular and ambient pCO_2 , e_i and e_a are the vapour pressures in the intercellular airspaces inside the leaf and in the surrounding air, respectively, and 1.6 is the ratio of binary diffusivity of water vapour in air to that of CO_2 in air (Farquhar et al., 1982). Transpiration rate depends on boundary layer conductance (wind speed), stomatal conductance (g_s) and the leaf to air vapour pressure difference, which is influenced by leaf

temperature and humidity of the surrounding air. The assimilation rate is also affected by g_s (as CO₂ and water vapour share the same diffusion path) and the underlying biochemistry of CO₂ fixation. In line with increased PNUE, C₄ species tend to have a greater A for a given g_s compared to C₃ species. This is illustrated in Fig. 8, where CO₂ assimilation rate is plotted as a function of leaf conductance in cotton and *Zea mays* plants grown under different environmental conditions but measured under identical gas exchange conditions (Wong et al., 1985). The higher W of C₄ species compared to C₃ species is illustrated by the fact that they operate at a lower C_i/C_a ratio than C₃ species, which has been demonstrated numerous times (Wong et al., 1979).

Increase in W can be achieved by either increased A or decreased g_s . Increased W in C₄ species is due to an increase in A per leaf area, which is achieved by the CO₂ concentrating mechanism. It is more difficult to establish whether the increase in A , accomplished through the CO₂ concentrating mechanism, has been accompanied by a concomitant evolutionary decrease in stomatal conductance. There are examples of co-occurring C₃ and C₄ species where the C₄ species has the

higher A and lower g_s (Knapp, 1993; Kalapos et al., 1996; Long, 1999). However Körner et al., (1979) compiled leaf conductance values of 246 plant species of different functional groups and they reported no differences between cultivated C₃ and C₄ grasses. In reports where gas exchange characteristics of C₃, C₄ or C₃–C₄ intermediate species from the same genera have been studied, C₄ species have very similar g_s values to their C₃ relatives. Examples can be found for *Flaveria* species (Monson, 1989), *Panicum* species (Bolton and Brown, 1980), *Heliotropium* species (Vogan et al., 2007) and two closely related *Alloteropsis* species (Ripley et al., 2007). In contrast, recent evidence obtained by screening C₃ and C₄ grasses belonging to independent origins in the Poaceae and grown under common conditions showed that increased W was due to both higher A and lower g_s (Taylor et al., 2010). This is an area of research that deserves further attention as it sheds light on the coordination between photosynthetic capacity and stomatal conductance.

B. Water Use Efficiency and Carbon Isotope Discrimination in C₄ Species

During photosynthetic CO₂ fixation, plants discriminate against the naturally occurring stable isotope ¹³C. In C₃ species, fractionation of carbon in the plant material is caused by the fractionation occurring primarily during carboxylation by Rubisco and during the diffusion of CO₂ from the atmosphere to the chloroplast (Farquhar et al., 1989). Farquhar et al. (1982) showed that carbon isotope discrimination (Δ) has a strong linear relationship to C_i/C_a because Rubisco has a large fractionation factor of 30‰ (Fig 9). Because of the link of C_i/C_a to both Δ and W (Eq. 1) the carbon isotope composition of leaf dry matter of C₃ plants has been used successfully as a screening tool for altered WUE (Farquhar and Richards, 1984; Brugnoli and Farquhar, 2000). Photosynthetic carbon isotope discrimination in C₄ species is more complex and reflects the fractionation of both carboxylation steps as well as their interconnectivity (Farquhar, 1983). The initial hydration of CO₂ to bicarbonate and PEP carboxylation has a combined fractionation of –5.7‰ at 25°C and is dependent on temperature and on the amount of carbonic anhydrase present (Henderson et al., 1992; Cousins et al., 2006).

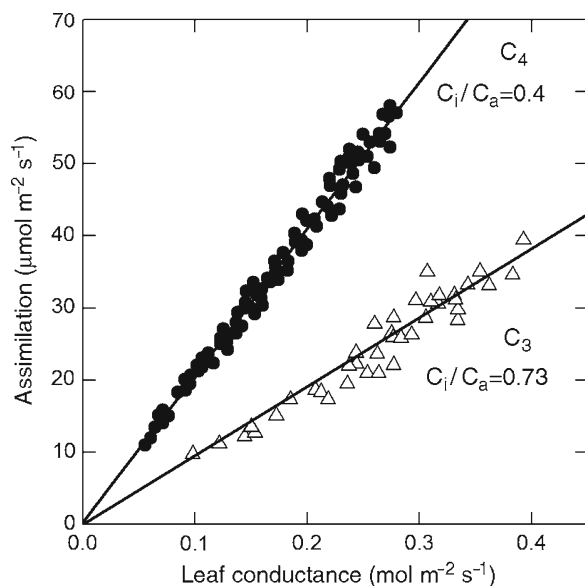


Fig. 8. Net CO₂ assimilation rate as a function of leaf conductance to CO₂ for *Zea mays* (C₄) and *Gossypium hirsutum* (C₃). Plants were grown under different nutrition, light or CO₂ environments and measurements were made at a leaf temperature of 25°C, high light and ambient p CO₂ (The data is redrawn from Wong et al., 1985).

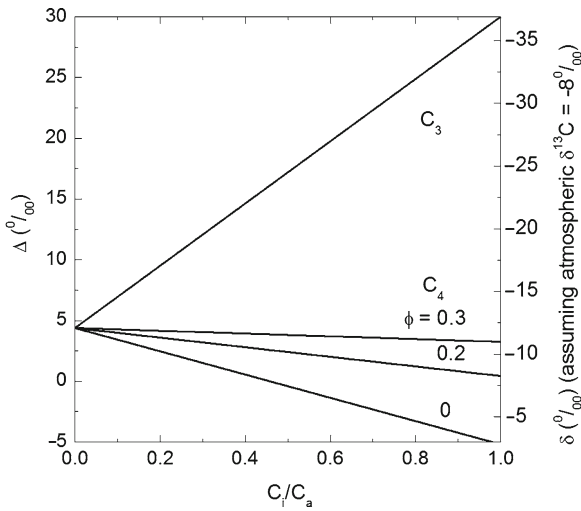


Fig. 9. Modeled photosynthetic carbon isotope discrimination as a function of the ratio of intercellular to ambient CO_2 (C_i/C_a). Carbon isotope discrimination is either referenced to the surrounding atmosphere (Δ) or the standard (PDB) (δ). $\Delta = R_{\text{air}}/R_p - 1$ and $\delta = R_p/R_{\text{(PDB)}} - 1$, where R stands for the ratio $^{13}\text{C}/^{12}\text{C}$ and the subscript p stands for photosynthetic product. $\Delta = (\delta_{\text{air}} - \delta)/(1 - \delta)$. The equations shown are $\Delta = 4.4 + 30 C_i/C_a$ for C_3 species and $\Delta = 4.4 + (-9.6 + \phi 28.2) C_i/C_a$ for C_4 species, where ϕ , the bundle sheath leakiness is defined as the ratio of the rate of CO_2 leakage out of the bundle sheath over the rate of CO_2 supply to the bundle sheath (Farquhar and Richards, 1984; Henderson et al., 1992).

The extent of Rubisco fractionation is dependent on bundle sheath leakiness (ϕ) defined as the fraction of CO_2 released in the bundle sheath by the C_4 cycle which is not fixed by Rubisco. The value of ϕ determines the slope of the relationship between Δ and C_i/C_a and this is illustrated in Fig. 9. Henderson et al. (1992) estimated ϕ to be about 0.2 under a range of environmental conditions and a number of C_4 species representing different decarboxylation types and these results have been confirmed for a number of C_4 grasses (Cousins et al., 2008). This means that the slope of the relationship between Δ and C_i/C_a has the opposite sign in C_4 compared to C_3 species. The change in Δ for a unit change in C_i/C_a is much less for C_4 compared to C_3 species. This together with the added complication of possible variations in ϕ make Δ less suitable for analysis of water use efficiency in C_4 plants (Henderson et al., 1998).

Consistent differences in the carbon isotope composition of plant dry matter between NAD-ME and NADP-ME species have frequently been reported and the depletion in ^{13}C in NAD-ME compared to NADP-ME species has been attributed to possible differences in leakiness (Hattersley, 1982; Ohsugi et al., 1988; Buchmann et al., 1996). It has been hypothesized that the presence of a suberised lamella in NADP-ME species may reduce ϕ by reducing the physical

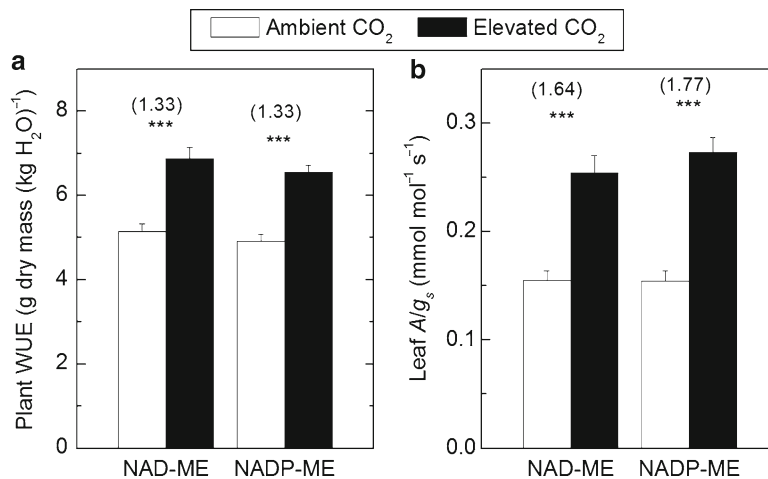


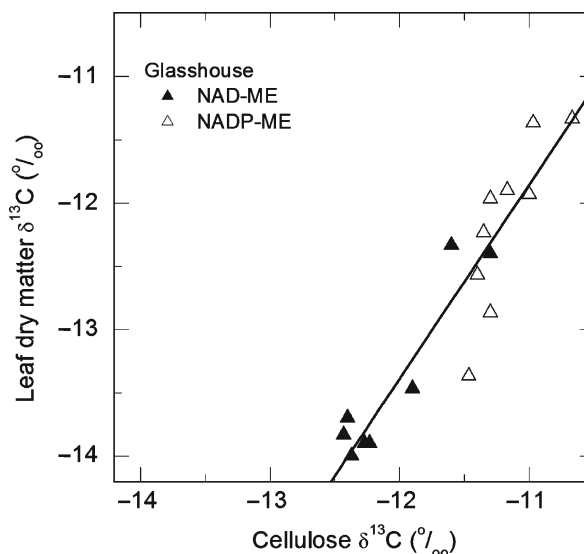
Fig. 10. (a) Mean whole plant water use efficiency (WUE) of 17 NAD-ME and NADP-ME grasses grown under ambient (420 μbar , open bars) or elevated (680 μbar , closed bars) $p\text{CO}_2$ in a naturally lit glasshouse. (b) The ratio of net CO_2 assimilation rate (A) to stomatal conductance (g_s), measured on young fully expanded leaves of 17 grasses grown as described in (a). Gas exchange measurements were made at high light at growth $p\text{CO}_2$. Values in parenthesis are the elevated/ambient ratios of the respective means. Statistical significance levels from a t-test for drought treatment are shown ***, $P < 0.001$ (Data was redrawn from Ghannoum et al., 2001b).

conductance to CO_2 diffusion across the bundle sheath. However the difference in dry matter Δ does not appear to be linked to differences in photosynthetic carbon isotope discrimination. Both Henderson et al. (1992) and Cousins et al. (2008) found no significant differences in ϕ or C_i/C_a between C_4 subtypes when gas exchange and carbon isotope discrimination were measured concurrently. A comparison of A/g_s (which is proportional to C_i/C_a) in 17 NAD and NADP-ME grasses also revealed no differences between the subtypes (Fig. 10b) suggesting that there are no differences in W (Ghannoum et al., 2001b). Indeed, there was no difference in plant WUE between the subtypes (Fig. 10a). Differences in quantum yield between the biochemical subtypes have also been ascribed to differences in leakiness (Ehleringer and Pearcy, 1983; Farquhar, 1983). However, Furbank et al. (1990) argued that there could be many other explanations for the variation in quantum yield. Cousins et al. (2008) examined photosynthetic discrimination at different light intensities and although Δ increased at lower light intensities suggesting an increase in ϕ , it did so similarly for both subtypes.

It is well-known that different carbon pools have different ^{13}C signatures (for example, lipids are depleted in ^{13}C). Hence, differences in leaf dry matter $\delta^{13}C$ can arise if the different biochemical subtypes partition their carbon in different proportions to the different pools. This can be seen by comparing the ^{13}C signature of dry matter with that of cellulose. While leaf and cellulose $\delta^{13}C$ were correlated in the various C_4 grasses, the difference between the two values was greater for the NAD-ME compared to the NADP-ME grasses (Fig. 11). This suggests that chemical composition is responsible for only part of the variation in $\delta^{13}C$ between NAD-ME and NADP-ME C_4 grasses. The reasons for the difference in dry matter and cellulose carbon isotope discrimination between NAD-ME and NADP-ME species therefore still need to be resolved.

C. Effects of Environmental Conditions on Water Use Efficiency of C_4 Grasses

Higher leaf W of C_4 species often translates into improved whole plant WUE (Osmond, 1982; Ehleringer and Monson, 1993; Long, 1999). Ghannoum and co-workers examined WUE in a number of wild, non crop C_4 grasses under different



to either no or little significant enhancement of leaf area in C_4 plants grown under relatively well-watered conditions. This is the case in both glasshouse (Ghannoum et al., 2001b; Siebke et al., 2002) or field free air CO_2 enrichment (FACE) (Leakey et al., 2006) experiments. Consequently, increases in A/g_s and W in C_4 grasses grown at elevated pCO_2 are not offset by increases in leaf area, and translate into greater WUE at the whole plant level (Fig. 10). Hence, increased WUE of C_4 grasses exposed to high pCO_2 are almost entirely driven by reduced stomatal conductance and transpiration, with no significant change in A (Ghannoum et al., 2001b). It is interesting to note that g_s continues to decrease with increasing pCO_2 beyond twice ambient concentrations, despite the fact that A is already CO_2 saturated at normal ambient pCO_2 (Siebke et al., 2002). This indicates that WUE of C_4 grasses is likely to continue improving with the steady rise in atmospheric pCO_2 .

In a drought experiment, WUE of NAD-ME and NADP-ME C_4 grasses was also increased, which was most likely due to decreased g_s as indicated by a more negative δ value (Figs. 9 and 12). Strikingly, NAD-ME grasses increased their WUE under drought to a greater extent than their NADP-ME counterparts (Ghannoum et al., 2002). This is in line with observations made by Kawamitsu et al. (1987) who found, in a small number

of C_4 grasses, that A/g_s and leaf W of NAD-ME types were higher than NADP-ME under high vapour pressure deficit. This means that under stress conditions these NAD-ME grasses were more water use efficient than the NADP-ME species examined, and maintained lower C_i/C_a . NAD-ME grasses are more abundant at the drier end of rainfall gradients than NADP-ME grasses (Ellis et al., 1980; Hattersley, 1992), and it is possible these WUE differences during drought may contribute to the differences in the distribution of these species along rainfall gradients.

C_4 photosynthetic pathways are common in the grass family with diverse taxonomic origins that also encompass all three of the classic decarboxylation subtypes (see Chapter 16, this volume). However, the physiological significance (if any) of this biochemical diversification of C_4 photosynthesis including the variation in Rubisco catalytic turnover rate discussed above, remains poorly understood. The global distribution of C_4 grasses is positively correlated with growing season temperature (Teeri and Stowe, 1976; Vogel et al., 1978; Hattersley, 1983), whereas the geographic distribution of the different C_4 subtypes is strongly correlated with rainfall. With increasing annual rainfall, the occurrence of NADP-ME and PCK species increases, whilst NAD-ME species are prevalent in low rainfall areas

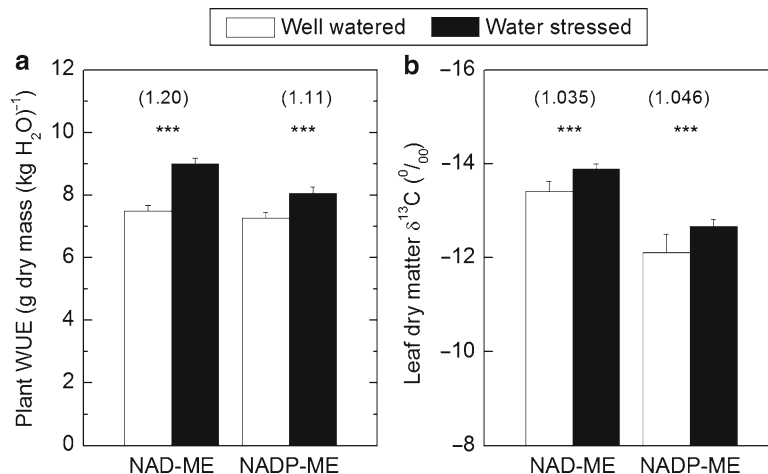


Fig. 12. (a) Mean whole plant water use efficiency (WUE) of 18 NAD-ME and NADP-ME grasses grown under well watered (open bars) or water stressed (closed bars) conditions in a naturally lit glasshouse. (b) Carbon isotope composition ($\delta^{13}C$) of leaf dry matter of 18 C_4 grasses grown under well-watered (open bars) or water stressed (closed bars) conditions as described in (a). The origin is drawn at -8‰ as this is close to the atmospheric composition. Statistical significance levels from a t-test for drought treatment are shown ***, $P < 0.001$ (Data was redrawn from Ghannoum et al., 2002).

(Ellis et al., 1980; Hattersley, 1992). One might expect that these distribution differences could link to differences in WUE. The convergence found in WUE and leaf gas exchange characteristics under most growth conditions between NAD-ME and NADP-ME grasses is a curious reminder that geographic distribution may not be related fully to the physiology of photosynthesis.

IV. Conclusions

The CO₂ concentrating mechanism operating in C₄ leaves allows Rubisco to operate at high $p\text{CO}_2$, leading to the CO₂-saturation of A in normal air. This allows C₄ plants to have higher leaf and whole plant nitrogen and water use efficiencies than their C₃ counterparts, when considered under their normal growth conditions. Greater leaf water use efficiency is largely due to increased A rather than reduced g_s . Greater leaf nitrogen use efficiency is the result of the C₄ concentrating mechanism allowing full expression of Rubisco k_{cat} . The advantages of greater NUE and WUE of C₄ relative to C₃ photosynthesis are fully realized at high light and temperature. Elevated $p\text{CO}_2$ does not usually affect the NUE of C₄ species. In contrast, WUE is greatly enhanced by high $p\text{CO}_2$ and water stress. In both cases, increased WUE results mainly from changes in g_s .

In addition to differences between C₃ and C₄ photosynthetic types, differences in NUE and WUE have also been observed between the C₄ biochemical subtypes. NUE is superior in NADP-ME relative to NAD-ME grasses because NADP-ME species have lower leaf N content and higher Rubisco k_{cat} . WUE is similar between the two subtypes under most environmental conditions, as long as plants are well-watered. Under water stress, NAD-ME type species, which are more abundant at the lower end of rainfall, have higher WUE than NADP-ME species. The significance of the intra-C₄ diversification is a research area worthy of further investigation.

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Development of Leaves in C_4 Plants: Anatomical Features That Support C_4 Metabolism

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Summary	147
I. Introduction	148
II. Overview of C_4 Biology and Leaf Anatomy.....	148
III. Quantitative Variation in Leaf Traits	149
A. Venation.....	149
1. Organizing Role of Venation	149
2. Formation of the Leaf Venation Pattern	149
3. Polar Auxin Transport and Vein Ontogeny in Arabidopsis.....	150
4. Vein Ontogeny in Other Species.....	151
5. Theoretical Relationships Between PAT, Leaf Cell Proliferation and Vein Pattern	151
6. Regulation of Density of Leaf Venation.....	151
B. Downstream from Vein Formation: Patterns of Cell Proliferation, Expansion and Differentiation for C_4 Cells.....	152
C. Barriers and Connections: Suberin Lamellae and Plasmodesmata	153
D. Environmental and Developmental Plasticity.....	154
IV. Complex Traits and Systems Analysis.....	154
Acknowledgments	155
References	155

Summary

C_4 metabolism is a metabolic cooperation between distinct sites for primary carbon assimilation and primary carbon reduction. In most C_4 species, the cooperating sites are in specialized cell types – mesophyll and bundle sheath – organized around a dense pattern of leaf venation and joined by abundant plasmodesmata. There is much recent information on the formation of venation, plasmodesmata, and barriers to gas diffusion in leaves. Recent evidence suggests that the specialized patterns of these features in C_4 leaves come from quantitative and spatial regulation of gene networks and protein interactions present in all higher plants. These networks and their regulatory points should emerge from the computational modeling of biological systems data from developing C_4 and C_3 leaves.

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I. Introduction

Leaf anatomical specializations for C_4 metabolism have evolved in many plant groups, in the context of many different underlying patterns of leaf shape and venation. Based on surveys to date, C_4 photosynthesis and Kranz anatomy are found in 16 eudicot families and in three monocot families (Kellogg, 1999; Sage et al., 1999; Sage, 2004; Muhaidat et al., 2007), representing numerous independent lineages of C_4 evolution. In both the C_4 dicots and the C_4 monocots that have Kranz anatomy, the leaves are specialized to provide four basic features: (1) high density of venation, (2) sequestration of the primary carbon reduction (PCR) tissues from the atmosphere, (3) extensive contact and communication between PCR and primary carbon assimilation (PCA) cells by means of cell arrangement and a high density of PCR–PCA plasmodesmata, and (4) complementary photosynthetic/metabolic specialization of PCA and PCR cells and organelles, commonly through extensive photosynthetic development of the bundle sheath (Dengler and Nelson, 1999; Dengler and Taylor, 2000; Muhaidat et al., 2007). The evolutionary convergence onto this suite of features probably reflects the common selection pressures provided by the environments in which each lineage evolved (Sage, 2001, 2004).

Numerous reviews and studies have documented the variations in the leaf anatomical features in each group containing C_4 species, including the frequent coevolution of certain combinations of C_4 biochemical type with particular cell arrangements (Hattersley and Watson, 1976; Dengler et al., 1994; Dengler and Nelson, 1999; McKown and Dengler, 2007; Muhaidat et al., 2007; Chapter 4, this volume). Such quantitative anatomical studies have thus far provided most of our understanding of structure–function relationships in C_4 leaves. To complement these studies and surveys, this chapter will focus on the current general understanding of the development and properties of veins, plasmodesmata, gas diffusion barriers, and specialization of the bundle sheath. The diverse schemes for C_4 carbon fixation all appear to rely on spatial and quantitative variations in the

regulation of resources present in the genomes of all higher plants. Veins, plasmodesmata, PCA and PCR functions, and gas diffusion barriers are likely to be produced by genetic and protein interaction networks that are similar among groups of higher plants. This implies that the recurring quantitative and spatial patterns of these features selected by C_4 evolution map to genes or other elements that regulate entire networks of structural genes that together produce the features.

II. Overview of C_4 Biology and Leaf Anatomy

In C_4 plants, PCA from intercellular CO_2 is spatially separated from the PCR process that employs Rubisco and the reductive pentose phosphate cycle (Edwards et al., 2001a). The sequestering of PCR processes into a subcellular or cellular compartment that restricts interaction with the atmosphere provides the conditions that give C_4 photosynthesis measurable advantages in environments that would otherwise favor photorespiration. Key photosynthetic advantages are the elevation of CO_2 concentration and the elimination of oxygen at the site of Rubisco (Kanai and Edwards, 1999; Leegood, 2002). Individual C_4 species, even those within the same families, exhibit a variety of anatomical and developmental strategies that achieve the compartmentalization of PCA and PCR and at the same time facilitate rapid metabolite fluxes to link these two processes (Sinha and Kellogg, 1996; Dengler and Nelson, 1999; Sage, 2001; Chapter 11, this volume).

The most common anatomy in support of C_4 biology is the wreath-like cellular arrangement, Kranz anatomy (Haberlandt, 1914), in which a highly developed photosynthetic bundle sheath (BS) layer and an adjacent mesophyll (M) cell layer are concentrically organized around a dense leaf venation. Variations exist in the number of cell layers from vein to mesophyll and in the ontological derivation of the PCR and PCA cells. Carbon fixation processes are compartmentalized such that PCA occurs in M cells and PCR occurs in BS cells, and each cell type is specialized for its share of these processes (Nelson and Dengler, 1992; Dengler and Nelson, 1999; Edwards et al., 2001a; Leegood, 2002). C_4 type carbon fixation is a metabolic cooperation between the two cells. A high density

Abbreviations: BS – Bundle sheath; DC – Distinctive cell; M – Mesophyll; PAT – Polar auxin transport; PCA – Primary carbon assimilation; PCR – Primary carbon reduction; PD – Plasmodesmata; PIN – PIN-LIKE (auxin efflux protein)

of BS-M plasmodesmata supports the intercellular fluxes of C₄ metabolites, and a high density of leaf venation maintains close proximity and roughly equal numbers of the two cell types – close to a 1:1 ratio in most C₄ grasses (Dengler and Nelson, 1999; Dengler and Taylor, 2000). There are many variations on this basic scheme for compartmentalization of PCA and PCR functions. In the leaves of certain C₄ species of Chenopodiaceae, cooperating PCA and PCR sites are within the same photosynthetic cells, in physically separate intracellular compartments (Edwards et al., 2001b, 2004); this particular variation is the subject of another chapter in this volume (See Chapter 4, this volume).

III. Quantitative Variation in Leaf Traits

In the evolution of C₄ leaves, the genetic resources that produce leaf anatomical features, such as plasmodesmata, venation and photosynthetic cell types, have become subject to specialized patterns of spatial and quantitative regulation (Gutierrez et al., 1974; Laetsch, 1974; Dengler and Nelson, 1999; Dengler and Taylor, 2000; McKown and Dengler, 2007; Muhaidat et al., 2007). Although the plasmodesmata and veins of C₄ leaves appear to be highly similar to those in their non-C₄ relatives in their ontogeny and basic structure, they differ greatly in density and abundance. Other features of C₄ leaf anatomy exaggerate features present in other plants. The bundle sheath, which makes minor if any contribution to the photosynthetic activity in leaves of non-C₄ species, is endowed with the key PCR role in C₄ leaves, and undergoes a much greater cell expansion and organellar development than occurs in the BS of non-C₄ species.

A. Venation

1. Organizing Role of Venation

Two aspects of leaf venation in C₄ plants are significant. First, nearly all C₄ plants exhibit a high density of venation and correspondingly small interveinal distance and cell number (Hattersley and Watson, 1975; Prendergast et al., 1987; Dengler and Nelson, 1999; Ueno et al., 2006; Muhaidat et al., 2007). Second, the leaf venation is the developmental and structural framework for Kranz anatomy and other C₄ anatomical

specializations, which vary in proportion to radial distance from the nearest vein (Hattersley, 1984; Dengler et al., 1994; Dengler and Nelson, 1999; Dengler and Taylor, 2000). In C₄ plants, PCA and PCR cells are organized in zones concentric with veins, regardless of the clonal derivation of these cells in a particular species. Numerous studies of the ontogeny of leaves of C₃ and C₄ monocot and dicot species have revealed that the establishment of a vein center in a developing leaf region precedes the differentiation of other anatomical and physiological specialization within that region, making it likely that subsequent cell division and differentiation events in the region are influenced by radial position relative to vein.

The radial organization of functions and cell types around C₄ veins is formally similar to the radial organization of the stem. Although it appears that the genetic effectors of the radial organization of xylem and phloem in the stem (e.g., Class III HD-zip transcription factors) have evolved into the adaxial/abaxial polarity system of leaves (Emery et al., 2003; Izhaki and Bowman, 2007), similar signals may be distributed radially around incipient vascular sites in leaves to effect the complementary cell differentiation of BS and M cells in successively distant zones (Langdale et al., 1988; Langdale and Nelson, 1991; Brutnell and Langdale, 1998). The local signals emanating from the developing vascular center might include hormones such as auxin, cytokinin, and brassinosteroids, as well as small RNAs, peptides, transcription factors, and metabolites. The same systems and effectors that regulate the venation pattern and vascular cell differentiation may influence the specialization of other cooperating leaf cells in the immediate region, based on their physical relationship to the venation. Thus, the formation of the leaf venation pattern may be the first manifestation of a local spatial regulation that organizes downstream the entire C₄ pattern of leaf development, including the local differentiation of bundle sheath and mesophyll cells, through integrated local signals for cell proliferation, polarity, and differentiation.

2. Formation of the Leaf Venation Pattern

The ontogeny of leaf venation has been described at the level of developmental anatomy in many monocot and dicot species, including several C₄ species (Dengler et al., 1990, 1996a,b, 1997;

Bosabalidis et al., 1994; Nelson and Dengler, 1997; Sud and Dengler, 2000; Dengler and Kang, 2001; McKown and Dengler, 2007). The hierarchical leaf venation patterns of both monocots and dicots are organized progressively during leaf development, coordinated with leaf initiation and growth (Nelson and Dengler, 1997; Dengler, 2001). In both monocots and dicots, the initiation of successive vein orders is highly coordinated with the general increase in cell number and leaf area in the blade (Dengler and Kang, 2001; Kang and Dengler, 2002, 2004; Kang et al., 2007). The pattern of venation appears to be influenced by the shape of the blade; treatments or mutations that limit vascular development are generally associated with reduction in the blade (Mattsson et al., 1999; Sieburth, 1999). The midvein and other major veins appear first, followed by lesser vein orders as the leaf expands. Recently, a substantial amount of experimental evidence has accumulated that supports the hypothesis proposed by Sachs (1991) that the formation of veins is a manifestation of dynamic sink-source relationships that orient polar auxin transport (PAT) within a developing tissue. In leaves, the ontogeny of venation pattern was hypothesized to reflect the dynamic shifts in paths linking auxin sources and sinks as cells in the developing leaf proliferate, expand and mature (Berleth et al., 2000a,b; Aloni, 2001; Aloni et al., 2003). The paths were hypothesized to be channeled in a self-reinforcing fashion that resists lateral spreading, through the induction of vascular cell differentiation by auxin (Sachs, 1991; Berleth et al., 2000a,b).

3. Polar Auxin Transport and Vein Ontogeny in Arabidopsis

Mechanistic genetic and molecular studies of vein patterning are most advanced in Arabidopsis, a C_3 species. The initiation of a midvein appears to be directly linked to leaf initiation, and may be a direct consequence of the primordium-initiating event and its effectors. It is notable that even the most severe of the many mutants that influence leaf or cotyledon venation pattern have little or no effect on the midvein, suggesting that most midvein-defective mutants fail to initiate organs (Carland et al., 1999; Koizumi et al., 2000; Sieburth and Deyholos, 2006). Auxin-responsive

reporters and the localization of PIN proteins reveal that a maximum of auxin is produced at the incipient leaf tip by transport of auxin originating outside the primordium. The primary route appears to be through the protoderm to the tip, oriented by the subcellular localization of the PIN auxin efflux proteins in cells of that layer (Benkova et al., 2003; Tanaka et al., 2006; Vieten et al., 2007). In Arabidopsis, the subsequent channeling of this auxin maximum away from the tip by PIN-guided polar auxin transport appears to guide the formation of tip-to-base procambial cells that subsequently proliferate and form the midvein. For later vein orders, the progressive appearance of procambial cell files for each order is nearly coincident with the presumed PAT paths identified by cellular alignment of PIN auxin efflux proteins, and with maxima for auxin-responsive gene expression along the path (Scarpella et al., 2006; Wenzel et al., 2007).

A substantial number of Arabidopsis mutants that are defective in venation pattern correspond to genes with demonstrated or implied roles in endomembrane traffic (Fukuda, 2004; Sieburth and Deyholos, 2006). It is likely that at least some of this traffic is dedicated to the subcellular alignment of the machinery for PAT within cells in the developing vein paths, as well as to other processes polarized along the vein paths. The differentiation of procambial cell files has been associated with the localized expression of transcription factors including class III HDZIP genes, and MONOPTEROS (Mattsson et al., 2003; Kang et al., 2003; Koizumi et al., 2005; Sawa et al., 2005). A large number of genes has been associated with further vascular cell differentiation, many of them first identified based on their cell-specific expression in Zinnia or Arabidopsis in vitro transdifferentiation systems, or by mutant phenotypes in Arabidopsis (Fukuda, 2004). Included among these are NAC-domain and HD-zip III transcription factors (Ohashi-Ito and Fukuda, 2003; Kubo et al., 2005; Ohashi-Ito et al., 2005), D/V polarity genes (for X/P), hormone signals (Fukuda, 2004; Yamamoto et al., 2007), perhaps acting in paracrine fashion to influence complementary neighbors, a xylogen continuity factor (Motosé et al., 2004), CLV3/ESR-related (CLE) peptides (Sawa et al., 2006) and putative vascular cell identity genes (Nishitani et al., 2001).

4. Vein Ontogeny in Other Species

Although the inhibition of PAT causes disturbances in the venation pattern of maize (Tsiantis et al., 1999) and other species (Mattsson et al., 1999) in addition to Arabidopsis, it is thus far unclear whether vein pattern formation is mechanistically similar in monocot and dicot leaves. Members of the class III HD ZIP, KANADI, G2-like, NAC, AP2, MADS, and MYB transcription factor families have been associated with vein formation and patterning in Arabidopsis (Baima et al., 1995; Mattsson et al., 2003; Scarpella et al., 2004; Zhao et al., 2005; Ko et al., 2006). In the monocot rice, specific members of all of the same transcription factor families are significantly expressed in seedling vascular tissues, based on transcriptional profiling of isolated cells (Rice Atlas of cell-specific transcriptomes, <http://plantgenomics.biology.yale.edu/riceatlas>; Jiao et al., 2009). A rice procambial-specific HD-zip transcription factor, Oshox1, influences polar auxin transport (Scarpella et al., 2002).

Vein pattern formation and vascular cell differentiation have been characterized in several C₄ species, using methods of quantitative anatomical and developmental analysis, including the grasses maize (Bosabalidis et al., 1994), *Stenotaphrum secundatum* (Sud and Dengler, 2000) and *Arundinella hirta* (Dengler et al., 1996a, b, 1997), and several species of *Flaveria* (McKown and Dengler, 2007), and *Cyperacea* (Ueno et al., 1989; Soros and Dengler, 1998, 2001). *Arundinella hirta* and certain other members of the genera *Arundinella*, *Garnotia*, *Microstegium* and *Arthraxon* are of particular interest with regard to the influence of the vein patterning process on C₄ differentiation, since they lack the high density of minor veins found in most C₄ grasses, and instead form files of “distinctive cells” (DC), PCR cells that cooperate with M cells but do not surround veins (Dengler et al., 1990, 1995, 1996b, 1997; Ueno, 1995; Wakayama et al., 2003, 2006). The observation that files of DC’s occupy sites that in related species develop into veins surrounded by photosynthetic BS cells suggests that positional signals for vein formation directly guide the formation of a provascular derivative cell type, the BS, in DC species. Studies of C₄ monocot and dicot leaf cellular patterns have been reviewed

previously (Dengler and Nelson, 1999; Muhaidat et al., 2007), including the many ontogenetic relationships that have evolved between vascular tissue, PCR, and PCA cells.

5. Theoretical Relationships Between PAT, Leaf Cell Proliferation and Vein Pattern

Whether the observed dynamic formation of auxin paths is the primary organizing process for leaf venation or is a key part of the downstream molecular machinery for the execution of an underlying pattern based on other effectors is the subject of ongoing experimentation and modeling. A variety of computational models have demonstrated that most observed features of leaf venation patterns (closed loops, freely ending veinlets, parallel veins) could be produced in a self-organizing ontogenetic sequence by PAT or another effector system. These models generate vein patterns similar to those observed, if programmed with the patterns of cell proliferation and expansion for the particular leaf and species. Most of these models assume that there exists feedback regulation between auxin flow and efflux carrier localization or other spatial limits on an effector (Meinhardt, 1996; Burton, 2004; Rolland-Lagan and Prusinkiewicz, 2005; Dimitrov and Zucker, 2006; Feugier and Iwasa, 2006; Fujita and Mochizuki, 2006a, b; Scarpella et al., 2006; Berleth et al., 2007). In the simplest scheme, procambial differentiation follows the resulting patterns of auxin flux or concentration. Experimental observations of PIN protein orientations and cell proliferation in Arabidopsis leaves support this view (Donnelly et al., 1999; Mattsson et al., 1999, 2003; Sieburth, 1999; Scarpella et al., 2006; Kang et al., 2007). A developing leaf appears to be regulated as a field that can in some cases balance disturbances in cell proliferation with compensatory changes in cell expansion to achieve its overall shape (Tsukaya, 2002, 2003, 2005, 2006; Ferjani et al., 2007). Despite this maintenance of leaf shape, disturbances in cell proliferation do result in vein pattern and density changes (Kang et al., 2007).

6. Regulation of Density of Leaf Venation

C₄ leaves are generally distinguished by a higher density of venation than their C₃ relatives. Within

taxonomic groups that include related C_4 and C_3 species, the C_4 species have a significantly smaller leaf interveinal distance (and number of cells) than their C_3 relatives (Crookston and Moss, 1974; Hattersley and Watson, 1975; Kawamitsu et al., 1985; Ueno et al., 2006; McKown and Dengler, 2007; Muhaidat et al., 2007). Presumably, the interveinal distance was minimized by selection to optimize the ratio of PCA and PCR cells and to minimize the potentially limiting distances for metabolite and photosynthate diffusion. The correlation of C_4 biochemical properties with vein density among C_3 , C_4 and C_3 - C_4 intermediate species in genera such as *Flaveria* support the proposal that increased vein density is a “precondition” for the evolution of other elements of C_4 physiology (Sage, 2004; McKown and Dengler, 2007). High vein density and accompanying reduced interveinal cell count are well correlated with degree of “ C_4 -ness” among the intermediate and C_4 species, and are predicted to provide physiological enhancements to C_4 species beyond a favorable BS/M ratio (Helliker and Ehleringer, 2000; Ogle, 2003). However, there are few experimental or genetic studies that demonstrate the consequence of altering vein density in C_4 species. Reduced vein-density variants of *Panicum maximum* produced by heavy mutagenesis were compromised in C_4 photosynthesis (Fladung, 1994). The sheath region of maize leaves, with a C_3 -like interveinal cell number, is C_3 in its photosynthetic gene expression pattern in cells distant from veins, but C_4 in cells adjacent (Langdale et al., 1988); the blade, which has a higher vein density, is entirely C_4 .

If the ontogeny and final pattern of leaf venation is a consequence of the patterns of leaf shape and expansion, through their influence on PAT paths, then it is the spatial and temporal regulation of cell proliferation, polarity and expansion that guides venation and C_4 cell differentiation. C_4 species that achieve a high density of venation appear to do so via a heterochronic regulation of the existing machinery for vein formation: (this is an awkward connection here, why not revise to have two sentences to improve clarity) a persistence of the initiation of minor veins beyond the developmental time at which it ceases in their non- C_4 relatives. This occurs without a substantial difference in the blade shape or overall change in cell division patterns. Vein density is a plastic

character that varies with environment, and light intensity in particular, in some species (Adams et al., 2007).

B. Downstream from Vein Formation: Patterns of Cell Proliferation, Expansion and Differentiation for C_4 Cells

Whatever the developmental linkage between vein initiation and the differentiation of surrounding cells, C_4 species that localize PCA and PCR functions in separate cell types have evolved a variety of ways of organizing and producing these cells among different lineages, summarized in a number of reviews (Dengler and Nelson, 1999; Soros and Dengler, 2001; Muhaidat et al., 2007). In species with typical Kranz anatomy, the PCR functions are specialized in the bundle sheath (BS) surrounding the vascular bundles (Fig. 1). The BS specializations include increased cell volume, increased chloroplast size and number, asymmetric arrangement of cytoplasmic contents, and a surrounding extracellular barrier to gas diffusion (e.g., suberin). Several maize mutations have been described that influence the photosynthetic specialization of the BS (Langdale and Kidner, 1994; Roth et al., 1996; Hall et al., 1998; Brutnell et al., 1999; Cribb et al., 2001). In all such mutants described to date, the BS is anatomically specialized in the C_4 pattern, except for defects in BS plastid development and morphology. Mesophyll (M) cells are often enlarged radially to enhance contact with BS cells. Numerous plasmodesmata link adjacent BS and M cells, facilitating the intercellular diffusion of metabolites. As noted above, the Kranz cell arrangement can be the result of various cell division and lineage patterns during leaf development. Many variations and alternatives to the Kranz scheme have been noted in both monocots and dicots, such as the occurrence of single-cell compartmentalization of PCA and PCR functions in the Chenopodiaceae (Edwards et al., 2004), the formation of BS-like “distinctive cell” files in certain grass species (Dengler et al. 1996a) and the radially extended files of BS cells in the maize *tangled* mutant (Jankovsky et al., 2001). This suggests that C_4 advantages can be obtained across a very broad range of supporting cellular arrangements as long as the PCA and PCR functions are sufficiently compartmentalized.

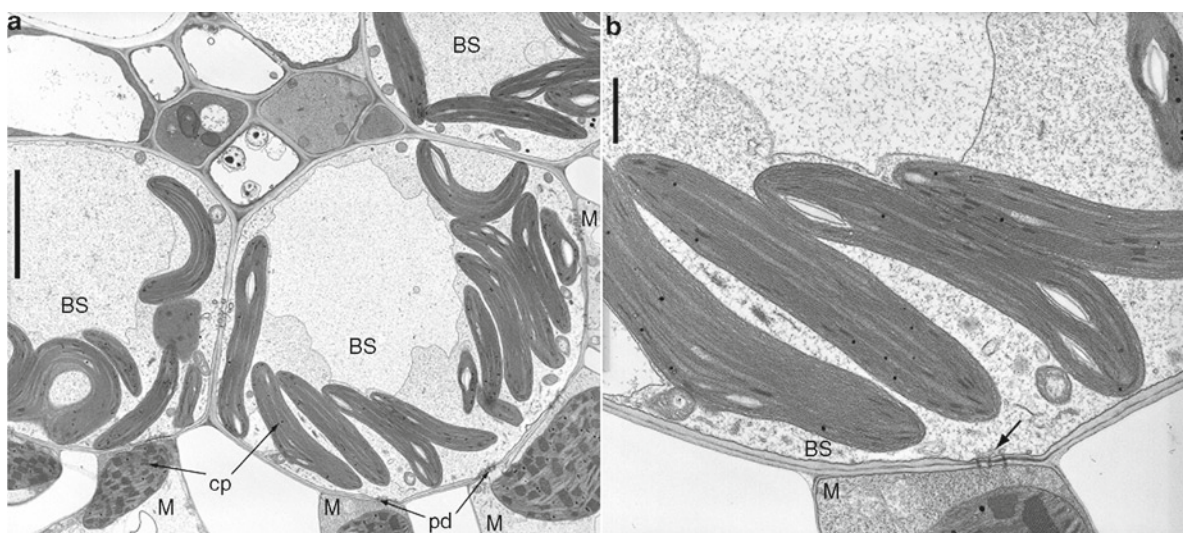


Fig. 1. Junctions of bundle sheath (BS) and mesophyll (M) cells in mature tip region of *Zea mays* inbred B73 18 cm leaf 3, grown under conditions of 12 h light (500–600 μ E metal halide + incandescent, 31°C), 12 h dark (22°C.), and 50% relative humidity. Standard glutaraldehyde fixation, osmium tetroxide postfixation, dehydration in acetone and embedding in Spurr's resin. Courtesy of Drs. Robert Turgeon (Cornell University) and Richard Medville (Electron Microscopy Services and Consultants, Colorado Springs, CO 80918). (a) and (b) are junctions from the same region; image (a) predominantly M cell, image (b) predominantly BS cell. Plasmodesmata (PD), suberin layer (S), and chloroplasts (cp) are labeled.

C. Barriers and Connections: Suberin Lamellae and Plasmodesmata

In two-cell C₄ schemes, the PCR cells (usually bundle sheath) are surrounded by a diffusion barrier that enables cells to exclude oxygen and to retain carbon dioxide, and are joined to neighboring PCA (mesophyll) and vascular cells by unusually abundant plasmodesmata (PD) (Fig. 9.1). Mechanically isolated bundle sheath strands from C₄ plants are capable of limiting gas diffusion and of selective permeability to metabolites (Weiner et al., 1988; Furbank et al., 1989, 1990; Jenkins et al., 1989). The diffusion barrier surrounds the cell wall and can consist of lamellae of suberin, the complex three-dimensional polyester that acts as an apoplastic water/solute barrier in roots, and in a variety of other roles (Kolattukudy, 2001; Franke and Schreiber, 2007; Graca and Santos, 2007). Recently, a suberin biosynthetic pathway based on products of numerous candidate genes, encoding membrane-associated P450 and fatty acid elongation complexes and other enzymes, was proposed for *Arabidopsis* (Franke and Schreiber, 2007), providing a basis for further genetic/genomic analysis of suberin biosynthesis and its regulation.

The plasmodesmata (PD) of C₄ leaves appear to be qualitatively similar to those present in C₃ leaves and elsewhere in the C₄ plant, based mostly on appearance and range of size-exclusion-limits (Evert et al., 1977, 1996; Robinson-Beers and Evert, 1991a, b; Botha, 1992; Botha et al., 1993). At the BS/M junction they are abundant enough to facilitate the metabolite fluxes of the C₄ pathway (Weiner et al., 1988; Robinson-Beers et al. 1991a,b). Although an increasing number of functions of PD in plant development and physiology have been studied in detail, including selective intercellular traffic of viruses, transcription factors, RNAs, and other signals (Haywood et al., 2002; Cilia and Jackson, 2004; Kim, 2005; Hofmann et al., 2007; Kim et al., 2007), it has proved exceedingly difficult to analyze the biogenesis and regulation of PD in more than a descriptive manner. Although it has long been clear that PD include a desmotubule core continuous with the ER of the adjoined cells, cytoplasmic sleeve, and a plasma membrane continuous with that of adjoined cells, only recently have specific proteins been associated with PD (exclusive of viral movement proteins), including a RabGT-Pase, centrin, calreticulin, and others (Cilia and

Jackson, 2004). The formation, location, and selectivity of PD is highly regulated during development of embryos and seedling organs, and it is reasonable to assume that the same or similar systems regulate the PD-producing gene/protein networks to produce the abundance, location and qualities of BS/M PD for C_4 metabolism. The abundance of PD appears to be a developmentally regulated and plastic character that responds to environmental factors such as light intensity at the time of leaf development (Ormenese et al., 2000; Roberts et al., 2001; Amiard et al., 2005; Adams et al., 2007).

D. Environmental and Developmental Plasticity

Some aspects of C_4 cellular differentiation appear to be plastic, and able to vary with environmental conditions, although plasticity overall seems to be less than in C_3 relatives (Sage and McKown, 2006). A few studies suggest that the C_4 system itself can be facultative and responsive to environmental demands and resources. In the C_3 -like sheath region of maize leaves, described above, photosynthetic differentiation of the M cells is in the C_4 pattern only in the vein-adjacent M cells, at a radius that depends on light intensity (Langdale et al., 1988). Vein-distant M cells differentiate in the C_3 pattern. The interveinal region may span up to 20 mesophyll cells, which can assume either the C_4 or C_3 pattern of mesophyll gene expression (e.g., absence or presence of RuBisCo) depending on the relative proximity to a vein and on light intensity. In higher light intensity, C_4 -type mesophyll cells extend farther from the nearest vein than when they develop in low light. Although this suggests there is an environmental regulation of the C_4 pattern of enzyme expression, the underlying anatomy that produces discrete adjacent compartments for PCA and PCR functions does not vary with light level. The sedge *Eleocharis vivipara* exhibits Kranz anatomy and C_4 metabolism in aerial leaves, but simplified leaf anatomy and C_3 metabolism in submerged leaves (Ueno et al., 1988; Ueno, 1996, 1998, 2001; Agarie et al., 1997, 1997; Ueno and Wakayama, 2004). The C_3 to C_4 transition can be influenced by abscisic acid (Ueno, 1998). However, the basic Kranz organization of most C_4 leaves, with specialized differentiation of adjacent and concentric BS and M cells, highly developed BS organelles and

high-density venation, does not appear to vary greatly with environment, at least in C_4 species characterized to date. In addition to this apparent homeostasis for potentially plastic anatomical features such as vein density, plasmodesmatal density and Kranz differentiation, a similar homeostasis is enforced for carbon metabolism. Many attempts have been made to modify carbon metabolism in rice through the introduction by transformation of C_4 pathway genes from maize (Matsuoka et al., 2001). Although some effects could be observed, in the majority of cases, an apparent metabolic homeostasis neutralized the effects of the additional and sometimes cell-specific enzyme activity introduced by the transgene (Matsuoka et al., 2001). The developmental plasticity of C_4 leaves is an area worthy of further study, since variation in a C_4 trait under environmental conditions would provide potential means of identifying its means of regulation (see Sage and McKown, 2006).

IV. Complex Traits and Systems Analysis

The leaf traits supporting C_4 biology appear to result from quantitative and spatial adjustments in the regulation of traits that exist in C_3 plants. Three predictions of this view are (1) the genetic and protein interaction networks that produce venation or plasmodesmata and other key leaf traits are the same in C_3 and C_4 plants, but with different patterns of regulation, (2) relatively few regulatory genes govern C_4 traits by acting on these networks, and (3) the networks are regulated quantitatively during development and in different environmental conditions in both C_4 and C_3 plants. That is, C_4 biology evolved primarily through the re-regulation of existing gene networks and traits, rather than through the evolution of novel genes and traits. This is consistent with the numerous independent lineages in which the conditions or stepwise “preconditions” for C_4 physiology were achieved in evolution, since relatively few regulatory factors can re-pattern entire downstream networks producing the traits. The efficiency of C_4 biochemistry has certainly been enhanced by the evolution of optimized isoforms of C_4 enzymes such as PEPC (Akyildiz et al., 2007; Gowik et al., 2004), but the repeated evolution of C_4 schemes is likely to have relied on altered patterns of the regulators farther upstream.

Recently, new tools have emerged for the analysis of complex quantitative traits, such as the gathering of systems data at the whole transcriptome, proteome, metabolome, and phenome levels, and computational approaches that model this data within networks of regulation, interaction, expression and metabolism (Hartwell et al., 1999; Barabasi and Oltvai, 2004; Yu et al., 2006; Zhu et al., 2007). Features that resisted genetic, biochemical, or developmental analysis because of their hierarchical complexity and/or redundant genetic basis may yield to systems approaches or to new genetic strategies capable of resolving multigenic quantitative traits (Yu et al., 2008). These emerging approaches of systems data-gathering and computational modeling should increasingly provide means to test the hypotheses stated above that C₄ leaves differ from C₃ leaves by relatively few quantitative traits. The potential benefit of this endeavor would be the identification of the means to re-regulate C₃ species into a C₄ pattern of development.

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Chapter 10

C₄ Photosynthesis and Temperature

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Summary	162
I. Introduction	162
II. The Temperature Responses of C ₄ Photosynthesis and Growth	163
A. Net CO ₂ Assimilation Rate	163
B. Interactions with CO ₂ and Light Intensity	166
C. Growth	166
III. The Biogeography of C ₄ Photosynthesis	168
A. Global Patterns	168
B. Cold-Adapted C ₄ Species	170
C. Evolutionary and Ecological Perspectives	170
D. Synopsis	175
IV. The Temperature Response of C ₄ Photosynthesis: Biochemical Controls	175
A. The Response of C ₄ Photosynthesis to Intercellular CO ₂ Partial Pressure	176
B. Photorespiration in C ₃ and C ₄ Plants	177
C. Quantum Yield	177
D. Rubisco Limitations	179
E. Rubisco Activase Limitations	180
F. C ₄ Cycle Limitations	181
1. Pyruvate-Pi-Dikinase	181
2. PEP Carboxylase	182
3. Other Enzymes	182
G. Electron Transport Limitations	183
V. Fluorescence at Low Temperature	184
VI. Stomatal Limitations	185
VII. Thermal Acclimation of C ₄ Photosynthesis	185

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VIII. Conclusion: Are C_4 Plants Inherently More Sensitive to Low Temperature Than C_3 Plants?	187
Acknowledgments	187
References	188

Summary

C_4 plants perform poorly at low temperature, in contrast to C_3 vegetation. As a consequence, low numbers of C_4 species occur at high latitude, high elevation, and during cooler growing seasons. The mechanisms explaining the poor performance of C_4 species in colder climates have not been clearly identified. Early physiological perspectives indicate that C_4 species fail at low temperature due to either low quantum yield of the C_4 relative to the C_3 pathway, or enzyme lability in the C_4 cycle, most notably at PEP regeneration by pyruvate-Pi dikinase (PPDK). Alternatively, recent phylogenetic surveys show that all C_4 lineages originated from C_3 ancestors adapted to warm climates, indicating the failure of most C_4 species in colder environments could reflect prior heat adaptation within their respective evolutionary lineages. Numerous C_4 species have independently evolved cold tolerance; these plants allow for examination of C_4 photosynthesis at low temperature without complications from chilling injury. Relative to ecologically-similar C_3 species, chilling-adapted C_4 species have similar or slightly reduced photosynthetic capacities below 10°C and, after cold acclimation, show no chilling lability of PPDK, PEP carboxylase or other enzymes of the C_4 cycle. Cold-adapted C_4 species have enhanced photoprotective capacity at low temperature as indicated by greater levels of antioxidants and carotenoid pigments. Rubisco capacity is similar to gross CO_2 assimilation rate below 20°C in cold-adapted C_4 species, indicating it is an important limitation on C_4 photosynthesis at cool temperature. Acclimation and adaptation of C_4 species to the cold does not overcome the apparent Rubisco limitation. It is likely that a ceiling on carbon gain by insufficient Rubisco capacity at low temperature may be a leading trait that maladapts the C_4 pathway to cold regions of the earth.

I. Introduction

C_4 photosynthesis is a complex evolutionary trait that has evolved over 50 times in 19 families of vascular plants, making it one of the most convergent of evolutionary phenomena (Sage, 2004; Muhaidat et al., 2007). Nearly all lineages are associated with species of tropical or sub-tropical origin, although species within some lineages have radiated into low-temperature environments in alpine habitats, along cool maritime coasts, and in high latitude marshes (Long, 1999; Sage

et al., 1999). Because C_4 plants are largely absent in cold climates, and C_4 photosynthesis is widely reported to be inferior to C_3 photosynthesis in cold climates (Berry and Bjorkman, 1980; Sage and Percy, 2000), a paradigm has arisen that the C_4 pathway is maladapted to the cold, and confers its greatest advantage in warm environments (for example, Black et al., 1969; Black, 1971; Doliner and Jolliffe, 1979; Jones, 1985; Raven et al., 1999). This view has been periodically challenged, most recently by authors who raise the possibility that the temperature requirements of C_4 plants reflect the evolutionary history of their ancestors, rather than a specific physiological requirement of the C_4 pathway for heat or drought (Long, 1983, 1999; Ehleringer and Monson, 1993; Edwards and Still, 2008). There are, however, clear physiological mechanisms behind the different temperature responses of C_3 and C_4 photosynthesis, and these responses correlate well

Abbreviations: A – Net CO_2 assimilation rate; C_i – Intercellular partial pressure of CO_2 ; C_a – Ambient partial pressure of CO_2 ; PEPC – Phosphoenolpyruvate carboxylase; PPDK – Pyruvate orthophosphate dikinase; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase.

with the distribution of C₄ species along thermal gradients (Ehleringer, 1978; Edwards and Walker, 1983; Pearcy and Ehleringer, 1984; Ehleringer et al., 1997; Sage et al., 1999).

Clarifying the degree to which the temperature response of C₄ photosynthesis affects the distribution of C₄ plants will have many benefits to society. For one, a better understanding of the limitations controlling the temperature response of C₄ photosynthesis could facilitate improved productivity of C₄ crops at a time when global food stocks are declining, and could be critical to the developing biofuel industry. C₄ crops are expected to provide a significant contribution to the future supply of bioenergy; however, much of the available land and markets for biofuels are in higher latitudes, where low temperature severely limits C₄ crop productivity (Clifton-Brown et al., 2001; Heaton et al., 2008). In addition, climate warming will disrupt current ecological relationships in the next century. C₄ species are often thought to respond more to rising temperatures than C₃ species; however, the seasonality of warming confounds such simple predictions (Sage and Kubien, 2003). Predicting responses of earth's vegetation to climate change will require a thorough understanding of the response of the C₄ pathway to temperature change (Sage and Kubien, 2003; von Fischer et al., 2008).

This chapter will re-evaluate the relationship between C₄ photosynthesis and temperature, by incorporating older understanding with recent developments that have broadened our perspectives and improved our mechanistic understanding of the biochemical controls over the temperature response of C₄ photosynthesis. We will first review the temperature responses of C₃ and C₄ photosynthesis and growth, and then discuss the biogeography of C₄ photosynthesis, with particular focus on the cold-tolerant outliers to general pattern of C₄ plant distribution. The second half of the chapter develops a mechanistic framework to interpret the temperature response of C₄ photosynthesis. To limit the chapter to a manageable size, we say little about chilling injury that is not directly linked to the C₄ pathway. We conclude with a synthesis of biochemical and ecological perspectives to argue that the C₄ pathway can be tolerant of cold climates, but it inherently requires warm conditions to be ecologically successful.

II. The Temperature Responses of C₄ Photosynthesis and Growth

A. Net CO₂ Assimilation Rate

Within a decade of the discovery of C₄ photosynthesis in the mid-1960s, it was realized that C₄ species exhibited higher rates of photosynthesis than C₃ species at warmer conditions, while C₃ species had higher rates of photosynthesis in cooler conditions (Black et al., 1969; Bjorkman and Pearcy, 1971; Black, 1971; Williams, 1974; Long et al., 1975; Ishii et al., 1977; Vong and Murata, 1977; Long and Woolhouse, 1978b; Kemp and Williams 1980; reviewed by Berry and Bjorkman, 1980; Jones, 1985; see also Pearcy et al., 1981; Monson et al., 1983; Henning and Brown, 1986; Fladung and Hesselbach, 1989). C₄ species typically exhibit a pronounced response of photosynthesis to increasing temperature up to a thermal optimum between 30°C and 40°C, while C₃ species have a broad thermal optimum of photosynthesis between 20° and 30°C (Ludlow and Wilson, 1971; Bjorkman and Pearcy, 1971; Bjorkman et al., 1970, 1972; Pearcy and Harrison, 1974; Long et al., 1975; Vong and Murata, 1977; Long and Woolhouse, 1978b; Berry and Bjorkman, 1980; Pearcy et al., 1981; Monson et al., 1983; Loomis, 1983; Tieszen and Detling, 1983). By the late-1970s, these patterns had become a textbook paradigm (for example, see Salisbury and Ross, 1978). A concern regarding this early paradigm was that many of these photosynthetic measurements did not compare species of similar ecological habitat, but instead, emphasized cool season C₃ crops, weeds and forage grasses versus warm season C₄ species (Percy and Ehleringer, 1984). Photosynthetic capacity can vary markedly in plants of different growth form and habitat such that these factors could influence results more than photosynthetic pathway.

To avoid some of these confounding effects, we measured the temperature response of the net CO₂ assimilation rate (*A*) from *Pectis multiflosculosa* (C₄, Asteraceae) and two ecotypes of the prostrate C₃ herb *Heliotropium currisavicum* (Boraginaceae, Fig. 1a). *Pectis multiflosculosa* is a prostrate dune species from the Pacific coast of subtropical Mexico, while *H. currisavicum* is a prostrate herb from coastal regions of North and South America. One population was from the

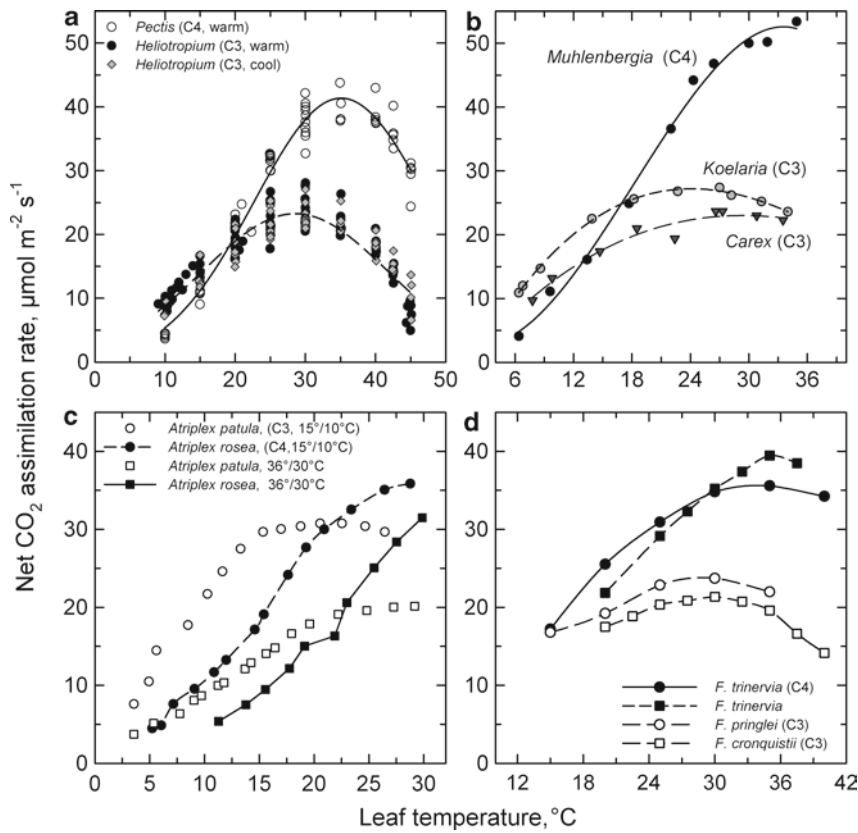


Fig. 1. Representative temperature response curves of net CO_2 assimilation rate in C_4 plants at the prevailing atmospheric CO_2 level during the time of measurement. (a) *Pectis multiflorescens* (C_4) and a cool and warm ecotype of *Heliotropium currisavicum* (C_3); (b) *Muhlenbergia richardsonis* (C_4), *Koeleria macrantha* (C_3) and *Carex helleri* (C_3); (c) *Atriplex rosea* (C_4) and *Atriplex patula* ssp. *spicata* (C_3 , now classified as *A. dioca*; Welsh 2003) from warm ($36^\circ/30^\circ\text{C}$ day/night) and cool ($15^\circ/10^\circ\text{C}$ day/night) growth conditions; and (d) *Flaveria trinervia* (C_4), *Flaveria pringlei* (C_3) and *Flaveria cronquistii* (C_3). Species in panel (a) and (b) were grown during summer in a greenhouse in Toronto, Canada and the net CO_2 assimilation rate measured with a steady-state gas exchange system. *Pectis multiflorescens* was collected on a beach dune near Manzanillo, Mexico. The warm ecotype of *H. currisavicum* was collected along the coast of Yucatan, Mexico; the cool ecotype at Pigeon Point, California. *Muhlenbergia richardsonis*, *K. macrantha* and *Carex helleri* were collected at 3,800 m in an alpine fellfield in the White Mountains of California. *Atriplex* data from panel (c) was recalculated from relative rates given by Bjorkman and Pearcy (1971), for plants grown in a growth chamber. *Flaveria* data in panel (d) are from Schuster and Monson (1990, squares) and Oberhuber and Edwards (1993, circles) using greenhouse grown plants (Data in panels (a) and (b) are published for the first time. *Flaveria* data are reprinted with permission).

cool coast of central California, while the other was from the hot subtropics of the Yucatan Peninsula, Mexico. In both of these situations, the classical pattern was apparent: the C_4 species outperformed the C_3 ecotypes at elevated temperature, while both the warm- and cool-climate ecotypes of the C_3 species had a higher photosynthetic capacity than the C_4 plants at cooler temperatures (Fig. 1a). Similar responses have been measured between ecologically similar C_3 and C_4 species from Mojave Desert shrubs

(Bjorkman et al., 1980), old fields (Percy et al., 1981), and graminoids from short-grass prairies (Monson et al., 1983; Tieszen and Detling, 1983). Growth temperature can complicate this general observation however; when grown at cool temperatures (20°C or less), C_3 species can have higher A than C_4 species across a wide range of temperature, while the reverse may be true in warm growth conditions (30°C or higher; Berry and Bjorkman, 1980; Bjorkman et al., 1980; Monson et al., 1983). Even in these situations,

however, the C₄ species still exhibited a steeper thermal response of *A* below the temperature optimum, and a higher thermal optimum than the C₃ species.

Because C₄ species are reputed to perform poorly in low temperature, we examined whether the classic temperature response of photosynthesis would also hold in the case of a C₄ plant that has evolved the ability to survive in cold climates. The C₄ grass *Muhlenbergia richardsonis* from the high alpine zone of western North America exhibited a much higher rate of photosynthesis at elevated temperature than the C₃ grass *Koeleria macrantha* and C₃ sedge *Carex helleri* from the same habitat; below 15°C, the two C₃ species had higher *A* than the C₄ species (Fig. 1b). Both species were grown in identical conditions in a greenhouse under moderate temperatures (about 20–25°C). A similar comparison between two ecological associates from high latitude fens in the boreal forest also demonstrated greater photosynthetic capacity of the C₄ grass (*Muhlenbergia glomerata*) than the C₃ grass (*Calamagrostis canadensis*) above 20°C but not below 15°C (Kubien and Sage, 2004a). These results demonstrate that even in plants from cold-climates, the typical C₃ and C₄ photosynthetic responses to temperature still prevail, although the thermal optimum of *A* and the C₃ versus C₄ crossover temperatures for *A* are shifted to lower temperatures.

Differences in environmental responses may also reflect phylogenetic history (Ackerly, 1999; Edwards et al., 2007), such that results attributed to photosynthetic pathway could instead reflect ancestral constraints. Few studies, however, have examined the photosynthetic temperature responses of C₃ and C₄ species that are phylogenetically closely related. Bjorkman and Pearcy (1971) studied the temperature responses of photosynthesis in *Atriplex rosea* (C₄) and *Atriplex patula* ssp. *spicata* (= *Atriplex dioca*, C₃; Welsh, 2003) from central California (see Osmond et al., 1980 for additional *Atriplex* comparisons). These closely related species are similar in growth form and morphology, and both grow in disturbed soils (Welsh, 2003; Zacharias, 2007). At a low growth temperature (15°/10°C day/night temperature), the C₃ species had superior *A* below 20°C, whereas *A* in the C₄ species was greater above 25°C (Fig. 1c; Bjorkman and Pearcy, 1971). At a warm growth temperature (36°/30°C day/night), the

same relative pattern prevailed, although both the C₃ and C₄ *Atriplex* species exhibited a higher thermal optimum and reduced *A* at each measurement temperature relative to the responses observed in cool-grown plants (Fig. 1c). In *Panicum* grasses, the C₄ species *P. maximum* (which is now *Megathyrsus maximus* and formerly *Urochloa maxima*, Barkworth et al., 2007) had over twice the rate of photosynthesis above 25°C, but half the rate below 15°C, than the C₃ species *P. bicusulatum*, attesting to the rapid drop in photosynthesis below 25°C in the C₄ species (Fladung and Hesselbach, 1989). In a separate *Panicum* study (Henning and Brown, 1986), *P. priontis* (C₄) had higher *A* from 10°C to 50°C than either the C₃ *P. laxum* (= *Steinchisma laxa*) or C₃ *P. boliviense* (= *P. polygonatum*). At lower measurement temperatures (10°C and 20°C), the C₄ species had a slightly higher *A*, but as temperature increased above 20°C, *A* became substantially larger in the C₄ than C₃ species, such that the C₄ species had more than twice the CO₂ assimilation rate at the thermal optimum of each species. In closely related *Flaveria* species, the C₃ *F. pringlei* and *F. cronquistii* have lower *A* than the C₄ species *F. trinervia* above 15°C (Fig. 1d); however, the differences between the C₃ and C₄ species decline with falling temperature. At 15°C, CO₂ assimilation rates are comparable between *F. trinervia* and *F. pringlei* (Fig. 1d; Schuster and Monson, 1990; Oberhuber and Edwards, 1993).

The most closely related C₃ and C₄ pair is perhaps the C₃ and C₄ subspecies of *Alloteropsis semialata*, a grass from the monsoon belt of South Africa, south Asia and Australia (Ibrahim et al., 2008, 2009; Osborne et al., 2008). In South Africa, the two subspecies co-occur, although the C₃ subspecies tends to be displaced towards cooler, drier upland habitats relative to the C₄ subspecies. The C₄ subspecies is not active in the winter, due to frost intolerance of its canopy (Ibrahim et al., 2008). The C₃ subspecies is frost tolerant, and thus remains winter active, allowing it to reduce the competitive pressure from the C₄ subspecies and other C₄ species. In a comparison of their relative photosynthetic responses to temperature (Osborne et al., 2008), the pattern was similar to that observed in *Flaveria* (Fig. 1d), and between *P. priontis* and *P. boliviense* (Henning and Brown, 1986). There was no photosynthetic advantage at low temperature for the C₃ *Alloteropsis* subspecies,

since the CO_2 assimilation rates were similar below 15°C ; however, the C_4 subspecies had a much stronger response to rising temperature, such that at $25\text{--}30^\circ\text{C}$, it had double the photosynthetic capacity of the C_3 subspecies (Osborne et al., 2008).

The results at saturating light intensities between closely related C_3 and C_4 genotypes conform to the general view that C_4 photosynthesis is more responsive to rising temperature and has a higher thermal optimum than observed in the C_3 plants. C_4 species exhibited higher A at the thermal optimum, often by a wide margin. The C_4 advantage is universally lost at lower temperatures, although C_4 plants do not always have a lower CO_2 assimilation rate at low temperature than their C_3 counterparts.

B. Interactions with CO_2 and Light Intensity

In both C_3 and C_4 species, the temperature response of A (the A/T curve) is influenced by atmospheric CO_2 content and light intensity. At low CO_2 values typical of the late Pleistocene ($180\text{--}200$ ppm about 20,000–30,000 years ago; Ward et al., 2008), the A/T response of C_4 plants is relatively shallow, with a broad thermal optimum (Fig. 2a). As CO_2 level increases to the current CO_2 value of 380 ppm, the rate of photosynthesis at the thermal optimum increases markedly in leaves of C_4 species, creating a curve with a narrower thermal optimum and greater responses to changing temperature (Fig. 2b). With further increases in CO_2 from 380 to 700

ppm, the C_4 A/T response is little changed, with only a small enhancement occurring near the thermal optimum (Fig. 2c). By contrast, C_3 species still show a substantial enhancement of A by rising CO_2 above 20°C , and as a result, can exhibit A/T responses at high CO_2 that mimic those of C_4 species (Fig. 2; see also Bjorkman et al., 1980).

At subsaturating light intensities, A in C_4 plants exhibits low sensitivity to variation in temperature, and thus the A/T curve becomes flattened with a broad thermal optimum (Fig. 3). Light requirements for A are highest at the thermal optimum, so the flattening of the A/T response with declining light begins at the optimal temperature, and then spreads to lower temperatures as light levels drop further (Berry and Bjorkman, 1980; Long and Woolhouse, 1978a). Consequently, the generalization that C_4 plants are more responsive to temperature and have a higher thermal optimum than C_3 plants only refers to the light saturated condition.

C. Growth

There are numerous studies examining C_3 and C_4 growth and competition responses to temperature, including many species of similar ecological requirements (Clements et al., 1929; Black, 1971; Bjorkman et al., 1974; Vong and Murata 1978; Percy et al., 1981; Christie and Detling, 1982; Loomis, 1983; Evans and Bush, 1985; Jones, 1985; Wall, 1993; Grise, 1997; Nord et al., 1999; Osborne et al., 2008; Ward et al., 2008). The majority of these studies show that warm growth temperatures above $25\text{--}30^\circ\text{C}$ favor the C_4 species,

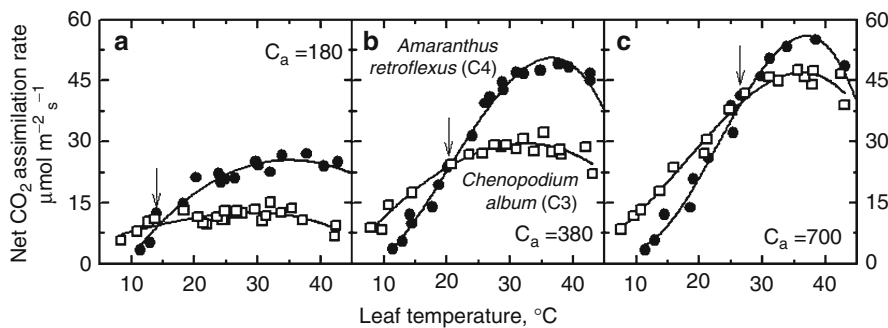


Fig. 2. The temperature response of C_4 photosynthesis in *Amaranthus retroflexus* (filled symbols) and of C_3 photosynthesis in *Chenopodium album* (open symbols) measured at ambient CO_2 levels (C_a) of 180, 380 and 700 ppm. Arrows indicate temperatures where photosynthesis rates are equivalent (Reprinted from Sage and Percy, 2000). Plants were grown at atmospheric CO_2 levels of 365 ppm.

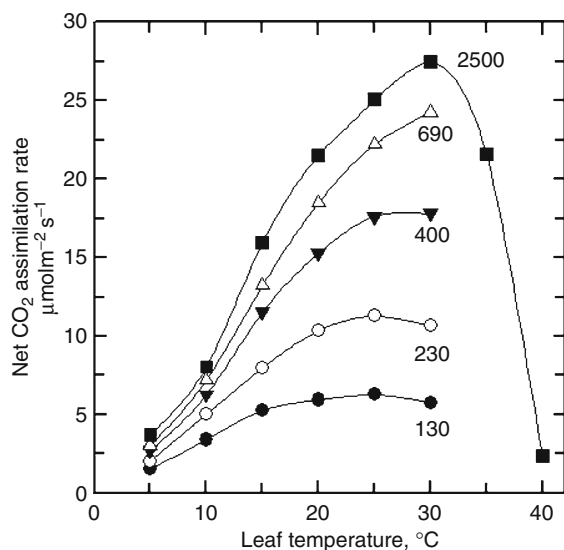


Fig. 3. The temperature response of C₄ photosynthesis at different light intensities in *Spartina townsendii* (= *S. anglica*) grown in a plant growth chamber at prevailing atmospheric CO₂ levels. Light intensities in μmol photons m⁻² s⁻¹ are given beside each respective curve (Redrawn from Long and Woolhouse, 1978b. With permission).

while the C₃ species predominate at temperatures below 20°C, as may occur early in the growing season (Percy et al., 1981; Christie and Detling, 1982; Wall, 1993; reviewed in Loomis, 1983; Tieszen et al., 1979; Jones, 1985).

Atmospheric CO₂ modulates the crossover temperature where the C₃ and C₄ performances are equal. Lower growth CO₂ reduces the temperature below which C₃ species show equivalent growth as the C₄ species, while high CO₂ raises this temperature (Grise, 1997; Ehleringer et al., 1997). Ward et al. (2008) compared growth of C₃ and C₄ annuals at atmospheric CO₂ levels of the late-Pleistocene (200 ppm or 20 Pa). The C₄ annual *Amaranthus retroflexus* had a large growth advantage over the C₃ species *Abutilon theophrastii* in a warm growth treatment (30°/24°C day/night temperature), but this advantage was markedly reduced in a cool treatment (22°/16°C). Using *Chenopodium album* (C₃) and *A. retroflexus* (C₄), Grise (1997) observed that the C₄ species lost its growth advantage at 23°/21.5°C when atmospheric CO₂ was increased two to three times. In a growth regime at 34°/31°C, increasing CO₂ to 750 ppm reduced by half a large growth advantage observed in *A. retroflexus* at current CO₂ levels. At 34°C and 1,000 ppm CO₂, the C₄ species still

grew faster than the C₃ species, although the growth rate difference was about 10% of that observed at current CO₂ levels.

Species and growth conditions selected for C₃-C₄ comparisons have a large influence over the outcome. The major rice weeds in the C₄ genus *Echinochloa*, for example, have superior growth relative to C₃ rice varieties above 15°C, in part because of the high chilling sensitivity of rice; however, when compared with wheat, which is chilling tolerant, daytime conditions must be warmer than 20–24°C for the *Echinochloa* spp. to exhibit greater growth (Evans and Bush, 1985). This highlights the need to compare ecologically similar species with similar growth form in order to isolate consequences of photosynthetic pathway.

A number of studies have examined competition between ecologically similar weeds (Black, 1971; Percy et al., 1981; Flint and Patterson, 1983; Ackerly et al., 1992; Grise, 1997; Ward et al., 2008) or prairie grasses (Christie and Detling, 1982) grown in contrasting thermal regimes. Increasing temperature universally enhanced C₄ species relative to the C₃ associates by reversing or reducing the C₃ advantage observed at the cooler growth conditions. Flint and Patterson (1983) observed the warm-season weed *Xanthium pennsylvanicum* (cocklebur, C₃) had a large initial advantage over the C₄ *Amaranthus hybridus* due to a much larger seed size. The initial size advantage of the C₃ species remained in place at a day/night temperature of 26°/17°C, but was greatly reduced at 32°/23°C.

Two sets of studies have examined growth responses of phylogenetically close C₃ and C₄ species. In *Atriplex*, habitat of origin played a major role in the relative performance. Warm-climate C₄ *Atriplex* species responded strongly to temperature, with three- to fivefold enhancements in biomass when grown at 36°/31° as compared to 15°/10°C (Osmond et al., 1980). The C₃ species (all from cool coastal climates) showed a 33–65% reduction in yield in the warm relative to cool temperature regime. Notably, a C₄ species from a cool coastal environment (*Atriplex sabulosa*) had a similar growth response to varying temperature as the C₃ species; it grew as well as the C₃ species in cool conditions, and also showed reduced growth in the 36°/31°C regime (Osmond et al., 1980). The other phylogenetically controlled

study was conducted with the C_3 and C_4 ecotypes of *Alloteropsis semialata* (Osborne et al., 2008). As observed with photosynthesis, the C_4 ecotype had substantially greater leaf expansion rate above 15°C than the C_3 ecotype, while below 15°C, the two ecotypes had similar leaf expansion rates.

III. The Biogeography of C_4 Photosynthesis

A. Global Patterns

C_4 species are completely absent from arctic and near-arctic latitudes (>65° latitude), and are rare in the high-latitude boreal zone between 50°N and 65°N (Teeri and Stowe, 1976; Takeda and Hakoyama, 1985; Collins and Jones, 1986a; Schwarz and Redmann, 1988; Sage et al., 1999; Collatz et al., 1998; Still et al., 2003). They are generally rare at high elevations, although they can be locally common in the treeless tundra of the alpine zone in dry mountain ranges (Pyankov, 1993; von B. Ruthsatz and Hoffmann, 1984; Sage et al., 1999; Sage and Sage, 2002; Wang, 2003; Wang et al., 2004). In these extreme cases, the upper elevation limit of the alpine C_4 species is lower than the local alpine C_3 vegetation (Sage and Sage, 2002).

The shift in dominance of C_4 taxa from low to high latitude has been well described in floristic analyses from most regions of the globe (Africa – Ellis et al., 1980; Akhani et al., 1997; eastern Asia and Japan – Takeda et al., 1985a; Takeda and Hakoyama, 1985; Ueno and Takeda, 1992; Australia – Hattersley, 1983; Takeda et al., 1985b; Egypt – Batanouny et al., 1988; Europe – Collins and Jones, 1986a; India – Takeda, 1985; Mongolia – Pyankov et al., 2000; North America – Teeri and Stowe, 1976; Stowe and Teeri, 1978; Li et al., 1999; Wan and Sage, 2001; South Africa – Vogel et al., 1978; Stock et al., 2004; reviewed in Long, 1983, 1999; Sage et al., 1999). Sage et al. (1999) summarized many of the prior efforts in one global map of C_4 grass distributions. In grass floristic studies, the percent of C_4 grass species in a local grass flora are estimated and then positioned on a continental-scale map. C_4 species dominate lowland grass floras. Greater than two-thirds of all grass species below 30° latitude are C_4 , while

C_3 species dominate all grass floras above 50°. The zone where grass floras are equally C_3 and C_4 corresponds to about 35–38° latitude. Sedge floras show C_4 dominance at lower latitudes than grass floras (Takeda et al. 1985a,b; Ueno and Takeda, 1992; Li et al., 1999; Sage et al., 1999).

In addition to the floristic studies, the contribution of C_4 biomass to local productivity as a function of temperature has been estimated using direct biomass measurements and carbon isotope ratios (Australia – Bird and Pousai, 1997; Wynn and Bird, 2008; Mongolia – Auerwald et al., 2009; central North America – Paruelo and Lauenroth, 1996; Epstein et al., 1996, 1997; Tieszen et al., 1997; von Fischer et al., 2008; South America – Paruelo et al., 1998; Murphy and Bowman, 2007). These data also show a decline of C_4 contribution to local productivity with increasing latitude that largely mirrors the change estimated with floristic data. In Australia, the shift from C_3 to C_4 dominance of grasslands occurs below 25–30° latitude, and C_4 species dominate grasslands across the northern half of the country. In central North America, the shift occurs at 40°N–45°N, the latitude of the state of Nebraska. Precipitation patterns modify these crossover points. Where winters are wet and summers dry, the crossover latitudes are reduced (Epstein et al., 1997; Still et al., 2003).

Numerous studies have plotted the C_4 grass representation as a function of altitude using floristic, biomass and carbon isotope assessments (Argentina – von B. Ruthsatz and Hoffmann, 1984; Cavagnaro, 1988; Cabido et al., 1997; Central America – Chazdon, 1978; East Africa – Tieszen et al., 1979; Livingstone and Clayton, 1980; Egypt – Sayed and Mohamed, 2000; Hawaii – Rundel, 1980; Japan – Nishimura et al., 1997; New Guinea – Earnshaw et al., 1990; Bird et al., 1994; Wyoming, USA – Boutton et al., 1980; reviewed in Sage et al., 1999). At temperate latitudes, C_4 grasses drop out of floras above about 3,500 m, while in tropical latitudes, they are absent from floras above about 4,000 m, with a few notable exception that will be discussed below. In subtropical Hawaii, C_4 biomass productivity is negligible above 2,000 m, while in equatorial Kenya, C_4 production becomes insignificant above 3,000 m (Tieszen et al., 1979; Rundel, 1980).

Human cropping systems also reflect the global pattern of C_4 grass dominance. Low latitude, low elevation grain crops are mainly C_4 except for

rice, a C₃ plant typically grown on flooded soils (Sage and Pearcy, 2000). At high latitudes, and in the tropical highlands, the cropping systems are exclusively C₃, with two major exceptions – maize, and the prospective biofuel species, *Miscanthus* × *giganteus* (Long, 1983, 1999; Clifton-Brown, et al., 2001). Maize cultivation at high latitude, however, occurs through the use of early-yielding varieties where the period of activity is compressed into the few warm months of summer. By contrast, *Miscanthus* appears to have substantial cold tolerance, derived from its origin in montane highlands of Taiwan, Japan and China (Beale and Long, 1995; Beale et al., 1996; Clifton-Brown et al., 2001).

In addition to variation along latitude and elevation gradients, the occurrence of C₄ species reflects a seasonal trend. In the temperate zone, C₄ species are largely active in the late spring and summer. C₃ species, by contrast, can be active year-round in mild temperate climates. In areas with harsh winters, C₃ species usually begin growth weeks before the C₄ species. In the North American plains grasslands, the major C₃ grasses begin growing March to April, while the C₄ grasses break bud 2–4 weeks later (Dickinson and Dodd, 1976; Kemp and Williams 1980; Ode et al., 1980; Monson and Williams, 1982; Monson et al., 1983; Tieszen and Detling, 1983; Sage et al., 1999). In the southwestern deserts of North America, most of the herbaceous C₃ productivity occurs in winter to spring, while the C₄ species are summer active where monsoon rains occur (Sage et al., 1999). In these deserts, winter annuals are exclusively C₃, while summer annuals are

mainly C₄ (Mulroy and Rundel, 1977; Kemp, 1983; Guo and Brown, 1996). In mild climates of the warm temperate zone, the differences in C₃ and C₄ periods of activity create difficulties for gardeners and landscapers (for example, R.F. Sage (2009) personal observation with lawns in Athens, Georgia, USA). Lawns that do well in summer consist of C₄ grasses such as *Zoysia japonica* or *Cynodon dactylon* (Bermuda grass). These grasses are dormant in the mild winters when C₃ weeds can infest the lawn, eventually ruining the C₄ turf. If instead the homeowner chooses a C₃ turf such as a fescue, the lawn initially does well in winter and spring but becomes infested with C₄ weeds (notably crabgrass, *Digitaria sanguinalis*) during the summer, which ruin the C₃ turf.

Temperatures thresholds for C₄ occurrence and dominance have been identified by regressing the presence of C₄ species versus climate data (Teeri and Stowe, 1976; Paruelo and Lauenroth, 1996; Epstein et al., 1996, 1997; Bird and Pousai, 1997; Sage et al., 1999; Pyankov et al., 2000; Wan and Sage, 2001; von Fischer et al., 2008). These trends consistently showed C₄ grass species becoming negligible within local floras when the minimum July temperature is below 6–12°C, and the minimum mean daytime high is below 12–14°C (Fig. 4; Sage et al., 1999). As shown in Fig. 4, these temperature trends are present on both the Pacific and Atlantic coasts of North America. The Pacific coast is cooler and drier than the Atlantic coast in the summer, and this difference separates the two plots of C₄ species representation as a function of latitude (Fig. 4a); however, when plotting the two distribution

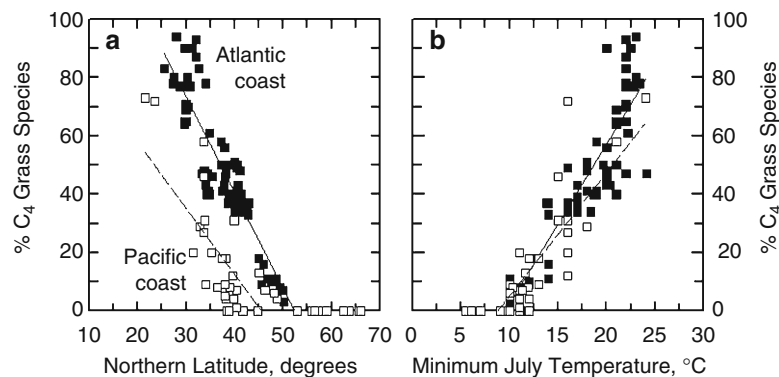


Fig. 4. The percentage of C₄ species in local grass floras from the Pacific (open symbols) and Atlantic (filled symbols) coastal regions of North America, as a function of latitude (panel a) and minimum July temperature (panel b) (From Wan and Sage, 2001. With permission).

curves as a function of mid-summer temperatures, both curves show the same relationship (Fig. 4b). While minimum summer temperature is frequently best correlated with C_4 occurrence in a flora, strong correlations are also shown for mean daily temperature, degree days, number of days over 32°C and for lower latitude sites, mean annual temperature. Mean annual temperature in temperate locations is often problematic because continental interiors can have very cold winters that skew the annual value. von Fischer et al. (2008) noted that C_4 dominance of soil carbon isotope ratios in central North America occurred when the following indices exceeded 22°C ± 2°C – May high temperature, July and August mean temperature, and high temperatures from April to August. In Australia, grasslands become C_4 dominated above a mean annual temperature of 23°C (Hattersley, 1983). In the highlands of Hawaii, Costa Rica and Kenya, C_4 dominance occurs above a mean daily maximum of 21–23°C, and a daily mean of 15°C (Chazdon, 1978; Tieszen et al., 1979; Rundel, 1980).

B. Cold-Adapted C_4 Species

While the vast majority of C_4 species occur in warm climates, many C_4 plants are cold tolerant. Hundreds of C_4 perennials from temperate latitudes survive winter temperatures, often below –20°C in the dormant state (Rowley et al., 1975; Rowley, 1976; Schwarz and Reaney, 1989; Sage and Sage, 2002; Walker et al., 2008). Dozens of C_4 species from distinct evolutionary lineages are able to withstand chilling and freezing conditions during the growing season (Table 1; Marquez et al., 2006; Liu and Osborne, 2008). For example, Andean C_4 grasses from the Venezuelan paramo survive temperatures as low as –18°C during the growing season (Marquez et al., 2006). C_4 species are also recognized to do well in cool environments along the coast of southern New Zealand and the Pacific northwest in the USA, in foggy coastal marshes along the north Atlantic in Britain and Canada, in boreal fens, meadows and marshes, and in early spring understories of forests along the central Mississippi river valley and the Sierra Nevada foothills of California (Long, 1983, 1999; Collins and Jones, 1986a; Smith and Wu, 1994; Wan and Sage, 2001; Kamler, 2004; Kubien and Sage, 2003). In the warm temperate zone, numerous C_4 species develop frost hardiness

and remain active during mild winter or spring periods that experience some subzero cold (Rowley et al., 1975; Rowley, 1976; Liu and Osborne, 2008). Cold arid regions also contain C_4 shrubs such as *Atriplex* species that are active during April, when snow and subzero temperatures can occur (Caldwell et al., 1977a, b).

Many C_4 grasses and dicots do well in alpine habitats, where they often exhibit the same specialized growth forms as C_3 species (Table 1). C_4 plants were generally not thought to occur in the true alpine zones until the mid-1990s when Pyankov and co-workers (Pyankov et al., 1992; Pyankov, 1993; Pyankov and Vosnesenskaya, 1995) described a number of C_4 grasses and dicots that occur above 3,500 m in the Pamir mountains of Central Asia (Table 1). These discoveries compliment descriptions of cold-adapted grasses from the highlands of New Guinea, Japan and Tibet, such as species in the genus *Miscanthus* (Earnshaw et al., 1990; Nishimura et al., 1997; Wang, 2003). The highest reported elevation for a C_4 species worldwide is 5,200 m, for the grass *Orinus thordii* from Tibet, growing on dry, gravelly steppe (Wang, 2003). In the Andes, numerous species extend into the alpine zone up to 4,800 m (von B. Ruthsatz and Hoffman, 1984; Boom et al., 2001; Sage et al., 2007). Long (1999) compiled a list of 34 species from ten genera of grasses occurring above 3,500 m in Peru where the average annual temperature is less than 9°C; eight species in three genera occur above 4,000 m where the average annual temperature is below 3–6°C. The genus *Muhlenbergia* has eight species collected above 3,500 m in Peru, while *Paspalum* has ten species occurring above 3,500 m. Above 3,500 m, alpine C_4 species survive subzero growing season temperatures that regularly occur at night and occasionally during the day (Sage and Sage, 2002; Marquez et al., 2006), and are tolerant of episodic snowfalls during midsummer.

C. Evolutionary and Ecological Perspectives

Since the realization that C_4 plants are predominantly warm climates species, there has been much consideration of the causative mechanisms, with some thought given to whether the C_4 pathway itself is responsible for the global pattern (Ehleringer 1978; Long 1983, 1999; Ehleringer and Monson, 1993; Ehleringer et al., 1997; Sage, 2002). The

Table 1. Selected cold-adapted C₄ species, their evolutionary lineage, location and habitat. Estimated age of the lineage, if known, follows the lineage name in parentheses. The '5,200 m!' indicates the maximum elevation recorded for C₄ plants. Poaceae lineages are from Christin et al. (2009). Cyperaceae lineages are from Besnard et al. (2009). Dicot lineages are from Muhaideb et al. (2007). Age estimates are from Christin et al (2009) for grasses.

Evolutionary lineage	Genus	Species	Location and habitat	Reference
Poaceae				
Andropogonae (17–22 mya)	<i>Andropogon</i>	<i>amethystinus</i>	Mt Kenya to 4,000 m	Long, 1983
	<i>Bothriochloa</i>	<i>ischaemum</i>	Widespread weed to 3,500 m in Tibet	Wang, 2003
	<i>Miscanthus</i>	<i>saccharoides</i>	Andean rocky slopes to 4,000 m	Brako and Zarucchi, 1993
		<i>nudipes</i> × <i>giganteus</i>	High altitude to 3600 m, Himalayas	Shenglian et al., 2006
		<i>sinensis</i>	High latitude, Europe	Clifton-Brown et al., 2001
		<i>adscensionis</i>	High altitude, East Asia	Shouliang et al., 2006a
	<i>Aristida</i>	<i>alpina</i>	Weedy habit to 4,000 m in S. America	Brako and Zarucchi, 1993
			Tibetan plateau, dry mountain slopes to 4,500 m	Bixing and Phillips, 2006
<i>Arundinella</i> (8–26.4 mya)	<i>Arundinella</i>	<i>yunnanensis</i>	Tibet, meadows to 4,170 m	Wang et al., 2004, Bixing and Phillips, 2006
Chloridoideae (25–32 mya)	<i>Bouteloua</i>	<i>gracilis</i>	Dry slopes, Rocky mountains USA to 3,200 m	R.F. Sage, (2009), personal observation, Bowman and Turner, 1993
	<i>Chloris</i>	<i>media</i>	Andean disturbed soils to 4,000 m	Brako and Zarucchi, 1993
		<i>halophila</i>	Andean grasslands to 4,000 m	Brako and Zarucchi, 1993
		<i>virgata</i>	Tibet, to 3,820 m	Wang et al., 2004
	<i>Distichlis</i>	<i>humilis</i>	Andean disturbed soils to 4,000 m	Brako and Zarucchi, 1993
		<i>spicata</i>	High latitude salt marshes and maritime beaches	Schwarz and Redmann, 1988, R.F. Sage, (2009), personal observation
	<i>Eragrostis</i>	<i>sorataensis</i>	Andean grasslands to 4,500 m	Brako and Zarucchi, 1993
	<i>Lycurus</i>	<i>phleoides</i>	Andean disturbed soils to 4,000 m	Brako and Zarucchi, 1993
	<i>Muhlenbergia</i>	<i>angustata</i>	Andean grasslands to 4,000 m	Brako and Zarucchi, 1993
		<i>fastigiata</i>	Andean grasslands to 4,500 m	Brako and Zarucchi, 1993, Boom et al., 2001
		<i>glomerata</i>	High latitude, Canadian boreal zone	Schwarz and Redmann, 1988
		<i>perviana</i>	Andean grasslands to 4,800 m	von B. Ruthsatz and Hoffman, 1984, Brako and Zarucchi, 1993
		<i>richardsonis</i>	High altitude and high latitude, North America	Schwarz and Redmann, 1988, Sage and Sage, 2002
	<i>Orinus</i>	<i>sobolifera</i>	N. America, early spring understories	Smith and Wu, 1994
		<i>thoroldii</i>	Tibet, arid sand and gravel steppes to 5,200! m	Wang, 2003, Wang et al., 2004, Shouliang et al., 2006a
	<i>Spartina</i>	<i>anglica</i>	High latitude salt marsh	Long, 1983
		<i>gracilis</i>	High latitude saline meadow	Schwarz and Redmann, 1988
	<i>Sporobolus</i>	<i>indicus</i>	Andean disturbed soils to 4,000 m	Brako and Zarucchi, 1993
	<i>Tripsacum</i>	<i>liouae</i>	Tibet, dry open spaces to 4,600 m	Shouliang et al., 2006a
	<i>Pennisetum</i>	<i>clandestinum</i>	Andean disturbed areas to 4,000 m	Brako and Zarucchi, 1993
<i>Panicum/Setaria</i> (16.4–18.5 mya)		<i>flaccidum</i>	W. Asia, dry sandy soils to 5,000 m	Wang, 2003, Wang et al., 2004, Shouliang et al., 2006c

(continued)

Table 1. (continued)

Evolutionary lineage	Genus	Species	Location and habitat	Reference
<i>Paspalum</i> (14.1–8.5 mya)	<i>Setaria</i>	<i>viridis</i>	Disturbed fields, high latitude to 60°N, to 3,700 m	Schwarz and Redmann, 1988, Wang, 2003
	<i>Paspalum</i>	<i>bonplandianum dilatatum pallidum</i>	Andean grasslands to 4,500 m Widespread weed, autumn cold tolerance Andean grasslands to 4,000 m	Brako and Zarucchi, 1993, Boom et al., 2001 Rowley et al., 1975 Brako and Zarucchi, 1993
Cyperaceae				
Abilgardieae	<i>Bulbostylis</i>	<i>juncoides longus</i>	Andean grasslands to 4,000 m High latitude weed, Europe	Brako and Zarucchi, 1993 Caldwell et al., 1977a
Cypereae	<i>Cyperus</i>			
Amaranthaceae				
<i>Alternanthera</i>	<i>Alternanthera</i>	<i>microphylla</i>	Andes, to 4,000 m	von B. Ruthsatz and Hoffman, 1984, Zuloaga and Morrone, 1999a, Sage et al., 2007
<i>Amaranthus</i>	<i>Amaranthus</i>	<i>peruviana retroflexus meyeniana</i>	Andes to 4,000 m Europe, high latitude Andean tundra to 4,600 m	Brako and Zarucchi, 1993 R.F. Sage, (2009), personal observation von B. Ruthsatz and Hoffman, 1984, Brako and Zarucchi, 1993, Zuloaga and Morrone, 1999a, Sage et al., 2007
<i>Gomphrena</i>	<i>Gomphrena</i>			Brako and Zarucchi, 1993
Asteraceae				
<i>Pectis</i>	<i>Pectis</i>	<i>sessiliflora</i>	Andean disturbed soils to 4,000 m Andes, rocky slopes to 3,500 m	Zuloaga and Morrone, 1999a
Chenopodiaceae				
<i>Atriplex</i>	<i>Atriplex</i>	<i>centralasiatica confertifolia laciniata pamirica</i>	Tibet and Pamirs to 3,900 m Early spring, North American cold deserts Norway coastline Pamirs (Asia), slopes to 4,500 m	Pyankov, 1993, Pyankov and Vosnesenskaya, 1995, Wang, 2003 Rowley et al., 1975 Hulten and Fries 1986 Pyankov, 1993, Pyankov and Vosnesenskaya, 1995
<i>Salsola</i>	<i>Climacoptera</i>	<i>lanata</i>	Pamirs to 4,125 m, arid soils	Pyankov, 1993, Pyankov and Vosnesenskaya, 1995
	<i>Halogeton</i>	<i>glomeratus</i>	Tibet and Pamirs, arid slopes and scree to 4,250 m	Pyankov, 1993, Pyankov and Vosnesenskaya, 1995, Wang, 2003
	<i>Salsola</i>	<i>kali monoptera paulsenii</i>	High latitude to 63°N, Europe Tibet to 4,800 m Pamirs to 3,900 m, rocky, sandy slopes	Collins and Jones, 1986a Wang, 2003 Pyankov, 1993
Euphorbiaceae				
<i>Chamaesyce</i>	<i>Chamaesyce</i>	<i>serpens</i>	Andean grasslands to 4,500 m	Brako and Zarucchi, 1993
Portulacaceae				
<i>Portulaca</i>	<i>Portulaca</i>	<i>fulgens pilosa</i>	Andes to 4,300 m Andes to 4,000 m as a widespread weed	Zuloaga and Morrone, 1999b Brako and Zarucchi, 1993
Zygophyllaceae				
<i>Tribulus</i>	<i>Tribulus</i>	<i>terrestris</i>	Tibet to 3,800 m as a widespread weed	Wang, 2003

various arguments put forward to explain the trends can be roughly segregated into two categories. First, C₄ species are intolerant of cold, and second, C₄ species require daytime warmth. Intolerance of cold is indicated by chilling and freezing injury that occurs at low temperature. By contrast, heat requiring C₄ species could be cold tolerant, but without warm growing conditions, they would fail to establish or would be excluded by adjacent C₃ species. Both cold injury and a heat requirement need not reflect inherent problems of the C₄ pathway, but could instead reflect non-photosynthetic adaptations to warm environments.

With the elucidation of phylogenies in the grass family and other families containing C₄ lineages (Christin et al., 2008, 2009; Vicentini et al., 2008), it is now apparent that all C₄ taxa arose from C₃ ancestors that currently occur in warm climates, usually in the tropics and subtropics (Sage, 2004; Edwards and Still, 2008; Edwards and Smith 2010). If a lack of cold tolerance due to ancestral adaptation explained the general absence of C₄ plants in low temperature, then in time, C₄ species might gradually radiate into cold climates (Long, 1983). Older C₄ lineages should then have more cold-adapted species than younger lineages. This pattern generally appears to be the case. Of the cold-adapted species listed in Table 1, most are from C₄ lineages that are estimated to be over 15 million years old, while the C₄ grass lineages less than 15 million years old lack high elevation and high latitude C₄ species, with the exception of the *Paspalum* clade (Christin et al., 2009). The Chloridoideae lineage, for example, which is estimated to be the oldest C₄ lineage, contains many cold-tolerant species and genera, including the highest elevation and latitude C₄ species in the world (Table 1).

While recognizing the possibility that ancestral cold intolerance could explain the failure of many C₄ species in cold climates, this argument does not address whether the C₄ pathway itself is inherently maladapted to low temperature, either because of a lesion in the pathway at low temperature, or simply poor performance. A number of lines of evidence indicate that the C₄ pathway is inherently problematic in cool climates relative to C₃ species, and these problems will contribute to the biogeographic pattern of C₄ distribution.

First, the evolution of cold tolerance has repeatedly occurred in the many C₄ lineages, indicating

it is not necessarily a difficult process. We estimate there are at least 16 independent origins of cold tolerance in C₄ lineages (Table 1), and more will be identified when species-level phylogenies become available for all C₄ lineages. Second, most of the C₄ taxa with cold tolerance, such as the temperate zone grasses of the North American prairie and high latitude marsh species, are largely summer active, breaking dormancy later than their C₃ associates. Although these species have evolved cold tolerance and winter survival, they still are restricted to warm periods of activity. Third, in the species *Alloteropsis semilata*, the C₃ ecotype is a revertant from the C₄ ecotype (Ibrahim et al., 2009). The C₃ ecotype has also evolved freezing tolerance, and an ability to acclimate to the cold that is not present in the C₄ ecotype (Osborne et al., 2008). The C₄ ecotype loses its leaf canopy following winter frosts, while the leaves of the C₃ ecotype persist and photosynthesize year round. This trait appears to have allowed the C₃ ecotype to radiate into cooler, upland habitats where the C₄ ecotype is not found. The failure of the C₄ ecotype to show the same pattern of cold adaptation as the C₃ ecotype indicates the C₄ pathway somehow limits the success of the C₄ ecotype in cooler habitats.

Finally, the microsite distribution of alpine and high latitude C₄ plants indicates there is a strong constraint associated with the C₄ pathway that reflects a performance limitation at low temperatures. In the alpine zone, growing season temperatures are low, typically less than 15°C during the day and frequently, below 0°C at night (Körner, 2003; Sage and Sage, 2002; Marquez et al., 2006). Light levels are very high on cloud-free days, but cloud-free nights experience regular frost events due to rapid loss of infrared radiation to the sky (Sage and Sage, 2002). The high solar insolation substantially warms alpine microsites during the day where wind is not great, and certain alpine plant morphologies, notably the cushion plant and prostrate mat morphology are noted to effectively capture solar heat and warm the canopy well above air temperature (Körner, 2003). C₄ plants of the alpine, such as the Andean dicot *Gomphrena meyeniana* that grows to 4,600 m and a number of species from the various C₄ grass lineages are noted to exhibit these heat-trapping morphologies (Sage and Sage, 2002; Sage et al., 2007). Other alpine C₄ species are restricted to arid slopes or salinized basins, such as *Salsola*

species of the high Pamirs of central Asia (Pyankov, 1993; Young and Young, 1983).

Sage and Sage (2002) describe the habitat of *Muhlenbergia richardsonis* in the Alpine region of the White Mountains of California. This species occurs as high as 3,960 m (13,000 feet) and is able to form dominant swards at 3,800 m (12,800 ft; Fig. 5). *Muhlenbergia richardsonis* exhibits a prostrate growth form that keeps the plants within the surface boundary layer, where intense solar heating can elevate the leaf temperatures 10–25°C above air temperatures, such that when the sun shines and the wind is low, the leaves generally operate between 25°C and 35°C. These conditions are common on most summer days in the White Mountains alpine zone between 9 a.m. and noon. At the high elevation limit of its distribution, *M. richardsonis* is restricted to locations where leaves can be warmed well above air temperatures, notably south-eastern slopes which face the mid-morning sun, and among rocks that break the wind. Wind speed usually is high in the

afternoon in the White Mountains, and clouds more common, such that heating by the afternoon sun on southwest facing slopes is less significant. Life in microsites where solar heat is trapped has its costs, however. At night, these same sites tend to be the coldest areas on the landscape, because convection cannot compensate for the high rate of infrared heat loss to the sky, due to a thick boundary layer at the earth's surface. Surprisingly, *M. richardsonis* is more likely to experience frost than other alpine vegetation due to its low location within the boundary layer. *Muhlenbergia richardsonis* is able to tolerate these cold nights with no apparent injury, leading Sage and Sage (2002) to conclude that this C_4 plants requires daytime heat to remain competitive, rather than avoiding nighttime cold. Where *M. richardsonis* is unable to acquire sufficient solar heating to routinely experience leaf temperatures above 25°C, it fails to occur in the community. As elevation increases above 3,000 m, it first disappears from the north facing slopes, then east and west faces with

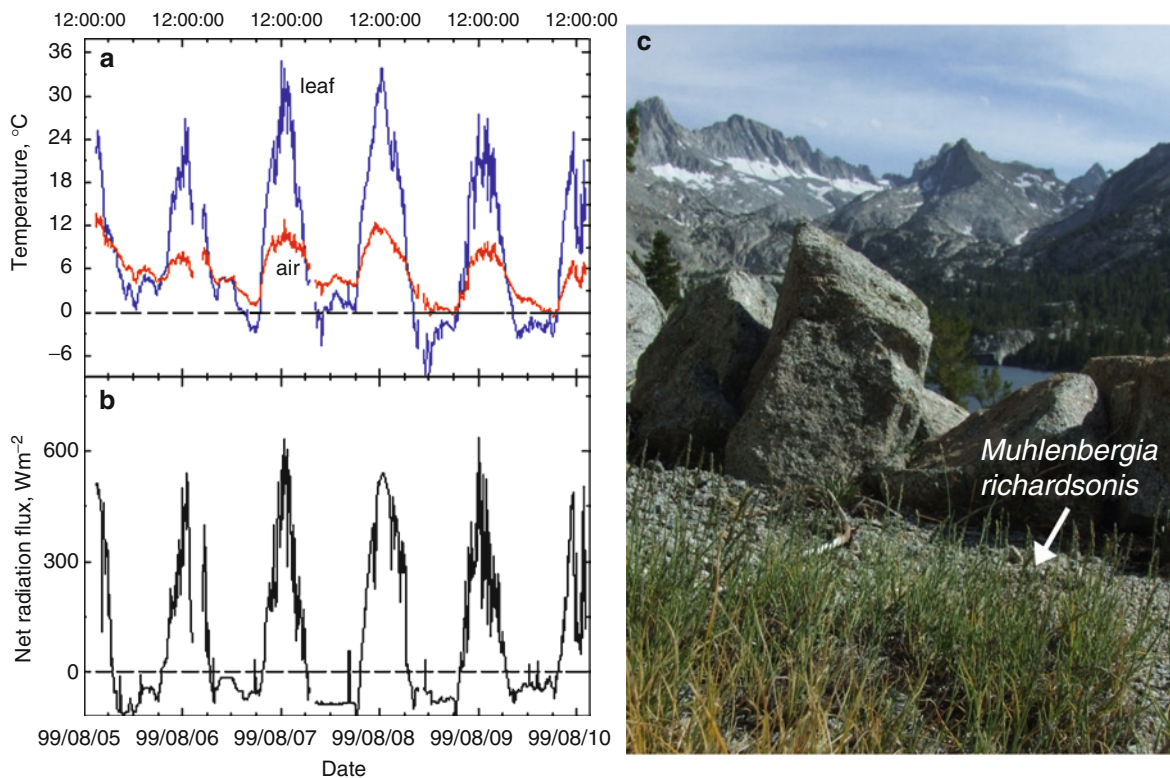


Fig. 5. (a) Leaf and air temperature profiles for *Muhlenbergia richardsonis* at 3,800 m in the White Mountains of California. (b) The corresponding net radiation flux above the *M. richardsonis* canopy during August 1999. (c) *M. richardsonis* in the Sierra Nevada mountains of California at 3,200 m elevation.

further increase in elevation, and is last observed on southeast faces at its upper elevation limit.

North of 60° in the Canadian boreal zone, six species of C₄ plants have been noted to prefer specific microsites, such as south-facing slopes or open, salinized and drought-prone sites (Schwarz and Redmann, 1988). *Muhlenbergia richardsonis* is the northernmost C₄ species known, occurring as far north as 65°N in the Northwest Territories of Canada on south-facing slopes. *Muhlenbergia glomerata* is commonly found on raised hummocks in fens; these likely experience severe episodic drought (Kubien and Sage, 2003). In Europe, the northernmost C₄ species are either *Spartina* and *Atriplex* species in coastal salt-marshes, or summer agricultural weeds (*Amaranthus retroflexus*, *Cyperus longus*, *Salsola kali*, and *Setaria viridis*) that exploit disturbed microsites as far north as 63° (Long, 1983; Jones et al., 1981; Collins and Jones, 1986a, b).

D. Synopsis

C₄ species commonly dominate herbaceous habitats at lower latitudes and altitude, but many have evolved cold tolerance and occur in higher latitude and altitude locations. These cold-adapted C₄ species, however, are still restricted to situations where daytime leaf temperatures are elevated, unless environmental stress (mainly salinity) or disturbance offset a C₃ advantage at low temperatures. Alpine C₄ species represent the most extreme cases of cold adaptation in the C₄ functional type, yet they still require warm microsites or exhibit canopy morphologies that trap solar radiation. These observations support the perspective that the C₄ pathway can function in the cold, but is superior to the C₃ pathway only in warm environments, unless environmental stress allows the higher WUE of C₄ plants to offset the cold advantage of the C₃ flora. Daytime heating, disturbance or stress are thus key requirements for C₄ success in these extreme environments. Only where sufficient daytime heating cannot occur, such as in the Arctic, are C₄ species completely absent.

With this understanding, we now discuss the biochemical processes controlling the response of C₄ photosynthesis to temperature, in order to evaluate underlying mechanisms controlling the geographic distribution of C₄ species.

IV. The Temperature Response of C₄ Photosynthesis: Biochemical Controls

The response of C₃ photosynthesis to temperature has been well studied and biochemical models of these responses exhibit good predictive power (Farquhar and von Caemmerer 1982; von Caemmerer, 2000; Bernacchi et al., 2001, 2003; Cen and Sage, 2005; Sage and Kubien, 2007). By contrast, the ability to model the temperature response of C₄ photosynthesis is incomplete (Collatz et al., 1992; von Caemmerer and Furbank, 1999; von Caemmerer, 2000; Massad et al., 2007). In C₃ species, the principle biochemical controls over photosynthesis are the capacity of Rubisco to consume RuBP (which reflects Rubisco content, activation state and CO₂ supply), and the capacity of light harvesting, electron transport, the Calvin cycle, and starch/sucrose synthesis to regenerate RuBP (von Caemmerer, 2000; Sage and Kubien, 2007). At lower levels of atmospheric CO₂, Rubisco capacity to consume RuBP is limiting for C₃ photosynthesis at the thermal optimum and moderately sub-optimal to supraoptimal temperatures. At temperatures further away from the thermal optimum, RuBP regeneration capacity can become limiting through either the capacity of starch and sucrose synthesis to regenerate P_i (at suboptimal temperatures) or the capacity of electron transport (at both high or low temperature extremes). As well, the capacity of Rubisco activase to maintain Rubisco in an active configuration may become limiting at high temperature (>35°C), particularly in plants from cooler climates. At elevated CO₂, RuBP regeneration capacity tends to limit C₃ photosynthesis at all temperatures, with P_i regeneration being the predominant limitation at cooler temperatures, and electron transport the predominant limitation at elevated temperature. At extreme temperatures, lesions in the photosynthetic apparatus develop, leading to photoinhibition and prolonged loss of carbon gain, even upon return to moderate conditions. Extreme temperature lesions tend to be associated with damage to light harvesting and electron transport components, or the dissociation of Rubisco activase (Berry and Bjorkman, 1980; Salvucci and Crafts-Brandner, 2004; Sage and Kubien, 2007).

In C₄ plants, Rubisco capacity, RuBP regeneration capacity, and the ability of Rubisco activase

to maintain Rubisco activation have also been proposed to be limiting photosynthesis across a range of temperatures (Pearcy, 1977; Sage, 2002; Kubien et al., 2003; Crafts-Brandner and Salvucci, 2002; Dwyer et al., 2007; Massad et al., 2007). In addition, the biochemical capacity of the C_4 cycle to deliver CO_2 to the bundle sheath can also control C_4 photosynthesis (Long, 1983; Potvin et al., 1986; Matsuba et al., 1997; Sage and Kubien, 2007; Wang et al. 2008b). Two leading limitations associated with the C_4 cycle are PEP regeneration by PPDK, and PEP carboxylase activity. A third potential limitation may arise at the decarboxylation step in the C_4 cycle, but this is not regarded as a major limitation in current models of C_4 photosynthesis. In addition, the rate of CO_2 leakage out of the bundle sheath can reduce the efficiency of nitrogen and light use in C_4 plants, and contribute to photosynthetic limitation (Siebke et al., 1997; Kubien et al. 2003, Kubien and Sage 2004a). Below, we will examine the significance of these biochemical limitations by first considering gas exchange approaches and then by addressing the role of the major enzymes hypothesized to control the temperature response of C_4 photosynthesis.

A. The Response of C_4 Photosynthesis to Inter-cellular CO_2 Partial Pressure

In both C_3 and C_4 plants, the response of net CO_2 assimilation to intercellular CO_2 (the A/C_i response) is widely used to evaluate the response of individual biochemical processes to temperature. This is because the stomatal effects are factored out in calculating intercellular CO_2 values, and specific biochemical limitations can be parameterized from the initial slope and CO_2 saturated regions of the A/C_i curve using theoretical models (von Caemmerer and Farquhar, 1981, von Caemmerer, 2000). In C_4 species, the initial slope of the A/C_i response shows a weak thermal dependence, while the CO_2 saturated plateau shows a pronounced response to rising temperature up to the thermal optimum of photosynthesis (Fig. 6, see also Ishii et al., 1977; Long and Woolhouse, 1978a; Laik and Edwards, 1997; Sage, 2002; Pittermann and Sage, 2000, 2001). As a consequence, the CO_2 saturation point rises with temperature. If the intercellular CO_2 level in air (the operating C_i) is not affected by temperature,

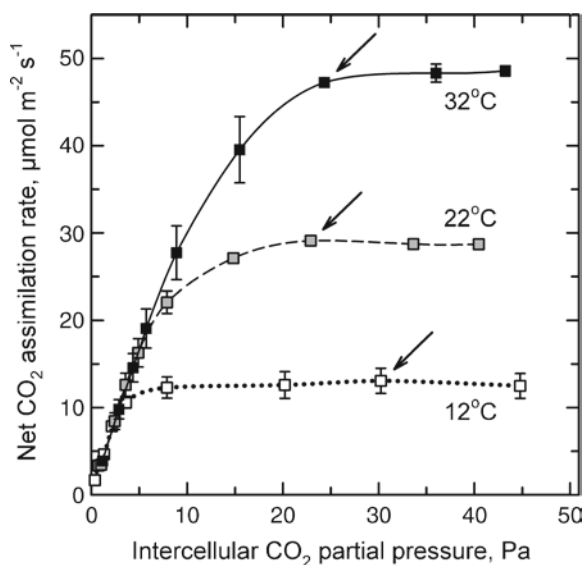


Fig. 6. The CO_2 response of C_4 photosynthesis at three temperatures in *Flaveria bidentis* plants grown in a plant growth chamber at 28°C. Measurement temperatures are beside each curve. Arrows indicate the operational C_i (the intercellular CO_2 partial pressure at prevailing atmospheric CO_2 levels) (From Kubien, 2003).

then the rise in the CO_2 -saturated plateau relative to the initial slope of the A/C_i response will cause CO_2 -saturated photosynthesis at low temperature to shift to CO_2 -limited photosynthesis at elevated temperature, which will have consequences for the A/T response (Fig. 6). At low CO_2 levels where the operating C_i falls on the initial slope, A will be relatively insensitive to the rise in temperature, and the A/T response will have a broad thermal optimum (Fig. 2a). If stomatal conductance were low enough that the operating C_i should fall below the CO_2 saturation point, then C_4 photosynthesis could lose thermal sensitivity, and exhibit a broader thermal optimum than it would if A were CO_2 -saturated. In the case of the responses in Fig. 2, the stimulation of A at the thermal optimum by increasing CO_2 is explained by increasing the operating C_i from the initial slope region to the CO_2 -saturated plateau. When the operating C_i is above the CO_2 saturation point, the A/T response is pronounced, reflecting the temperature stimulation of the CO_2 -saturated plateau (Fig. 2c).

Unlike C_3 photosynthesis, where the initial slope of the CO_2 response curve at light saturation generally reflects Rubisco capacity, the A/C_i initial slope of C_4 photosynthesis is modeled to largely reflect the activity of PEPC

(von Caemmerer and Furbank, 1999, von Caemmerer, 2000). At low CO₂, PEPC operates below its K_m for CO₂, and thus has a weak response to temperature (Laik and Edwards, 1997); hence, the initial slope is also insensitive to temperature. The CO₂ saturated plateau is modeled to reflect the minimum of either Rubisco capacity, RuBP regeneration capacity, or PEP regeneration in most situations (von Caemmerer and Furbank, 1999; von Caemmerer, 2000; Sage, 2002). At cooler temperatures, the reduction in the CO₂-saturated plateau can lower the CO₂ saturation point below the operational C_i, so photosynthesis becomes limited by one of the temperature-sensitive processes that determine the CO₂-saturated rate of *A*. Once this occurs, photosynthesis exhibits a steep decline with further reductions in temperature.

Recent examinations of the thermal response of CO₂-saturated *A* in cold-tolerant C₄ plants indicate Rubisco capacity is an important limitation on the CO₂ saturated plateau at cooler temperature (<20°C), but not at warm temperatures near the thermal optimum (Sage and Kubien, 2007). Instead, RuBP regeneration, PEP regeneration or PEP carboxylase capacity appears to limit *A* at warmer temperatures in C₄ plants, although it is not clear which is the most important. A challenge for the future will be developing a capability to distinguish between these possible limitations over C₄ photosynthesis at elevated temperature.

B. Photorespiration in C₃ and C₄ Plants

Photorespiration occurs because Rubisco can both oxygenate and carboxylate RuBP. The oxygenation of RuBP produces phosphoglycolate, which must be converted back to PGA through the expenditure of photosynthetic energy and the loss of previously fixed CO₂. Thus, the oxygenation of RuBP and the associated photorespiratory metabolism significantly inhibit C₃ photosynthesis in warm, low CO₂ conditions favoring photorespiration (Sharkey, 1988). In hot environments where C₄ photosynthesis is most productive, inhibition due to photorespiration in C₃ species is 30–50% (Ehleringer et al., 1991; Sage and Pearcy, 2000). Photorespiration explains much of the difference in the responses of C₃ and C₄ photosynthesis to temperature. Under non-photorespiratory conditions (2% oxygen or high

CO₂), the *A/T* responses of ecological-similar C₃ and C₄ species become similar (Pearcy and Ehleringer, 1984). For example, the thermal optimum of C₃ photosynthesis increases towards the C₄ value when photorespiration is reduced by O₂ reduction or CO₂ enrichment (Pearcy and Ehleringer, 1984; Sage and Kubien, 2007).

C₄ plants also experience photorespiration, but it is generally small, estimated to be 3–16% of *A* under physiological conditions in NADP-ME and NAD-ME subtypes (Volk and Jackson, 1972; Ku and Edwards, 1980; Furbank and Badger, 1982, 1983; de Veau and Burris, 1989; Laik and Edwards, 1998; von Caemmerer, 2000; Yoshimura et al., 2004; Ueno et al., 2005), or 20% of the C₃ value (Dai et al., 1993, 1996). C₄ species express a full complement of photorespiratory enzymes, but at levels much lower than observed in C₃ species (Ku and Edwards, 1975; Yoshimura et al., 2004; Ueno et al., 2005). Photorespiration is difficult to observe in C₄ species, because photorespiratory CO₂ release occurs in the bundle sheath cells. This CO₂ is either quickly re-assimilated by bundle sheath Rubisco or, should it escape the bundle sheath, is captured by PEP carboxylase and sent back to the bundle sheath (Volk and Jackson, 1972; Ku and Edwards, 1980; Dai et al., 1993). Hence, there is little observed photorespiration response to temperature, unless the CO₂ level in the bundle sheath is reduced, for example by drought stress, or O₂ level is increased (Ku and Edwards, 1980; Dai et al., 1993). In maize leaves at 40% O₂, the quantum yield of photosynthesis declines about 20% from 15°C to 40°C, indicating a progressive rise in photorespiration with rising temperature (Dai et al., 1993). At 21% O₂, by contrast, the quantum yield of CO₂ uptake in maize is unaffected by temperature above 25°C, indicating that photorespiration rates remain low in C₄ plants in current atmospheric conditions.

C. Quantum Yield

The maximum quantum yield of photosynthesis is equal to the initial, linear slope of the response of *A* to absorbed light intensity. Maximum quantum yield is closely associated with the biogeographic distribution of C₄ versus C₃ grasses, and approximates the relative photosynthetic and growth performance of C₃ and C₄ species across a range of temperatures (Ehleringer, 1978, 2005;

Ehleringer et al., 1997). The maximum quantum yield of C_4 photosynthesis is typically greater than C_3 photosynthesis above 30°C , and less than C_3 photosynthesis below 20°C (Ehleringer and Bjorkman, 1977; Ehleringer and Pearcy, 1983). Between 20°C and 30°C , a crossover typically occurs between the C_3 and C_4 quantum yields that depends upon CO_2 level and the sub-type of C_4 photosynthesis (Ehleringer and Bjorkman, 1977; Ehleringer, 1978; Ehleringer and Pearcy, 1983; Ehleringer et al., 1997). NADP-ME sub-types have a higher quantum yield than NAD-ME C_4 species, and thus a lower cross-over temperature of C_4 versus C_3 quantum yield (Ehleringer and Pearcy, 1983). Quantum yield-based models have been frequently used to predict both the distribution of C_4 species across the globe, and the change in distribution with past and future climate change (Ehleringer, 1978, 2005; Ehleringer et al., 1997; Collatz et al., 1998; Still et al., 2003). For example, the transition temperature between C_3 and C_4 dominance of the North American grass flora corresponds to the crossover temperature for C_3 and C_4 quantum yields (Ehleringer, 1978).

The close association between maximum quantum yield and performance parameters, such as net CO_2 assimilation rate, growth or ecological dominance, have led to the hypothesis that quantum yield differences are causal mechanisms that explain the relative performance of C_3 versus C_4 vegetation (Ehleringer and Bjorkman, 1977; Ehleringer, 1978; Collatz et al., 1998; Still et al., 2003). This hypothesis has been questioned, because maximum quantum yield differences would have physiological significance only at very low light levels (Sage and Kubien, 2003). In most C_4 plants, the vast majority of carbon is absorbed at light intensities above the range where quantum yield differences would be significant, indicating maximum quantum yield is a small contributor to differences in C_4 performance (Long, 1999; Sage et al., 1999; Kubien and Sage, 2004b). However, as demonstrated by Fig. 7, maximum quantum yield is inversely related to the photorespiration rate in C_3 plants, and thus is a robust index of the relative drag on C_3 photosynthesis caused by photorespiration.

Maximum quantum yield is just one measure of quantum yield that has significance for understanding relationship between temperature and the performance of C_4 relative to C_3 plants. As

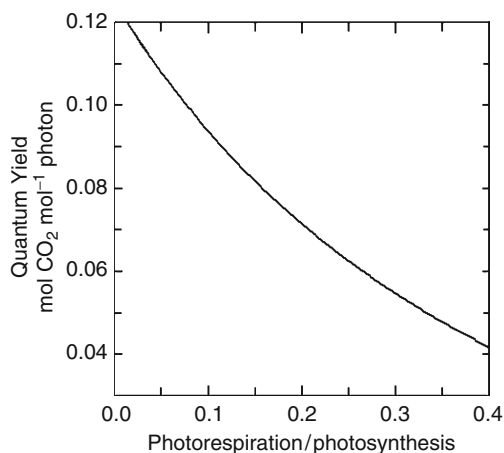


Fig. 7. The relationship between the maximum quantum yield of photosynthesis and the ratio of photorespiration to photosynthesis for a C_3 plant. Modeled according to Sage and Kubien (2003) using the equation $\frac{dA_g}{dI_a} = \frac{0.125 - 0.0625V_o/V_c}{1 + V_o/V_c}$ where dA_g/dI_a is the initial slope of the light response of A versus absorbed photons (I_a), and V_o/V_c is the oxygenation to carboxylation rate of Rubisco.

light intensity increases above $100\text{--}200 \mu\text{mol m}^{-2} \text{s}^{-1}$, photoprotection mechanisms are activated that reduce the quantum efficiency of photosynthesis, causing the instantaneous quantum yield to decline below the maximum quantum yield observed at low light. Temperature affects the engagement of photoprotection mechanisms and thus the instantaneous quantum yield at any given light intensity (Kubien and Sage, 2004b; Farage et al., 2006). At cool temperatures, photoprotection is greater in both C_3 and C_4 species, because the temperature lowers the capacity of the carbon fixation reactions to use absorbed light energy (Labate et al., 1990; Haldimann, 1998; Kubien and Sage, 2004b; Savitch et al., 2009). C_4 plants at low temperature appear to maintain greater levels of photoprotection, due to a greater restriction in demand for energy (Labate et al., 1990; Kubien and Sage, 2004b). In two boreal grass species, Kubien and Sage (2004b) observed that relaxation of photoprotective quenching following shading was faster in a C_3 grass (*Calamagrostis canadensis*) than a co-occurring C_4 grass (*Muhlenbergia glomerata*) below 20°C (Kubien and Sage, 2004b). This lag in recovery of the instantaneous quantum yield

further reduces the overall light use efficiency of C₄ photosynthesis in a dynamic light environment on a cool day. Due to photoprotection, the actual quantum yield differences between C₃ and C₄ species in the field may not reflect maximum quantum yield differences measured in the lab.

D. Rubisco Limitations

In C₄ plants, the temperature response of the fully activated capacity of Rubisco in vitro is nearly identical to the temperature response of gross photosynthesis below 15–22°C (Fig. 8). Below about 17°C, similar values of Rubisco activity in vitro and CO₂ assimilation rate have been demonstrated for the C₄ dicot weed *Amaranthus retroflexus* originating from northern Europe (Sage, 2002), alpine grasses from the Rocky Mountains of North America (Pittermann and Sage, 2000, 2001), the C₄ dicot *Flaveria bidentis*, and the boreal C₄ grass *Muhlenbergia glomerata*. Bjorkman and Pearcy (1971) and Pearcy (1977) also noted similar changes in Rubisco activity and photosynthetic capacity in *Atriplex* shrubs below 20°C, leading them to suggest that Rubisco may limit C₄ photosynthesis at low temperature. Because Rubisco operates near CO₂ saturation in the C₄ bundle sheath at cooler temperatures (Sage, 2002), the identical temperature response

of Rubisco and carbon assimilation is strong evidence that Rubisco capacity is an important limitation for photosynthesis at cooler temperatures in C₄ plants. Above 25°C, Rubisco capacity in vitro exceeds the observed rate of photosynthesis, indicating Rubisco is not a major limitation, unless the bundle sheath CO₂ levels become low enough to greatly limit RuBP carboxylation. This possibility appears unlikely given the low level of photorespiration estimated for C₄ leaves (Dai et al., 1993).

To further examine the possibility that Rubisco controls C₄ photosynthesis at cooler temperature, Kubien et al. (2003) examined the relationship between gross photosynthesis and Rubisco capacity in *Flaveria bidentis* plants transformed with an antisense construct against the small subunit of Rubisco. Antisense *F. bidentis* had anywhere from 30% to 80% less Rubisco than wild type lines (Furbank et al., 1997). If Rubisco was limiting at low temperature in the wild type, then reducing Rubisco via an antisense construct would increase the temperature at which the in vivo k_{cat} (gross CO₂ uptake/ number of Rubisco active sites) would diverge from the in vitro k_{cat} (rubisco activity/number of active sites). Consistently, the in vivo k_{cat} of the wild type diverged from the in vitro response at about 15°C, while the antisense lines did not statistically diverge until above 25°C (Kubien et al., 2003). Furthermore, the ratio of gross CO₂ assimilation in the wild type to antisense line was equal to the ratio of rubisco content in the two lines below 18°C, which should be the case if Rubisco is limiting. Above 20°C, the ratio of gross CO₂ assimilation in the wild-type versus antisense lines declined below the content ratio, reflecting the appearance of a non-Rubisco limitation on photosynthesis in the wild type line.

In C₄ plants, there is generally little or no change in the ratio of the quantum yield of photosystem II to the quantum yield of gross CO₂ fixation (Φ_{PSII}/Φ_{CO_2}) from moderate to high temperature (Oberhuber and Edwards, 1993; Edwards and Baker, 1993); however, the ratio increases as leaf temperature declines below 16–20°C. This increase in Φ_{PSII}/Φ_{CO_2} begins at a higher temperature in the antisense *F. bidentis* lines with reduced Rubisco content, and reaches a greater value at cooler temperatures (Kubien et al., 2003). In C₄ plants, Φ_{PSII}/Φ_{CO_2} is affected by the rate of CO₂ leakage out of the bundle sheath cell, because

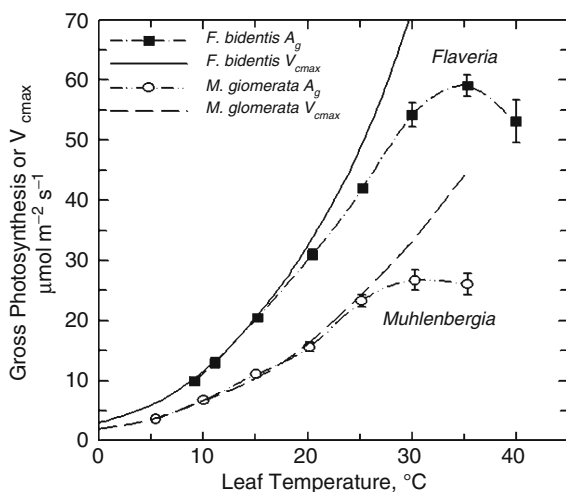


Fig. 8. The temperature response of gross photosynthesis (A_g) and the maximum Rubisco activity in vitro (V_{cmax}) in the C₄ plants *Flaveria bidentis* and *Muhlenbergia glomerata*. Rubisco samples were collected from the same plants in which A was measured (From Kubien, 2003).

leakage reduces Φ_{CO_2} , but does not affect Φ_{PSII} (Siebke et al., 1997). Kubien et al. (2003) interpreted the rise in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ at low temperature as evidence for increased CO_2 leakage. If Rubisco capacity is reduced by declining temperature to a greater degree than the C_4 cycle activity, the bundle sheath CO_2 level should increase, creating a greater CO_2 gradient between the mesophyll and bundle sheath cells, and thus driving a faster leak rate. Increased photorespiration can also increase $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$, but this should not be a concern at low temperature due to an increase in the specificity of Rubisco for CO_2 relative to O_2 (Jordan and Ogren, 1984).

Why does Rubisco become limiting in C_4 species at cooler temperatures? The ability of C_4 plants to concentrate CO_2 around Rubisco to near the CO_2 saturation point, and the higher k_{cat} of C_4 Rubisco, allows C_4 species to utilize the RuBP generated by the light reactions with a third to a quarter of the Rubisco as C_3 species (Schmitt and Edwards, 1981; Osmond et al., 1982; Sage et al., 1987; Sage, 2002). At elevated temperature, the catalytic capacity of Rubisco is more than sufficient to meet the CO_2 and RuBP supply provided by the C_4 cycle and light reactions, respectively. However, Rubisco has a Q_{10} near 2.2, so its turnover capacity declines rapidly with temperature. At cooler temperatures, the Rubisco capacity declines below the capacity of the C_4 cycle and RuBP regeneration, simply because of the low amount of Rubisco in the C_4 relative to a C_3 leaf.

C_4 plants could conceivably compensate for cooler temperatures by producing more Rubisco relative to other leaf photosynthetic enzymes. There is little evidence to support this hypothesis in C_4 species adapted to cool climates; however, few studies have directly assessed Rubisco content in cool- and warm-adapted C_4 species under similar growth conditions. In one such comparison, Pittermann and Sage (2000) examined high and low elevation ecotypes of *Bouteloua gracilis* and observed no clear difference in Rubisco content. During acclimation to cold, increased Rubisco content could be expected to relieve a Rubisco limitation; however, there is no evidence to support such a possibility. In the chilling tolerant C_4 species *Miscanthus* $\times$ *giganteus*, *Muhlenbergia montana*, and *M. glomerata*, Rubisco content was similar in plants grown in chilling conditions (10–15°C) compared to warm conditions

(Pittermann and Sage, 2001; Kubien and Sage, 2004a; Naidu et al., 2003; Naidu and Long, 2004; Wang et al., 2008a). In contrast to responses in chilling tolerant plants, chilling-sensitive C_4 plants such as maize show a prolonged decline in Rubisco content following growth in chilling conditions (Naidu et al., 2003). Also, growth at temperatures above 30°C reduced Rubisco content in *Atriplex lentiformis* and three warm adapted C_4 species relative to Rubisco level in leaves grown below 30°C (Pearcy, 1977; Dwyer et al., 2007). At low temperature where Rubisco is potentially limiting, however, there are few studies describing this phenomenon.

The lack of an obvious ability of cool-tolerant C_4 species to increase Rubisco following cold acclimation or adaptation indicates that the amount of Rubisco C_4 plants may contain is evolutionarily constrained (Sage, 2002). One possible constraint may be the structural properties of the C_4 leaf. In C_4 leaves, Rubisco is compartmentalized into a fraction of the bundle sheath volume, either at the periphery in classical NADP-ME grasses, or along the inner half of the bundle sheath cells in most of other C_4 types (Dengler and Nelson, 1999; Sage and McKown, 2006). This localization places a volume limit on how much Rubisco could be packaged into a C_4 leaf. There are also ecological constraints to consider. Investing in a large amount of Rubisco at low temperature would reduce the nitrogen use efficiency advantage of C_4 plants during warmer temperatures later in the growing season, when the C_4 pathway is most adaptive.

E. Rubisco Activase Limitations

As observed in C_3 plants, the activation state of Rubisco declines above the thermal optimum of photosynthesis in maize, in close proportion to the decline in photosynthesis (Crafts-Brandner and Salvucci, 2002). Crafts-Brandner and Salvucci argued that Rubisco deactivation is due to heat lability of Rubisco activase, and the loss of Rubisco activation is responsible for the decline in *A* above the thermal optimum. Hendrickson et al. (2008) found no evidence for a limitation by Rubisco activase at elevated temperature in the C_4 species *Flaveria bidentis* with genetically reduced levels of Rubisco activase. They observed

no relationship between activase content and *A*, nor between activase content and Rubisco carbamylation state, between 20% and 100% of wild-type activase levels. The carbamylation state of Rubisco was reduced at 40°C compared to 25°C, but this change was not correlated with activase content. These results support the possibility that deactivation of Rubisco at elevated temperatures does not reflect a heat lability of activase, but instead may be a response to other limitations in the photosynthetic apparatus.

F. C₄ Cycle Limitations

1. Pyruvate-Pi-Dikinase

Pyruvate-Pi-dikinase (PPDK) regenerates PEP in the C₄ cycle using the equivalent of two ATP molecules (Kanai and Edwards, 1999). In temperature studies, attention has focused on PPDK in C₄ plants because it can show substantial cold lability, and the activity in vivo is reported to be close to or below observed photosynthetic rates (Taylor et al., 1974; Sugiyama and Boku, 1976; Sugiyama et al. 1979; Long, 1983; Usuda et al., 1984; Potvin et al., 1986; Simon and Hatch, 1994; Matsuba et al., 1997; Du et al., 1999a). The cold lability is twofold: the active enzyme is a tetramer that dissociates into inactive monomers in vitro between 10°C and 15°C in most C₄ species examined, and the rate of activation slows, while deactivation accelerates, at cool temperatures, particularly in C₄ plants from warm climates (Hatch, 1979; Wang et al., 2008b). Circumstantial evidence for PPDK having a major limiting role over *A* in C₄ plants at low temperature has been the close association between the temperature for cold lability of PPDK and the mean minimum growing season temperature where C₄ species drop out of regional floras (Long, 1983). While there is some evidence for a PPDK limitation in certain species, for example, in warm ecotypes of barnyard grass (Potvin et al., 1986), maize (Wang et al., 2008b), sugarcane (Du et al., 1999a), and sorghum (Taylor et al., 1974) particularly after a sudden chill, there is little evidence that cold-tolerant C₄ plants are PPDK limited when acclimated to the cold (Simon and Hatch, 1994; Du et al., 1999a, b; Pittermann and Sage, 2000, 2001; Kubien and Sage, 2004a; Wang et al., 2008b). In the cold-tolerant bioenergy crop

Miscanthus × giganteus, plants acclimate to the cold by increasing PPDK content to a greater degree than other enzymes, potentially overcoming a PPDK limitation which may initially occur upon chilling (Naidu et al., 2003; Wang et al., 2008b). In the cold tolerant C₄ grass *Muhlenbergia montanum*, transfer of plants to 4°C nights led to a drop in photosynthesis at all measurement levels of CO₂ at both 23°C and 33°C, indicating enzymes in the C₄ cycle had been impaired. After 4 weeks of acclimation to 4°C nights, photosynthesis had fully recovered to the pre-chilling values (Pittermann and Sage, 2001).

The lability and activation characteristics of PPDK at low temperature depend upon total protein and solute concentrations. Increased PPDK, and higher polyol and divalent cation concentrations, stabilize the PPDK tetramer in cold environments in vitro (Hatch, 1979; Shirahashi et al., 1978; Yamazaki and Sugiyama, 1984; Krall et al., 1989). In many species, cold adaptation of PPDK activity is not associated with changes to PPDK itself, but instead reflect an altered cellular environment. Simon (1996) noted that PPDK from a cold and warm ecotype of *Echinochloa crus-galli* exhibited identical kinetic properties. Sugarcane varieties differing in cold tolerance also express PPDK forms with similar thermal responses in vitro (Du et al., 1999b).

Some forms of PPDK have greater cold stability. *Flaveria brownii* expresses a PPDK that exhibits greater cold stability than the PPDK from its close relative, the C₄ plant *Flaveria bidentis* (Burnell, 1990; Usami et al., 1995; Ohta et al., 1997). This difference is attributed to variation in as few as three amino acid residues near the carboxy-terminus (Ohta et al., 1997). Recently, cold tolerant segments of PPDK genes have been transformed into parts of the maize gene for C₄ PPDK, leading to a hybrid gene that coded for a cold tolerant form of PPDK in maize (Ohta et al., 2004, 2006). The resulting impact on photosynthesis was small, however. The authors claimed to have detected a slight enhancement of photosynthesis by the chimeric protein at 8°C, but inconsistently, there was no enhancement at any other temperature, including 13°C, which is close to temperatures commonly used as chilling treatments in many studies (Ohta et al., 2006).

An important tool in evaluating the control an enzyme has over photosynthesis is to develop

antisense constructs against the enzyme of interest, which then allows for selective reduction of that enzyme. Using an antisense approach, Furbank et al. (1997) estimated control coefficients for PPDK in *Flaveria bidentis* at 25°C were 0.2–0.3, while the control coefficient for Rubisco was estimated with Rubisco antisense lines to be 0.7. Unfortunately, there has been no follow-up work with these PPDK antisense lines at lower temperature, and they are no longer viable (R.T. Furbank (2009), personal communication). Follow-up work with the anti-Rubisco line of *F. bidentis* at lower temperature shows the Rubisco control coefficient approaches unity below 10°C, and CO₂ leakiness increases, which reflects a greater CO₂ gradient between mesophyll and bundle sheath cells (Kubien et al., 2003). If relative PPDK limitations had increased, leakiness should have declined since the CO₂ delivery to the bundle sheath would slow.

In summary, there is little direct evidence that PPDK is a major limitation in cold-tolerant plants that have acclimated to cooler temperatures. PPDK limitations may be important in cold-sensitive genotypes in barnyard grass, maize, sugarcane and sorghum; however, in some of these species, PPDK shows increased cold stability in varieties from cooler climates (Simon and Hatch, 1994; Du et al., 1999b). Thus, evolutionary adaptation to cooler conditions includes stabilization of PPDK. There is no clear evidence that cold lability of PPDK is the problem that explains the rarity of C₄ species in cold climates.

2. PEP Carboxylase

PEP carboxylase (PEPC), like PPDK, exhibits cold lability in C₄ plants from warm climates, while C₄ plants from cool climates exhibit a form of PEP carboxylase that remains stable at low temperature (Krall and Edwards, 1993, for two species of *Panicum*; Matsuba et al., 1997 for *Spartina* and *Zoysia* grasses). In *Zoysia*, PEPC activity declined by two-thirds upon exposure to 10°/7°C day/night conditions, leading to the conclusion it could be an important limitation in this cold-intolerant plant after prolonged cold exposure (Matsuba et al., 1997). In the cold-tolerant species *Spartina anglica*, PEPC levels fell 30% in the first week of cold exposure but then stabilized (Matsuba et al., 1997). In sugarcane following cold exposure, PEPC activity is relatively

stable at low temperature (Du et al., 1999a, b). Cold-adapted ecotypes of *Echinochloa crus-galli* differed from warm ecotypes by having a lower k_{cat} across a range of assay temperatures; otherwise the enzymes were similar and did not account for differences in low temperature performance of the ecotypes (Hamel and Simon, 2000).

PEPC thus seems to have little control over *A* at cooler temperatures in cold tolerant species, except perhaps, shortly after following transfer from warm to cool conditions. Analyses of the *A/Ci* response in a range of chilling-tolerant C₄ species show the initial slope to be insensitive to differences in measurement temperature, except shortly after chilling, and the photosynthesis rate in air typically falls on the CO₂ saturated region of the curve where Rubisco, PPDK or light harvesting are thought to be limiting (Pittermann and Sage, 2000, 2001; Sage, 2002; Kubien and Sage, 2004a). Thus while some warm-adapted C₄ species can show a decline in PEPC activity that could limit photosynthesis in cold growth conditions, it appears that PEPC is also not an enzyme that would exclude C₄ photosynthesis from cold climates.

3. Other Enzymes

NADP-MDH catalyzes the conversion of OAA to malate in the C₄ cycle (Kanai and Edwards, 1999), and has been noted to have no control over C₄ photosynthesis in antisense *Flaveria bidentis* plants unless the content is reduced by about 90% (Furbank et al., 1997; Trevanion et al., 1999). Hence, this enzyme would not be expected to become a major limitation unless it had a much higher thermal dependency than Rubisco and PPDK, which may occur if it was extremely cold sensitive. Du et al. (1999a, b) observed a large reduction in initial NADP-MDH activity with low temperature exposure in a cold sensitive lines of sugarcane, but not a cold tolerant line; total NADP-MDH activity in both lines was little affected. These results indicate regulation of this enzyme is sensitive to chilling in maladapted plants.

Because they lack PPDK, it has been suggested that PEP carboxykinase (PCK) type of C₄ plants may be inherently more tolerant of the cold than NADP-ME and NAD-ME types of C₄ plants that rely on PPDK for PEP regeneration (Matsuba et al., 1997). In a comparison of two PCK-type of

C₄ species, the cold tolerant variety (*Spartina anglica*) maintained steady levels of PCK following transfer to 10°C growth conditions, whereas PCK activities declined by over 50% in the cold-sensitive *Zoysia japonica*. In *Zoysia*, PEPC levels declined by two-thirds, while Rubisco levels fell about 40% compared to the initial control condition at 27°C. These results support the view that lability of PCK and PEPC are not problems in cold-adapted C₄ species, but can limit *A* in cold-sensitive species exposed to low temperature.

Other enzymes of photosynthesis appear to be stable in cold tolerant C₄ plants following chilling, although they may decline in cold intolerant plants (Leegood and Edwards, 1996; Matsuba et al., 1997; Du et al., 1999a; Naidu et al., 2003). In *Zea mays*, for example, there is a prolonged decline in a wide range of proteins following exposure to low temperature, indicating many problems arise in chilled maize as a result of a general cold intolerance, rather than a lesion specific to the C₄ pathway (Naidu et al., 2003; Wang et al., 2008b).

G. Electron Transport Limitations

In C₄ plants, limitations in electron transport are difficult to discern because the mesophyll and bundle sheath chloroplasts can differ in their electron transport capacity, and the energy requirements of the C₃ and C₄ cycles differ. As a result, few studies report electron transport as a function of temperature in C₄ plants. From what is reported, it is known that electron transport capacity decays above the thermal optimum in a manner that indicates it could become an important limitation at high temperature (Bjorkman et al., 1976; Berry and Bjorkman, 1980). Consistently, increasing temperature above the thermal optimum reduces the CO₂ saturated rate of photosynthesis in maize, but has little effect on the *A*/C_i initial slope, at least to 40°C (Massad et al., 2007).

In maize, changes in levels of metabolites from the C₃ and C₄ cycles indicate there could be a limitation in energy supply at 8°C (Labate et al., 1990). From 20°C to 10°C, the PGA to triose-P ratio declined, which is consistent with a reduction in energy utilization, as may occur if Rubisco or PPDK were limiting. However, at 8°C, PGA/TP ratios increased sharply, indicating energy utilization is no longer limiting, but energy supply

may be (Labate et al., 1990). Low energy supply in maize could result from extensive photoinhibition this species experiences at low temperature (Haldimann, 1998). Detailed metabolite assays on other C₄ species across a range of temperatures are lacking, so further evidence is not available. In *Atriplex* species grown at 8°C, Caldwell et al. (1977b) noted that malate and aspartate pools increase relative to the respective pool sizes in plants at warm temperatures. This result is consistent with a limitation in Rubisco at low temperature, but could also indicate a problem with decarboxylation or electron transport capacity.

In contrast to the metabolite literature, the fluorescence literature examining C₄ responses to low temperature is comparatively rich, particularly with studies of maize, a chilling-sensitive species prone to photoinhibition in low temperatures (Labate et al., 1990; Fryer et al., 1995, 1998; Loreto et al., 1995; Massacci et al., 1995; Haldimann, 1996; Kingston-Smith et al., 1997; Kubien et al., 2003; Kubien and Sage, 2004a, b; Naidu and Long, 2004; Farage et al., 2006; Savitch et al., 2009). Much of this literature describes the responses of photochemical and non-photochemical fluorescence quenching at low temperature. These studies generally confirm that photochemical quenching declines with temperature and non-photochemical quenching markedly rises, mainly reflecting a photoprotective response by the xanthophyll cycle (Fryer et al., 1995; Massacci et al., 1995; Haldimann, 1996; Leipner et al., 1997; Kubien and Sage, 2004b; Farage et al., 2006). Additional photoprotective mechanisms complement zeaxanthin dependent quenching of fluorescence in maize at low temperature, although these remain to be identified (Savitch et al., 2009). The fluorescence results are generally consistent with a limitation in carbon metabolism rather than the light reactions at suboptimal temperature (Kubien et al., 2003; Kubien and Sage, 2004b). Using whole leaf fluorescence to estimate electron transport capacity *in vivo* in the cold tolerant C₄ grass *Muhlenbergia glomerata*, Kubien and Sage (2004a) estimated that electron transport capacity was non-limiting below the thermal optimum, but it could be limiting for photosynthesis above the thermal optimum in both warm and cool grown plants (Fig. 9). Electron transport capacity shared the same thermal optimum as CO₂ assimilation in *M. glomerata*,

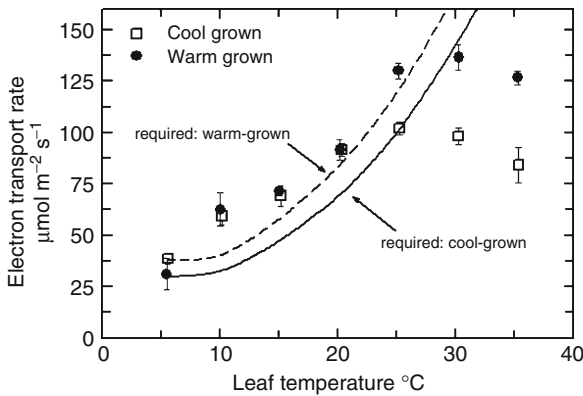


Fig. 9. The response of electron transport to temperature in the C_4 grass *Muhlenbergia glomerata* grown in a cool ($14^\circ/10^\circ\text{C}$ day/night) and warm ($26^\circ/22^\circ\text{C}$) growth chamber. Symbols are from measurements using chlorophyll *a* fluorescence at a PPFD of $1,300 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 370 ppm CO_2 . The solid and dashed lines indicate the theoretical rate of electron transport required to support the observed RuBP carboxylation capacity in cool- and warm-grown plants, respectively. Where measured electron transport rates exceed the theoretical requirements, electron transport capacity is non-limiting. Where observed rates are below theoretical requirements, an electron transport limitation is possible (From Kubien and Sage, 2004a).

and the reduction in the estimated electron transport rate above the thermal optimum was similar to the reduction in photosynthesis (Kubien and Sage, 2004a).

V. Fluorescence at Low Temperature

One of the common responses observed in C_4 plants at low temperature is a rise in the ratio of $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$, which reflects the electron requirement for CO_2 fixation. In warm conditions, including supraoptimal temperatures, $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ is near the theoretical value of 12; however, below 20°C , $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ increases to 20–25 (Edwards and Baker, 1993; Oberhuber and Edwards, 1993; Fryer et al., 1998; Kubien et al., 2003; Kubien and Sage, 2004a; Farage et al., 2006; Baker, 2008). This rise in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ is also observed as a shift of the response of Φ_{PSII} versus Φ_{CO_2} from their theoretical relationship, particularly in warm-adapted species exposed to chilling (Fig. 10). While plants grown in warm conditions tend to track the theoretical response, C_4 plants grown in cool conditions often show an enhanced Φ_{PSII} versus Φ_{CO_2}

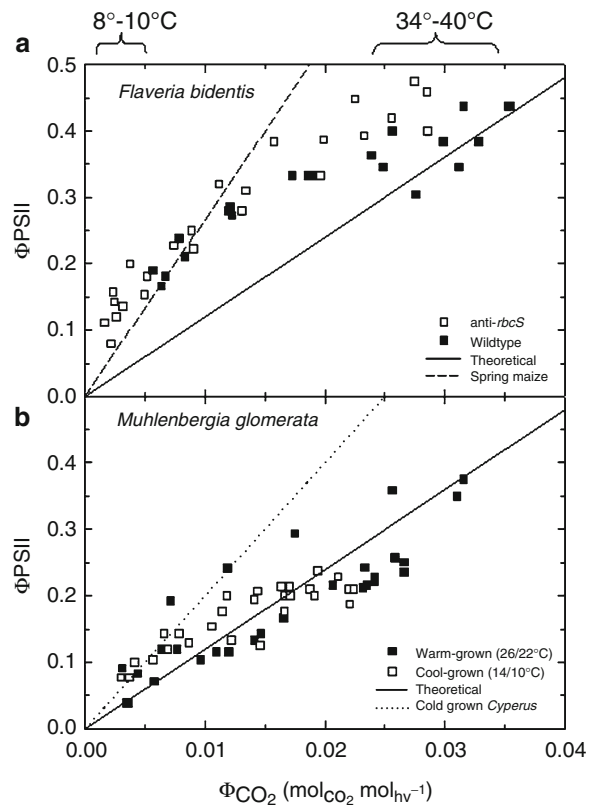


Fig. 10. The relationship between the instantaneous quantum yield of photosystem II (Φ_{PSII}) and the instantaneous quantum yield of CO_2 assimilation (Φ_{CO_2}) in the C_4 plants *Flaveria bidentis* (panel a) and *Muhlenbergia glomerata* (panel b). For *F. bidentis* and *M. glomerata*, the responses were generated by varying leaf temperature (Kubien, 2003), and the corresponding temperature ranges are shown above the graph. *Flaveria bidentis* plants were from a Rubisco antisense line (open squares) or wild-type line (filled squares). Plants of *M. glomerata* were grown in a warm ($26^\circ/22^\circ\text{C}$ day/night – filled symbols) or cool ($14^\circ/10^\circ\text{C}$ day/night – open symbols) regime in plant growth chambers. The solid lines show theoretical responses between Φ_{PSII} and Φ_{CO_2} assuming 12 photons are required per CO_2 fixed. The dashed lines show the response slope for measured responses from spring-grown maize (panel a, from Fryer et al., 1998) and cool-grown *Cyperus longus* at 17°C (panel b, from Farage et al., 2006). Summer treatments of maize and 25°C grown *C. longus* produced relationships similar to the theoretical responses shown.

response relative to the theoretical response (Fig. 10; Fryer et al., 1998; Farage et al., 2006).

A rise in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ above the theoretical value indicates that an increased fraction of electrons moving through PSII are not being utilized by carbon fixation (Kubien et al., 2003; Farage et al., 2006).

This could occur if (A) photorespiration increases at low temperature, due to low CO₂ in the bundle sheath (as would occur if PPDK was the limiting step); (B) low temperature induces an alternative electron sink, such as oxygen through the Mehler reaction; or (C) CO₂ leakage from the bundle sheath increases. Increased photorespiration is unlikely to be the cause of increased $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ at low temperature, because the Rubisco specificity for CO₂/O₂ rises substantially at cooler temperatures, and there is little detectable sign of increased oxygen sensitivity of photosynthesis in C₄ plants at normal O₂ levels (Jordan and Ogren, 1984; Ku and Edwards, 1980; Dai et al., 1993). Also, the operating C_i is greater than the CO₂ saturation point, and there is little reduction in the initial slope of photosynthesis, which should occur if CO₂ supply to the bundle sheath is impaired (Sage, 2002). In C₄ plants, cyclic or pseudocyclic electron flow through PSI is an important means to produce extra ATP required by the C₄ cycle (Edwards and Walker 1983; Furbank et al., 1990; Maroco et al., 1997). At low temperatures, increased flow of electrons to O₂ via PSI can generate additional reactive oxidative species which in turn consume NADPH, thereby reducing the flow of electrons to CO₂ reduction. This would reduce Φ_{CO_2} , resulting in a rise in $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ (Fryer et al., 1998; Farage et al., 2006). Increased CO₂ leakage from the bundle sheath is a likely possibility because an increase in the degree to which Rubisco limits photosynthesis at low temperature would allow CO₂ levels to accumulate in the bundle sheath, thereby driving increased diffusive efflux of CO₂ (Kubien et al., 2003).

VI. Stomatal Limitations

In C₄ plants, low stomatal conductance limits photosynthesis only when it reduces the C_i below the CO₂ saturation point. Unless the stomatal conductance is substantially reduced by low humidity or abiotic stress, stomatal limitations are unlikely to occur below 20°C, because the operating C_i is above the CO₂ saturation point in most C₄ plants at these temperatures (Long and Woolhouse, 1978a; Pittermann and Sage, 2000, 2001; Sage, 2002). In addition, the maximum

vapor pressure difference (VPD) between leaf and air is reduced in low temperature because the saturation vapor pressure of the air declines. High VPD induces stomatal closure in most situations, but below 20°C, the highest VPD possible is about 1.5 kPa, which is generally too low to substantially reduce stomatal conductance in non-droughted plants (Long and Woolhouse, 1978a; Postl and Bolhar-Nordenkamp, 1993).

At the thermal optimum and above, high VPD can occur, particularly for those C₄ species in semi-arid and arid habitats. With the rise in the CO₂ saturation point with increasing temperature, the operating C_i could fall below the CO₂ saturation point on the A/C_i curve. Because the initial slope of the A/C_i curve in C₄ species is typically very steep, the stomatal limitation could be large if the operating C_i falls on the initial slope. This phenomenon has been infrequently studied, however, so there are few case studies of stomatal limitation to highlight for C₄ species. In high elevation C₄ grasses, Pittermann and Sage (2000, 2001) observed no stomatal limitation in well watered plants at moderate VPD levels at measurement temperatures of 13°C, 23°C and 33°C. Bunce (1982, 1983) noted little change in A in *Amaranthus hypochondriacus* and *Portulaca oleracea* with increasing VPD at a range of air temperatures between 26° and 36°C, despite a high VPD induced decline in stomatal conductance. In maize, the reduction in A with rising VPD from 10 to 40 mbar was about half as great as observed in the C₃ species castor bean and tobacco (Dai et al., 1992). These results support the hypothesis that in general, C₄ species are less likely to experience stomatal limitations in well-watered conditions than C₃ species.

VII. Thermal Acclimation of C₄ Photosynthesis

Acclimation refers to a series of adjustments in the leaf that compensate for the change in environmental conditions and allow for performance in the new environment that is greater than would occur had there been no compensatory adjustments. This contrasts with injury responses, where the new environment reduces carbon gain potential by damaging one or more components of the

photosynthetic apparatus. Many studies describe the thermal acclimation of CO₂ fixation in C₄ plants (Fig. 1c; Bjorkman and Percy, 1971; Caldwell et al., 1977b; Percy and Harrison, 1974; Percy, 1977; Bjorkman et al., 1980; Kemp and Williams 1980; Percy et al., 1981; Bowman and Turner, 1993; Du et al., 1999b; Pittermann and Sage, 2001; Naidu et al., 2003; Naidu and Long, 2004; Kubien and Sage, 2004a, b; Dwyer et al., 2007). Except for a change in the thermal optimum of photosynthesis, no general pattern of acclimation can be summarized, as the acclimation response depends upon the thermal environment to which the plants are adapted. One pattern of acclimation is observed in C₄ plants adapted to and grown in warm environments. In this group, plants grown above 30°C show a strong enhancement of photosynthesis at high temperature and a pronounced decline in photosynthesis at low temperature relative to species grown at cool temperature. In *Atriplex lentiformis* and *A. hymenalytra*, growth in high temperature shifts the thermal optimum to warmer temperatures, but does not change the shape of the *A/T* curve (Percy and Harrison, 1974; Percy, 1977; Bjorkman et al., 1980). The shift to higher temperatures of the initial slope of the *A/T* response in *A. lentiformis* with an increase in growth temperature is associated with a 60% reduction in Rubisco activity in vitro, and only a slight decline in PEP carboxylase activity (Percy, 1977). In *Tidestromia oblongifolia*, which is adapted to hot summers in the Mojave desert, growth at low temperature (16°C) causes a great reduction in photosynthesis at all measurement temperatures (Berry and Bjorkman, 1980; Bjorkman et al., 1980), similar to what is observed in chilling sensitive C₄ species such as maize that are grown at 10–15°C.

A second pattern of acclimation commonly observed is for growth at warmer temperatures to increase *A* at the thermal optimum and warm measurement temperatures, while at cool measurement temperatures, *A* is little changed between warm- and cool-grown plants (Caldwell et al., 1977b for *Atriplex confertifolia* and *A. vesicaria*; Bowman and Turner, 1993 for *Bouteloua* species; Pittermann and Sage, 2001 for *Muhlenbergia montanum*; Naidu et al., 2003 for *Miscanthus × gigantea*; Kubien and Sage, 2004a for *Muhlenbergia glomerata*). In the C₄ shrub *Atriplex confertifolia*, for example, growth at 8°C caused a

drop in photosynthesis at all but the coolest measurement temperatures relative to the rates measured in plants grown at 25°C (Caldwell et al., 1977a). The lack of acclimation in the photosynthesis rate at lower temperature in *Miscanthus* and *Muhlenbergia* is associated with a lack of any change in Rubisco content, which is consistent with a Rubisco limitation on CO₂ uptake at low temperatures in these C₄ species. This apparent inability to adjust photosynthesis and Rubisco content at low growth temperature led Sage and McKown (2006) to suggest that cold-adapted C₄ species such as *Bouteloua*, *Miscanthus* and *Muhlenbergia* may have a low capacity for photosynthetic acclimation to cooler conditions.

A third pattern of acclimation was observed by Dwyer et al. (2007) in three warm-climate C₄ species (the grasses *Cenchrus ciliaris* and *Pennisetum coloratum*, and the dicot *Flaveria bidentis*) grown at 25°/20°C and 35°/29°C day/night temperatures. In each species, the warm grown plants exhibited lower *A* at the cooler measurement temperatures than the cool-grown plants; however, there was little change in *A* above the thermal optimum between the treatments. In all three species, these changes in CO₂ assimilation rate in warm compared to cool growth conditions were associated with a drop in the amount of Rubisco, carbonic anhydrase, cytochrome *f* and leaf nitrogen. PEPC levels did not change. *Atriplex rosea* also exhibits this type of acclimation response (Fig. 1c).

In summary, C₄ species can show substantial adjustments in the photosynthetic response to temperature following growth in different thermal environments; however, these changes appear to be most pronounced in warmer conditions, particularly in plants from warm environments. C₄ plants from cool environments appear to be limited in their ability to acclimate to varying thermal conditions below the thermal optimum. Most studies of growth at low temperature have focused on stressful chilling responses (notably in maize), where prolonged exposure to low temperature is accompanied by photoinhibition, a loss of leaf protein content and eventual leaf death. However, most of these species are of tropical or subtropical origin, and the chilling responses are not necessarily indicative of problems with the C₄ pathway, as similar responses are observed in C₃ species from low latitudes following chilling. Chilling-tolerant C₄ species show a good ability to

overcome short term limitations in the C₄ cycle and photosystems following growth at chilling temperatures, and appear to maintain a stable photosynthetic capacity after an acclimation period. Where examined, Rubisco capacity appears to control *A* in these species at low temperature.

VIII. Conclusion: Are C₄ Plants Inherently More Sensitive to Low Temperature Than C₃ Plants?

The lack of C₄ plants in polar, boreal and most cool temperate habitats demonstrates that C₄ plants are less ecologically successful in colder climates. However, the occurrence of dozens of C₄ species in cold climates at high elevation and in specialized microsites of the boreal and cool temperate regions demonstrates that the C₄ pathway can be tolerant of low temperature, including freezing conditions during the growing season. C₄ species have evolved from C₃ lineages of warm climates, leading to the hypothesis that they are excluded from cold areas due to prior adaptations to higher temperature. Certainly, failure of many C₄ species in cold climates is due to a general cold intolerance, just as failure of low-latitude C₃ taxa in the cold reflects chilling sensitivity. However, the repeated evolution of cold tolerance in numerous C₄ grass, sedge and dicot lineages indicates that acquisition of cold tolerance is not an overwhelming barrier to the radiation of C₄ plants into cold environments. As well, the rapid acquisition of freezing tolerance in the C₃ ecotype of *Alloteropsis semialata* indicates cold tolerance can evolve quickly in evolutionary time.

Of particular note is the local distribution of C₄ species adapted to cold regions. Alpine C₄ species are restricted to microsites that are warm during the day or have growth forms that efficiently trap solar heat. At high latitudes in the northern boreal region, many species occur on south facing slopes, where solar heating is high. In cases where there is no identifiable restriction to warm microsites, the C₄ species from high latitude are restricted to drought or saline microsites, where the higher water use efficiency of the C₄ pathway may compensate for the abiotic stress. In these situations, the vegetation away from the stressed habitats is completely C₃, indicating that the stress offsets an advantage the C₃ species may have in the cold.

Ecological disturbance may also create temporary niches for C₄ species in cold regions by checking competitive pressure from C₃ plants.

The conclusion from the species distribution is that C₄ plants can evolve tolerance of cold conditions, but still require warm microsites, disturbance or abiotic stress to realize ecological success. This view is consistent with our physiological understanding. While C₄ photosynthesis can perform as well as C₃ species at low temperature, the universal pattern is for C₄ species to perform better at high temperatures than C₃ species of similar growth form and ecological habit. This difference in performance is due in large part to the ability of C₄ plants to raise CO₂ levels around Rubisco high enough to suppress most photorespiration, and to allow Rubisco to operate at high efficiency. At low temperature, the physiological explanations are less clear, although recent progress suggests why the C₄ pathway may do less well in the cold. The idea that superior C₃ quantum yields explain C₃ success at low temperature is inadequate in a direct sense, because maximum quantum yield differences do not relate to conditions under which the vast majority of daily carbon is assimilated. Limitations in C₄ cycle enzymes such as PEPC or PPDK are not likely except shortly after exposure to chilling; cold-adapted C₄ species have cold-stable forms of these enzymes, and synthesize sufficient quantity to overcome any short term limitation. Instead, the common pattern of limitation appears to be a low capacity of Rubisco. At low temperature, the low amount of Rubisco in C₄ plants imposes a ceiling on photosynthesis that adaptation and acclimation appear unable to overcome. This low ceiling may in turn restrict the ability of C₄ species to compete against their C₃ associates in perennially cold climates, and to deal with other ecological challenges that may be present. An inflexible ceiling on carbon gain imposed by a low Rubisco capacity may thus be the key trait that maladapt the C₄ pathway to low temperature environments.

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Part III

Molecular Basis of C₄ Pathway

Transport Processes: Connecting the Reactions of C₄ Photosynthesis

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Summary	199
I. Introduction	200
II. Intercellular Fluxes	203
III. Transport Processes in the NADP-Malic Enzyme Type	203
A. PEP Export from PCA Type Chloroplasts	204
B. Oxaloacetate and Malate Exchange in PCA Type Chloroplasts	205
C. Malate Import into PCR Chloroplasts	206
D. Pyruvate Export from PCR chloroplasts	207
E. Pyruvate Import into PCA Type Chloroplasts	207
F. Auxiliary Transport Processes	208
IV. Transport Processes in the NAD-Malic Enzyme Type	209
A. PEP Export from PCA Chloroplasts	210
B. Dicarboxylate Transport in PCR Mitochondria	210
C. Pyruvate Export from PCR Mitochondria	210
D. Pyruvate Import into PCA Chloroplasts	211
V. Transport Processes in the PEP Carboxykinase (PEP-CK) Type	211
A. PEP Export from PCA Chloroplasts	212
B. Oxaloacetate Malate Exchange in PCA Chloroplasts	212
C. Malate Import into PCR Mitochondria	212
D. ATP/ADP Translocation to Supply PEP-CK with ATP	212
VI. Transport Processes in Single Cell C ₄ Metabolism	212
VII. Future Prospects	213
A. Discovering the Molecular Identity of C ₄ -Adapted-Transport Proteins	213
B. Prospects for Engineering C ₄ Photosynthesis into C ₃ Crop Species	214
Acknowledgments	214
References	215

Summary

The C₄ cycle requires immense metabolite fluxes. The spatial separation of initial carbon fixation by phosphoenolpyruvate carboxylase and entry in the photosynthetic carbon reduction cycle through Rubisco requires metabolites to shuttle not only between cells but also across intracellular membranes. C₄ photosynthesis is a highly compartmentalized process. Atmospheric CO₂ is fixed into C₄ acids (photosynthetic carbon assimilation, PCA) in one tissue and C₄ acids donate CO₂ to Rubisco in another (photosynthetic carbon reduction, PCR). PCA occurs in chloroplasts and cytosol; PCR occurs in

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chloroplasts, and depending on the subtype of C_4 photosynthesis may involve the mitochondria and cytosol. Intercellular transport likely occurs symplastically but the intracellular transport processes across the organellar membranes are at least in part mediated by specific transport proteins. These transport processes are of particular interest because metabolites have to be transported at the rate of carbon assimilation; each carbon which is shuttled as a C_4 acid necessitates distinct transport processes as does the C_3 acid which returns to recycle the initial carbon acceptor. Currently, it is not fully understood how the organellar membranes accommodate the high volume and velocity of the necessary flux.

This chapter will review the different types of C_4 cycle reactions and the transport processes required for each sub-type based on the localization of the enzymes involved in the C_4 cycle. For each transport process the current knowledge about the transport proteins involved is stated in detail, including discussion of candidate transport proteins characterized in C_3 systems. Finally, novel strategies for identifying and characterizing molecular candidates for transport proteins and their importance for engineering a C_4 cycle in C_3 crop plants are described.

I. Introduction

One of the highest steady state fluxes of metabolites across organellar membranes known to date originates from the process of C_4 photosynthesis. In C_4 photosynthesis, the production of the primary carbon acceptor phosphoenolpyruvate (PEP) and fixation of atmospheric CO_2 by phosphoenolpyruvate carboxylase (PEPC) occurs in the photosynthetic carbon assimilation (PCA) compartment. In the photosynthetic carbon reduction (PCR) compartment, CO_2 is released from C_4 acids and enters the Calvin Cycle (Hatch, 1987; Edwards et al., 2001a). In most C_4 species, the PCA and PCR compartments are located in two distinct cell types, with PCA cells comprising leaf mesophyll tissue and PCR cells surrounding the veins as a bundle sheath. This arrangement is called Kranz anatomy. Metabolite transport between the cells is not completely understood but it likely involves symplastic connections (Craig and Goodchild, 1977; Hattersley and Browning, 1981; Sowinski et al., 2008). In contrast, intracellular transport requires specific transport proteins in the membranes surrounding the organelles since the reactions of the C_4 cycle occur in three subcellular compartments,

the cytosol, the chloroplasts, and the mitochondria (Hatch, 1987). Both chloroplasts and mitochondria are separated from the cytosol by two membranes. In chloroplasts, the outer envelope membrane which faces the cytosol is relatively permeable to small metabolites because it contains a number of porins with broad substrate specificities (Pohlmeyer et al., 1997, 1998; Bolter et al., 1999; Goetze et al., 2006; Murcha et al., 2007). The inner envelope membrane between the intermembrane space and the stroma is the diffusion barrier and contains specific transport proteins (Weber, 2004; Weber et al., 2005; Weber and Fischer, 2007). In mitochondria, the outer membrane is also relatively permeable because of a number of porins (Benz, 1994) and the inner membrane represents the selectivity filter. Most specific transport proteins residing in the inner envelopes catalyze the movement of two molecules, operating either in counter-exchange or co-transport mode (Weber et al., 2005). When the transport of a solute is coupled to that of another ion or solute it is not only possible to avoid creating electrochemical gradients but the solute can even be transported against its concentration gradient at the expense of the gradient of the second molecule.

Abbreviations: 2-OG – 2-Oxoglutarate; 3-PGA – 3-Phosphoglycerate; AAC – ATP ADP carrier; DHAP – Dihydroxyacetone phosphate; DIC – Dicarboxylate carrier; DiT – Dicarboxylate translocator; DTC – Di- and tricarboxylate carrier; MCF – Mitochondrial carrier family; MDH – Malate dehydrogenase; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; OAA – Oxaloacetate;

PCA – Photosynthetic carbon assimilation; PCR – Photosynthetic carbon reduction; PEP – Phosphoenolpyruvate; PEPC – Phosphoenolpyruvate carboxylase; PPT – Phosphoenolpyruvate phosphate translocator; PPDK – PEP phosphate dikinase; PEP-CK – PEP carboxykinase; Rubisco – Ribulosebiphosphate carboxylase oxygenase; TPT – Triose-phosphate phosphate translocator;

For each carbon atom that is assimilated and reduced in C_4 species, the C_4 cycle performs at least one full turn, and the metabolites involved in C_4 photosynthesis are transported across different organellar membranes at the rate of carbon assimilation (Fig. 1–3). Overcycling to enrich CO_2 within the PCR tissue, which has been estimated at 10–40% of the apparent rate of CO_2 assimilation (Henderson et al., 1992; Laisk and Edwards,

2000; Kubasek et al., 2007), further increases the load on the metabolite transport systems. Compared to the most abundant transport protein in C_3 chloroplasts, the triose-phosphate phosphate translocator (TPT), each metabolite transport protein involved in the C_4 cycle has to carry at least three times more load per unit of time. In *Zea mays*, a NADP-malic enzyme (NADP-ME) type C_4 plant, for each carbon assimilated,

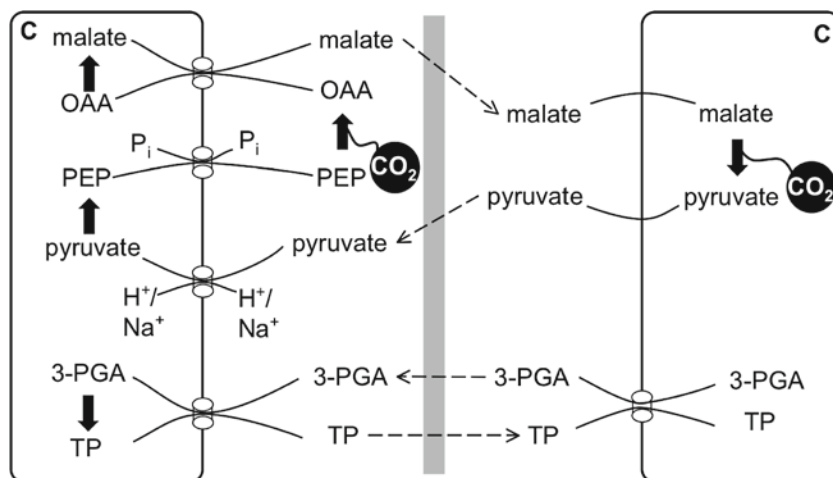


Fig. 1. A scheme outlining the reactions of C_4 photosynthesis and the connecting transport processes for the NADP-malic enzyme type of C_4 photosynthesis in both the PCA (left) and the PCR (right) compartment; the cell wall is represented by a grey bar; intracellular transport by arrows; intercellular transport by dashed arrows; metabolic reactions by broad arrows; C – chloroplast, for additional abbreviations see text.

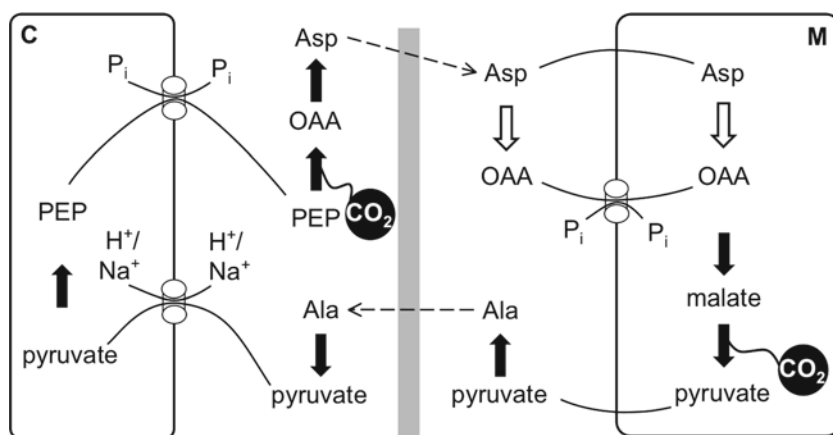


Fig. 2. A scheme outlining the reactions of C_4 photosynthesis and the connecting transport processes for the NAD-malic enzyme type of C_4 photosynthesis in both the PCA (left) and the PCR (right) compartment; the cell wall is represented by a grey bar; intracellular transport by arrows; intercellular transport by dashed arrows; metabolic reactions by broad arrows; C – chloroplast, M – mitochondrion; reactions processes which are not resolved are represented by open arrows; for additional abbreviations see text.

major transport substrates of the corresponding C_4 plant transporter. Therefore, whenever possible, evidence from C_3 plants is considered when transport proteins for C_4 species are discussed.

II. Intercellular Fluxes

All C_4 plants that rely on two different cell types to enrich CO_2 in the vicinity of RubisCO need to transport the metabolites of the C_4 cycle between these cell types. The two photosynthetic cell types involved are connected by an unusually high number of plasmodesmata (Craig and Goodchild, 1977; Evert et al., 1977; Hattersley and Browning, 1981). Based on the frequency of plasmodesmata and the diffusion surface area it was calculated that concentration gradients of 10 mM are necessary to drive efficient transport of metabolites by diffusion (Hatch, 1987). Indeed, concentration gradients of 5–10 mM for many metabolites involved in the C_4 cycle appear to be present in C_4 species (Leegood, 1985; Stitt and Heldt, 1985; Furbank and Hatch, 1987). The transport of metabolites between cells is thus dependent on Brownian motion, which is limited by cytoplasmic viscosity and temperature (Leegood and Edwards, 1996). The concentration gradient for pyruvate, however, which was experimentally determined between whole mesophyll and bundle sheath cells in *Zea mays*, is not steep enough to drive efficient diffusion (Flügge et al., 1985; Stitt and Heldt, 1985). It was concluded that the sequestration of metabolites into subcellular compartments such as chloroplasts might provide the driving force for intercellular transport (Flügge et al., 1985). Studies of plasmodesmatal architecture (Overall et al., 1982; Ding et al., 1992; Roberts and Oparka, 2003) have also questioned the assumptions of the simple diffusion model (Sowinski et al., 2008). In particular, the plasmodesmata contain internal structures, most notably the desmotubule, which connect the endoplasmic reticulum of the adjacent cells and limits the cross section available for diffusion. Plasmodesmata also have variable size exclusion limits for molecules (Erwee and Goodwin, 1985). Recent estimates for the concentration gradients needed to drive metabolite transport are three orders of magnitude higher than previously assumed (Sowinski et al., 2008). Sowinski et al. (2008) propose alternative transport mechanisms

such as diffusion through the desmotubule or vesicle transport of metabolites but the membrane continuity between the endoplasmic reticulum and chloroplasts or mitochondria required for either of the alternatives proposed has never been conclusively demonstrated. Although symplastic transport by diffusion remains the accepted model for intercellular transport (Sowinski et al., 2008), it is currently not known how the steep metabolite gradients needed to drive diffusion are generated. Cytoplasmic streaming may support both the transport of metabolites within the cytoplasm by mass flow as well as the transport between cells, especially if streaming in adjacent cells is countercurrent. Assuming a model of countercurrent exchange similar to that developed for fish gill gas exchange, steep gradients for metabolites are created at the site of exchange.

III. Transport Processes in the NADP-Malic Enzyme Type

Operation of the C_4 cycle in the NADP-Malic type involves the cytosol and chloroplast of the PCA tissue and the chloroplasts of PCR tissue (Fig. 1). This type of the C_4 cycle necessitates at least seven metabolite transport steps for each molecule of CO_2 assimilated (Fig. 1). The CO_2 acceptor PEP is first generated within chloroplasts of the PCA tissue and then exported to the cytosol by a phosphoenolpyruvate/phosphate translocator (PPT). Oxaloacetate (OAA) is produced from PEP and CO_2 in the carboxylation reaction in the cytosol of PCA cells and then imported into the chloroplasts of these cells. There it is reduced to malate and then exported to cytosol again. The import and export of these C_4 acids are assumed to occur through a single transport protein, the OAA/malate exchanger (Hatch et al., 1984; Taniguchi et al., 2004). The reduction of OAA to malate enables the C_4 acid to carry not only CO_2 but also one reducing equivalent to the PCR tissue. After import into the PCR type chloroplasts, malate is oxidatively decarboxylated to yield pyruvate, NADPH, and CO_2 , the latter two entering the photosynthetic carbon reduction (or Calvin) cycle. Pyruvate is exported back out of the PCR chloroplasts and returns to the PCA tissue where it is imported back into the chloroplasts to

Table 1. Summary of transport processes necessary in C₄ photosynthesis in NADP-ME plants; for abbreviations see text.

Tissue	Substrate	Co-substrate	Name	Evidence	References
PCA	PEP	P _i	PPT	Biochemical and molecular, supported by proteomics	Day and Hatch 1981; Fischer et al. 1997; Bräutigam et al. 2008a; Majeran et al. 2008
PCA	OAA	Malate	OAT DiT	Biochemical Biochemical and molecular, supported by proteomics	Hatch et al. 1984 Weber et al. 1995; Taniguchi et al. 2002, 2004; Renne et al. 2003
PCR	Malate	?	?	Inferred from localization of soluble proteins	—
PCR	Pyruvate	?	?	Inferred from localization of soluble proteins	—
PCA	Pyruvate	H ⁺ Na ⁺	? ?	Biochemical Biochemical	Flügge et al., 1985; Aoki et al. 1992 Aoki et al. 1992
PCA	3-PGA	DHAP	TPT	Biochemical and molecular, supported by proteomics	Day and Hatch, 1981; Rumpho and Edwards, 1985; Bräutigam et al. 2008a
PCR				Inferred from localization of soluble proteins	Majeran et al. 2008

regenerate the CO₂ acceptor PEP (Hatch, 1987). The transport processes are summarized in Table 1. The best studied C₄ species are of the NADP-ME subtype and they include species from the genera *Zea*, *Sorghum*, and *Flaveria*.

A. PEP Export from PCA Type Chloroplasts

The primary CO₂ acceptor PEP is regenerated from pyruvate by pyruvate phosphate dikinase (PPDK). This enzyme is localized in PCA type chloroplasts, both in single-cell and Kranz-type C₄ photosynthesis, necessitating the export of PEP across the chloroplast envelope (Fig. 1) (Hatch, 1987; Voznesenskaya et al., 2001, 2002). It was initially characterized biochemically using isolated PCA chloroplasts of *Zea mays* and *Digitaria sanguinalis* (Huber and Edwards, 1977b; Day and Hatch, 1981). Both inorganic phosphate (P_i) and 3-PGA but not pyruvate applied externally lead to export of PEP from chloroplasts in vitro in *D. sanguinalis* (Huber and Edwards, 1977b). The transport protein has high affinity for P_i with an apparent K_M of 200 μM and an equally high affinity for PEP, determined as the apparent K_i = 450 μM for inhibition of P_i transport by PEP. Likewise, PEP formation of intact chloroplasts in the light could be stimulated by external application

of P_i but not pyruvate, and presumably P_i serves both for export of PEP and as a substrate for ATP formation needed as a substrate for PPDK (Huber and Edwards, 1977b). In *Zea mays*, the exchange of PEP and 3-PGA for PEP, 3-PGA, and P_i (but not pyruvate or malate) and the exchange of 3-PGA for dihydroxyacetonephosphate (DHAP) across intact chloroplast envelopes was demonstrated (Day and Hatch, 1981). The exchange of PEP for DHAP was not tested. From the biochemical data it was concluded that all substrates are exchanged by the same phosphate translocator protein.

The first transporter capable of exchanging phosphorylated sugars for phosphate was identified at the molecular level as a triose-phosphate phosphate translocator (TPT) in C₃ chloroplasts of spinach (Flügge and Heldt, 1984). It accepts triose-phosphates, 3-PGA, and P_i but not PEP as substrates. Based on earlier biochemical evidence with intact chloroplasts (Day and Hatch, 1981; Rumpho and Edwards, 1984, 1985), tests were performed to determine whether the TPTs of C₄ plants, unlike those of the C₃ plant spinach, were capable of mediating PEP transport. The TPT from the C₄ species *Flaveria trinervia* and *Zea mays* both accepted PEP in contrast to the spinach TPT, which has a low affinity for PEP (Fischer et al., 1994). However the transport characteristics of C₄ TPT cannot explain the transport

rates for PEP of intact C_4 chloroplasts (Fischer et al., 1994). A phosphate translocator specific to PEP transport in exchange for P_i , the phosphoenolpyruvate phosphate translocator (PPT), was identified on the molecular level from several tissues and species, including maize endosperm, and was characterized in detail from cauliflower bud envelopes (Fischer et al., 1997). When heterologously expressed, this protein has a high affinity for PEP ($K_i = 300 \mu\text{M}$ for inhibition of P_i transport) whereas 3-PGA and triose-phosphates are only poorly bound and transported ($K_i = 8,000$ and $4,600 \mu\text{M}$, respectively). RNA gel blot analysis showed that the PPT identified in maize endosperm is expressed at high levels only in non-green tissues (Fischer et al., 1997).

Phosphate translocators are evolutionary ancient proteins, and the split into the PPT and TPT subfamilies of transporters occurred in the common ancestor of the red and the green lineage (Weber et al., 2006). Consequentially, PPTs are present in all plant and algal genomes sequenced to date. The genome of the C_3 plant *Arabidopsis thaliana* harbors two PPT genes. The AtPPT1, like the protein from spinach, accepts both PEP and 2-phosphoglycerate whereas AtPPT2 accepts PEP rather than 2-phosphoglycerate (Knappe et al., 2003). In C_3 plants, the PPT imports PEP into chloroplasts as a substrate for the shikimate pathway since chloroplasts lack the activities of enolase and phosphoglyceromutase that are required to produce PEP from 3-phosphoglycerate; therefore PPT provides the substrate for aromatic amino acid biosynthesis (Knappe et al., 2003; Voll et al., 2003). The import of PEP into chloroplasts is a minor flux in C_3 plants compared with the export of assimilates, for example, mediated by TPT. PPT activity is also required in bundle sheath chloroplasts of C_3 plants to generate a signal that is required for proper mesophyll development (Streatfield et al., 1999; Voll et al., 2003). Recently, a PPT protein abundant in mesophyll chloroplast envelopes of leaves was identified in maize (Bräutigam et al., 2008a) and shown to be mesophyll specific (Majeran et al., 2008); however, its activity has not been tested biochemically. Compared to expression in C_3 chloroplasts envelopes, this PPT is vastly increased in abundance indicating that the high PEP export rates are supported at least in part by increasing the amount of transport protein present at

the envelope (Bräutigam et al., 2008a). Possibly, based on the results by Huber and Edwards (1977b) outlined above, the C_4 PPT from *Zea mays* can also exchange 3-PGA for PEP, which is not the case for any of the C_3 PPTs characterized to date.

B. Oxaloacetate and Malate Exchange in PCA Type Chloroplasts

After PEP is exported from the PCA chloroplast, it is carboxylated to OAA by PEP carboxylase in the cytosol. Since carbon is shuttled to PCR cells in the form of malate in NADP-ME type C_4 plants, OAA and malate need to be exchanged across the chloroplast envelope. Isolated chloroplasts from both C_3 and C_4 plants produce malate and evolve oxygen when externally supplied with OAA (Heber, 1974; Anderson and House, 1979; Day and Hatch, 1981). In maize and spinach chloroplasts a high velocity OAA transporter was characterized biochemically both by OAA-dependent oxygen evolution and radiolabeled precursor uptake studies (Hatch et al., 1984). In *Zea mays* PCA chloroplasts, this transport protein binds OAA with an apparent K_M between 53 and 71 μM , depending on the method used for measuring, and the corresponding apparent inhibitory binding constants for malate are $K_i = 7,300$ and $7,500 \mu\text{M}$. In spinach chloroplasts, the $K_M(\text{OAA})$ is 9 μM and $K_i(\text{malate})$ is 1,400 μM . The high affinity for OAA compared to malate is crucial if, like in *Zea mays*, the malate pools are 10–100 times greater than those of OAA (Hatch et al., 1984). The reaction equilibrium constant of malate dehydrogenase favors the reaction in the direction of malate, rather than OAA (Hatch et al., 1984). Finally, the velocity of OAA transport in *Zea mays* is sufficiently high to supply the C_4 cycle metabolites, and orders of magnitude higher than the velocity of transport in the C_3 chloroplasts of spinach (Hatch et al., 1984).

In C_3 plants, the OAA/malate exchanger is hypothesized to be involved in a reducing equivalent shuttle (Scheibe, 2004; Scheibe et al., 2005). An OAA/malate exchanger working in concert with malate dehydrogenases in the chloroplasts, the cytosol and the peroxisomes can balance reducing power throughout the cell. The molecular identity of the OAA malate transport protein has not been unequivocally established in either

C₃ plants or C₄ plants. A small family of transport proteins capable of transporting dicarboxylates (dicarboxylate translocators, DiTs) was biochemically identified in spinach chloroplasts (Woo et al., 1987) and later identified at the molecular level (Weber et al., 1995). The protein initially characterized transports malate, fumarate, succinate, and 2-oxoglutarate (2-OG), but not glutamate and was named DiT1 (Weber et al., 1995). Its transport characteristics fit well with the model of two translocators which, in concert, import 2-OG into the chloroplast and export glutamate while the counter-substrate malate cycles between compartments (Woo et al., 1987). A second translocator, called DiT2, was also identified at the molecular level and characterized (Taniguchi et al., 2002; Weber and Flügge, 2002; Renne et al., 2003). Proteins of the DiT2 family of transporters have a high affinity for glutamate and aspartate in addition to 2-OG (Taniguchi et al., 2002; Renne et al., 2003). In C₃ plants, a knock down and a knock out for one member of each family have been analyzed. In tobacco, a knockdown of the sole representative of the DiT1 family of transport proteins causes a photorespiratory phenotype with dramatic metabolic changes in precursors and products of ammonia fixation as well as decreases in photosynthesis rate and sugar pools (Schneidereit et al., 2006). In Arabidopsis, the knockout of one of the two members of the DiT2 family results in a photorespiratory phenotype (Somerville and Ogren, 1983; Renne et al., 2003). Changes in OAA and malate pools or redox status have not been reported (Renne et al., 2003; Schneidereit et al., 2006).

All DiTs also transport OAA in vitro. The affinity for OAA of both, C₃ DiT1 and C₄ DiT1, is similar (Taniguchi et al., 2002, 2004). For the Arabidopsis protein the apparent K_M for malate is 700 μ M and the corresponding apparent K_i for OAA is 70 μ M (Taniguchi et al., 2002). For the maize protein the K_M for malate is 610 μ M and the corresponding K_i for OAA is 90 μ M (Taniguchi et al., 2004). It was proposed that DiT1 is capable of exchanging OAA and malate in vivo (Taniguchi et al., 2004), although the kinetic constants of recombinant reconstituted DiTs are different from those determined with transport experiments using intact isolated chloroplasts (Hatch et al., 1984). The discrepancies between transport experiments with intact chloroplasts and isolated

proteins have yet to be resolved. Proteomic analysis of mesophyll and bundle sheath chloroplast membranes also indicates DiT1 may indeed be the OAA/malate exchanger. A DiT1 homologue (called OMT in maize) was enriched in mesophyll compared to bundle sheath chloroplast membranes (Majeran et al., 2008). The proteome analysis solves controversial evidence for the expression pattern of this DiT1 family protein, which is reported with different expression patterns in *Zea mays* and *Sorghum bicolor* (Renne et al., 2003; Taniguchi et al., 2004; Sawers et al., 2007). Despite mounting evidence, the differences between in vitro and in vivo data are unresolved and therefore it remains to be determined whether DiT1 exchanges OAA and malate in either C₃ or C₄ chloroplasts in vivo (Table 1).

C. Malate Import into PCR Chloroplasts

Malate is produced in PCA cells from OAA, and it moves to PCR cells by diffusion through plasmodesmata. The decarboxylation enzyme, NADP-ME, is localized in chloroplasts and malate is imported into PCR chloroplasts (Fig. 1). The transport capacities of chloroplasts in PCR tissues are less well understood compared to chloroplasts in PCA tissues. Most species with Kranz-type C₄ photosynthesis have re-enforced cell walls around the PCR cells, likely to prevent CO₂ from leaking. These cells are therefore less amenable to isolation and characterization in comparison to PCA cells. In *Zea mays*, malate import was studied as a function of CO₂ fixation and as a function of pyruvate generation (Boag and Jenkins, 1985). Malate import was shown to be affected by application of aspartate (Boag and Jenkins, 1985) or aspartate and glutamate (Boag and Jenkins, 1986). Application of aspartate lowered the binding constant and increased the maximal velocity of transport. The only candidate transport proteins currently known of being capable of transporting malate into chloroplasts are the DiTs. DiT2 family proteins from *Zea mays* (Taniguchi et al., 2004) and *Sorghum bicolor* (Renne et al., 2003) are expressed at a higher level in the bundle sheath. A recent proteomic analysis identified a DiT2 family member, ZmDCT2/3, as bundle sheath specific (Majeran et al., 2008). However, DiTs are antiporters that exchange a dicarboxylic acid

for malate (Weber et al., 1995; Taniguchi et al., 2002, 2004; Renne et al., 2003). Therefore DiTs cannot catalyze the net import of a C_4 acid in a uniport mode. The decarboxylation of malate produces the three carbon monocarboxylate pyruvate. Pyruvate has been tested as a counter-substrate for malate transport for a DiT2 protein from *Flaveria bidentis*, a NADP-ME species. It was shown that exchange of pyruvate with malate was negligible in vitro (Renne et al., 2003). It is therefore unlikely that DiT2 proteins catalyze the exchange of malate and pyruvate across PCR chloroplast envelopes (Renne et al., 2003). It thus remains currently unknown how the net transport of C_4 acids across the chloroplast envelopes of PCR cells is achieved.

D. Pyruvate Export from PCR chloroplasts

After import and decarboxylation of malate in PCR chloroplasts of NADP-ME species, the resulting pyruvate is exported to allow the recycling of the initial carbon acceptor in the PCA compartment (Fig. 1). Pyruvate export from PCR type chloroplasts has only been studied in a NAD-ME species, *Panicum miliaceum*, in which the majority of pyruvate is generated in mitochondria. Pyruvate uptake into those PCR chloroplasts was markedly different both in speed and binding affinity from pyruvate uptake into PCA chloroplasts, and was not affected by light (Ohnishi and Kanai, 1987b). In NADP-ME species, pyruvate transport across the PCR type chloroplast envelope has not been studied (Flügge et al., 1985; Ohnishi and Kanai, 1987a; Aoki et al., 1992). Possibly, the high concentration of pyruvate that is generated by malate decarboxylation drives the export of pyruvate out of the PCR chloroplasts. It remains to be determined whether the export of pyruvate from the PCR chloroplasts is mediated by the same or by a different transport protein than the one which imports pyruvate into PCA chloroplasts.

E. Pyruvate Import into PCA Type Chloroplasts

Finally, the pyruvate produced in the PCR compartment needs to be recycled to the CO_2 acceptor PEP in the PCA compartment (Fig. 1). The overall

gradient for pyruvate between PCA and PCR tissue opposes the actual direction of transport (Stitt and Heldt, 1985), thus leading to the hypothesis that pyruvate is actively transported to sequester it in PCA chloroplasts, hence displacing it from the equilibrium (Flügge et al., 1985). In C_4 plants pyruvate import was characterized as a slow process in the dark as a pyruvate anion symport in *Digitaria sanguinalis* (Huber and Edwards, 1977a). Later, light-driven active pyruvate transport was characterized in *Zea mays* (Flügge et al., 1985) and *Panicum miliaceum* (Ohnishi and Kanai, 1987b). In *Zea mays*, light-driven pyruvate transport is dependent on proteins, and it is inhibited by protonophores (Flügge et al., 1985). Transport can be initiated in the dark by applying a pH gradient between the stroma of isolated chloroplasts and the external medium in vitro (Aoki et al., 1992). A second, sodium-dependent mode of active pyruvate import into PCA chloroplasts was discovered in *Panicum miliaceum* (Ohnishi and Kanai, 1987a). Several other species, such as *Urochloa panicoides* and *Panicum maximum*, but not *Zea mays*, also exhibit sodium-dependent pyruvate transport (Ohnishi et al., 1990). A systematic evaluation of more than forty C_4 species revealed no correlation between the mode of pyruvate transport (i.e. sodium- or proton-dependent) and biochemical C_4 subtype (Aoki et al., 1992). In all species tested, light driven pyruvate uptake into PCA type chloroplasts could be mimicked either by a sodium or by a proton gradient in vitro but not by both for any given species (Aoki et al., 1992).

In C_3 plants, pyruvate transport was analyzed biochemically with isolated pea chloroplasts. Pyruvate uptake followed saturation kinetics at low external concentrations and linear kinetics at higher pyruvate concentrations. Based on these results, carrier mediated transport was proposed at low substrate concentrations and transport by diffusion at high concentrations (Proudlove and Thurman, 1981). Pyruvate transport into the chloroplast of C_3 species may be relevant for fatty acid production in certain tissues but not in seeds (Andre and Benning, 2007; Andre et al., 2007), and when serving as a substrate for branched chain amino acid biosynthesis or isoprenoid production (Singh and Shaner, 1995; Schwender et al., 1996). At the molecular level, the protein or proteins which catalyze pyruvate import remain unknown both in C_3 and in C_4 species. Possibly, independent

evolution of C_4 photosynthesis recruited two different types of pyruvate transport proteins, using either a sodium or a proton gradient as the driving force. Passive diffusion of pyruvate across the chloroplast envelope appears unlikely since the metabolite gradient for pyruvate between PCA and PCR tissue requires active pyruvate import into PCA chloroplasts for C_4 photosynthesis, and pyruvate transport is dependent on intact proteins in the chloroplast envelope (Flügge et al., 1985). Establishing either sodium or proton gradients to drive pyruvate transport across the envelope membrane of PCA chloroplasts requires the input of energy. To date, energy requirements for driving metabolite transport have not been included in the overall ATP balance required for driving the C_4 biochemical CO_2 pump. Molecular identification and biochemical characterization of the respective transporters will be required to address this question.

The knowledge about transport proteins involved in the C_4 cycle of NADP-ME plants is summarized in Table 1.

F. Auxiliary Transport Processes

In addition to the C_4 cycle metabolites, pyruvate, PEP, OAA, and malate, several other metabolites are also moved between PCA and PCR cells and compartments. The C_4 cycle serves to enrich the PCR tissue in CO_2 and creates an environment that minimizes photorespiration at the site of Rubisco. To further reduce photorespiration, in some species of the NADP-ME subtype, the partial pressure of O_2 is minimized. PCR tissue chloroplasts have minimal photosystem II activity (Leegood et al., 1983; Walker and Edwards, 1983; Rumpho et al., 1987; Meierhoff and Westhoff, 1993) and consequentially minimal production of reducing equivalents through linear electron transport. To supply the reactions in PCR tissue with reducing power, a reducing equivalent shuttle is hypothesized to operate. This shuttle transports 3-PGA from PCR chloroplasts (Fig. 1) to PCA chloroplasts where 3-PGA is reduced to triose-phosphate and moved back to the PCR tissue. It has been proposed that in *Zea mays*, the PCR cycle is compartmented between PCA and PCR chloroplasts with part of the Calvin cycle relegated to the PCA chloroplasts (Majeran et al., 2005). The shuttling of 3-PGA across two

chloroplast envelopes into the PCA chloroplasts, and the return of triose-phosphate to the PCR chloroplasts, requires the concerted action of two triose-phosphate phosphate translocators. High activity of a transport protein exchanging 3-PGA, triose-phosphates, and P_i has been demonstrated in PCA chloroplasts of *Zea mays* (Day and Hatch, 1981; Rumpho and Edwards, 1985; Rumpho et al., 1987).

In C_3 plants, the TPT is the most abundant transport protein of the chloroplast envelope and it serves to export triose-phosphate from the chloroplast while inorganic phosphate is imported (Flügge and Heldt, 1984, 1991). A knock out in TPT causes massive accumulation of transitory starch but no dramatic visible phenotype since the chloroplasts are able to export assimilated carbon during the night in the form of glucose and maltose (Schneider et al., 2002). The transport characteristics determined in vitro reveal that the TPT of higher plants is also capable of exchanging 3-PGA for triose-phosphates. It has been proposed that TPT functions both as a reducing equivalent shuttle and as the exporter of carbon in C_4 PCR chloroplasts (Flügge and Heldt, 1991). It is unknown whether the inverse direction of 3-PGA transport in comparison to C_3 plants (i.e., export of 3-PGA and import of DHAP) requires specific adaptations of the TPT protein, especially since the stromal pH likely causes 3-PGA to have three negative charges.

Both enriching CO_2 and minimizing O_2 in the vicinity of Rubisco reduces photorespiration in C_4 plants, yet it is not absent. Moreover, the separation of the photorespiratory pathway into different tissues has been hypothesized to predate a true C_4 cycle and indeed be an evolutionary intermediate of true C_4 photosynthesis (reviewed in Chapter 6, this volume). It has been demonstrated that the final step of the photorespiratory pathway, the phosphorylation of glycerate to 3-PGA by glycerate kinase, is localized exclusively in PCA chloroplasts of different C_4 plants belonging to all three subtypes (Usuda and Edwards, 1980a). For maize, the localization was independently confirmed by proteomics (Majeran et al., 2005). In *Panicum capillare*, it was demonstrated that the PCR tissue photosynthetically produces glycerate, which is metabolized in PCA tissue (Usuda and Edwards, 1980b). Photorespiration itself is a highly compartmentalized process involving

not only the chloroplasts but also peroxisomes and mitochondria (Chapter 6, this volume). Even in C_3 plants, the transport proteins involved are unknown at the molecular level. There is biochemical evidence for glycolate and glycerate transport through the same transport protein at the chloroplast envelope (Howitz and McCarty, 1986, 1991; Young and McCarty, 1993). Glycine and serine import into mitochondria was also shown biochemically (Yu et al., 1983). It is currently unknown whether the transport of photorespiratory intermediates in C_4 plants occurs through the same transport proteins as in C_3 plants, especially since in C_4 plants the export of glycolate from the chloroplast and the import of glycerate into chloroplasts are located in different tissues.

Enzymes involved in lipid biosynthesis, nitrogen fixation, tetrapyrrol and isoprenoid biosynthesis accumulate preferentially in mesophyll chloroplasts of *Z. mays* whereas enzymes for sulfur import accumulate preferentially in bundle sheath chloroplasts (Majeran et al., 2005). Presumably, the preferential localization of pathways necessitates transport of the pathway products to the other compartment but specific adaptations of transport proteins are not known to date.

Finally, all metabolites entering the chloroplasts do not only need to cross the specificity barrier, the inner envelope, but also the outer envelope. It has recently been demonstrated that specific outer envelope porins are increased in abundance in *Z. mays* chloroplast envelopes compared to *Pisum sativum* chloroplast envelopes (Bräutigam et al., 2008a). The outer envelope porins OEP37 and OEP24 were increased in abundance and OEP21 was decreased. Apparently, the increased amount of metabolite exchange across the outer envelope is mastered by an increase in specific outer envelope porins demonstrating the importance of the outer envelope for total metabolite traffic to the chloroplast (Bräutigam et al., 2008a).

IV. Transport Processes in the NAD-Malic Enzyme Type

The operation of the C_4 cycle in the NAD-malic enzyme (NAD-ME) subtype of C_4 photosynthesis is compartmentalized between the PCA chloroplasts and cytosol and the PCR mitochondria

(Fig. 2). As is the case in the NADP-ME subtype, the initial carbon acceptor PEP is exported from the chloroplasts and carboxylated in the cytosol. The carboxylation product is OAA, which is subsequently transaminated in the cytosol to yield aspartate. The C_4 amino acid aspartate then moves to the PCR tissue. The compartmentation of the subsequent transamination to OAA is unresolved (Fig. 1). Aspartate may either be imported into the mitochondria and transaminated in the mitochondrial matrix. Alternatively, it may be transaminated to OAA in the cytosol before it is imported. OAA is reduced to malate, probably within the mitochondria (Hatch, 1987; Edwards et al., 2001a), although the localization of the corresponding malate dehydrogenase activity has not been demonstrated. Malate is subsequently decarboxylated to yield pyruvate, CO_2 , and NADH by NAD-ME. The resulting CO_2 enters the PCR cycle where it is fixed by Rubisco. There are no specific transport proteins for either CO_2 or HCO_3^- at either the mitochondrial or the chloroplast envelopes known to date. Pyruvate is exported from mitochondria and transaminated to alanine in the cytosol. If the transamination of aspartate to OAA is localized in the mitochondria (Fig. 2), a 2-oxoglutarate (2-OG)-glutamate shuttle is required to connect the transamination of alanine in the cytosol with the one of aspartate in the mitochondria via the amino-donor/-acceptor pair, glutamate and 2-OG. If the transaminations of aspartate to OAA and pyruvate to alanine both occur in the cytosol (Fig. 2), they can either be directly coupled or connected by the amino-donor/-acceptor pair 2-OG and glutamate. Alanine moves back to the PCA cytosol where it is converted to pyruvate in a transamination reaction, and pyruvate is imported back into PCA chloroplasts where the carbon acceptor PEP is regenerated by the action of pyruvate phosphate dikinase (Hatch, 1987). In addition to this major route, there is a minor pathway in which malate is formed in and exported from PCA chloroplasts and decarboxylated in PCR mitochondria but this pathway accounts for less than 10% of the total C_4 acids moved (Kagawa and Hatch, 1975; Hatch, et al., 1988). This C_4 subtype has also evolved multiple times independently and includes species from the genera *Amaranthus*, *Cleome*, *Digitaria*, and *Atriplex* (Sage, 2004; Muhaidat et al., 2007).

A. PEP Export from PCA Chloroplasts

As with NADP-ME plants the primary carbon acceptor PEP is recycled in PCA chloroplasts by PPDK (Hatch, 1987). PEP export is thought to be catalyzed by a member of the same transport protein class, the PPTs, in NAD-ME plants. A detailed description can be found in [Section 3.1](#).

B. Dicarboxylate Transport in PCR Mitochondria

PEP is carboxylated and the resulting OAA transaminated to aspartate in the cytosol of PCA cells. Aspartate diffuses into the PCR tissue. Unlike in NADP-ME plants, in NAD-ME plants CO₂ is liberated from malate in the mitochondria (Fig. 2). It remains unclear whether aspartate or OAA is the C₄ acid imported into mitochondria but like in chloroplasts of NADP-ME plants a net import and not exchange of C₄ acids is necessary. Generally, it is assumed that aspartate is the imported C₄ acid in NAD-ME plants (Hatch, 1987, 2002; Hatch et al., 1988; Edwards et al., 2001b) although the metabolite fluxes across the mitochondrial membrane have not been established for any NAD-ME species. In C₃ plants, metabolite transport of malate, OAA, aspartate, 2-OG, and glutamate across the mitochondrial membrane has been demonstrated (Desantis et al., 1976; Zoglowek et al., 1988; Hanning et al., 1999) and several proteins catalyzing di- and tricarboxylate exchange have been characterized biochemically and at the molecular level in C₃ plants (Laloi, 1999; Picault et al., 2002, 2004; Palmieri et al., 2008).

The di- and tricarboxylate carrier (DTC) transports a broad spectrum of organic acids. While 2-OG, malate, succinate, OAA, citrate, isocitrate, and sulfate are transported at high levels by DTC from both tobacco and Arabidopsis, both transport proteins do not accept pyruvate or glutamate as a substrate (Picault et al., 2002). DTC depends on a strict counter exchange of two molecules with each other (Picault et al., 2002). The malate transport protein initially identified as the malate transporter from the C₄ grass *Panicum miliaceum* (Taniguchi and Sugiyama, 1996) is an ortholog of the DTCs characterized from the C₃ plants, Arabidopsis and tobacco (Picault et al., 2002).

Although the spatial and temporal expression pattern of this transporter agrees well with a role in C₄ photosynthesis (Taniguchi and Sugiyama, 1997), the substrate specificity of DTC makes it an unlikely candidate for a major role in C₄ photosynthesis. The dicarboxylate carrier (DIC) transports a similar spectrum of dicarboxylic acids. DICs from the C₃ plant Arabidopsis transport 2-OG, OAA, succinate, malate, phosphate and sulfate (Palmieri et al., 2008). Like DTCs, DICs do not accept three carbon organic acids such as PEP and pyruvate, or amino acids such as glutamate or aspartate (Palmieri et al., 2008). In contrast to DTC, the DIC is capable of transporting inorganic anions such as phosphate and DIC can therefore catalyze the net import of a C₄ acid into the mitochondrial matrix by exchanging phosphate for a dicarboxylic acid (Palmieri et al., 2008). The transport creates a phosphate imbalance, which could be compensated by a phosphate/proton transporter in the mitochondrial inner membrane (McIntosh and Oliver, 1994). Neither DTC nor DIC accepts glutamate or aspartate as a substrate. Based on the transport specificities of the proteins known to transport dicarboxylates, we posit that OAA is the main metabolite imported into mitochondria in NAD-ME plants.

C. Pyruvate Export from PCR Mitochondria

Like the import of the C₄ acid into mitochondria for decarboxylation, the export of the resulting C₃ acid, pyruvate, has not been characterized biochemically in C₄ plants of the NAD-ME type (Fig.). In C₃ plants, pyruvate is imported into, rather than exported from, mitochondria since it is one of the substrates for mitochondrial respiration. Isolated mitochondria of the C₃ plant pea import pyruvate with saturation kinetics (Proudlove and Moore, 1982) and the transport depends on a pH gradient, as protonophores efficiently inhibit transport (Proudlove and Moore, 1982). The molecular identity of the mitochondrial pyruvate carrier from plants and other eukaryotes is unknown. Free diffusion of protonated pyruvic acid is an alternative to carrier mediated transport. Small organic acids including pyruvic acid can diffuse through biomembranes (Bakker and Vandam, 1974; Proudlove and Thurman, 1981;

Benning, 1986). Possibly, the irreversible decarboxylation of malate creates sufficient amounts of pyruvate within C_4 PCR mitochondria to drive export by free diffusion of pyruvate, which is further promoted by active uptake of pyruvate into C_4 PCA chloroplasts (Flügge et al., 1985). Alternatively, pyruvate might be transaminated to alanine in the mitochondria and the neutral amino acid may be exported; neutral amino acids are known to permeate the mitochondrial membrane without specific transport mechanisms (Halling et al., 1973; Wiskich, 1977).

D. Pyruvate Import into PCA Chloroplasts

As with NADP-ME plants, after pyruvate has moved to the PCA compartment in NAD-ME plants, it reenters the PCA chloroplasts to serve as the substrate for PEP regeneration. For a detailed discussion of the transport protein involved, see Section 3.5. In addition to the metabolites involved in the C_4 cycle of NAD-ME plants (summarized in Table 2) several additional metabolites likely have to be moved. For example, the liberation of CO_2 in the mitochondria and the localization of Rubisco in the chloroplasts possibly requires an inorganic carbon transport protein at either or both organellar membranes. Aquaporin-like proteins in organellar membranes might act as dedicated CO_2 transport proteins, thereby facilitating intracellular CO_2 flux (Prasad et al., 1998; Uehlein et al., 2003). Unlike in NADP-ME plants (Majeran et al., 2008), knowledge about the spatial distribution of reactions like nitrogen and sulfur fixation or isoprenoid biosynthesis in NAD-ME

species is limited and consequently, no specific hypothesis about additional transport proteins can be put forward.

V. Transport Processes in the PEP Carboxykinase (PEP-CK) Type

The core reactions of the C_4 cycle involving PEP-CK as the decarboxylation enzyme require the fewest transport steps of the three C_4 subtypes (Fig. 3, black part) (Hatch, 1987). In this C_4 cycle, the initial carbon acceptor PEP is in part recycled in the cytosol of PCR tissue. In the PCA cytosol, PEP is carboxylated to OAA, which is subsequently transaminated to aspartate. This C_4 amino acid moves to the PCR tissue where another transamination takes place and the resulting OAA is decarboxylated by PEP-CK in the cytosol. The CO_2 produced enters the PCR cycle of the chloroplasts possibly by free diffusion; whereas PEP moves back to the PCA tissue where it can again serve as the primary carbon acceptor. The cycling metabolites aspartate and PEP transport one aminogroup to PCR cells during each turn of the cycle. The activity of PEP-CK in the cytosol requires ATP, which is produced in the mitochondria through NADH oxidation and exported from the mitochondria in counter-exchange with ADP (Hatch, 1987; Hatch et al., 1988) (Fig. 3). It has been demonstrated that PEP-CK is not the sole decarboxylation enzyme in PEP-CK type plants. NAD-ME is proposed to contribute about equally to C_4 photosynthesis (Fig. 3, grey part) (Burnell and Hatch, 1988a, b; Hatch et al., 1988). Malate is probably the C_4 acid for this part of the cycle.

Table 2. Summary of transport processes necessary in C_4 photosynthesis in NAD-ME plants; for abbreviations see text.

Tissue	Substrate	Co-substrate	Name	Evidence	References
PCA	PEP	P_i	PPT	Biochemical and molecular	Huber and Edwards, 1977b, Fischer et al. 1997
PCR	Aspartate	?	?	Inferred from presumed localization of soluble proteins	—
PCR	OAA	P_i	DIC	Molecular in C_3 plants	Palmieri et al. 2008
	Pyruvate	?	?	Inferred from localization of soluble proteins	—
PCA	Pyruvate	H^+	?	Biochemical	Aoki et al. 1992
		Na^+	?	Biochemical	Aoki et al. 1992

It is probably produced in PCA chloroplasts and exported in a manner similar to that in NADP-ME plants (Fig. 1 and 3). After diffusing to the PCR compartment, malate enters the mitochondria for decarboxylation. The C_3 acid produced through NAD-ME in the mitochondria is exported, either as pyruvate or possibly after being transaminated to alanine, and re-imported as pyruvate into PCA chloroplasts to serve as the substrate for recycling the primary carbon acceptor PEP. The cycling of the metabolites malate and alanine transport one amino group to the PCA tissue in each cycle and therefore balance the amino group moved by aspartate and PEP if both pathways contribute about 50% of the total decarboxylation reactions. The C_4 cycle involving PEP-CK does not rely on intracellular transport except for the provision of ATP, but the C_4 cycle involving the NAD-ME in mitochondria relies heavily on intracellular transport.

A. PEP Export from PCA Chloroplasts

PEP export from PCA chloroplasts is reduced in PEP-CK plants by at least half (Burnell and Hatch, 1988a, b; Hatch et al., 1988) compared to other C_4 species since part of the PEP is recycled from the cytosol of PCR tissue where it is generated by PEP-CK. PEP recycled in the PCA chloroplasts by PPDK is likely exported through the PPT described in Section 3.1.

B. Oxaloacetate Malate Exchange in PCA Chloroplasts

Up to 50% of the fixed carbon is transported to the PCR tissue as malate (Burnell and Hatch, 1988a, b; Hatch et al., 1988), which is probably produced in PCA chloroplast from OAA. The candidate transport protein is described in detail in Section 3.2.

C. Malate Import into PCR Mitochondria

As with NAD-ME plants, part of the CO_2 liberated in PCR cells is produced in the mitochondria by NAD-ME (Burnell and Hatch, 1988a, b; Hatch et al., 1988). Since malate is the C_4 acid transported to PCR cells, no transamination reactions are required and malate can directly be imported

in counter-exchange with an inorganic anion such as phosphate or sulfate. For a detailed analysis of the transport process see Section 3.2.

D. ATP/ADP Translocation to Supply PEP-CK with ATP

The import of malate may not only serve to supply NAD-ME with its substrate but also to drive the tricarboxylic acid cycle which produces ATP for the cell. The ATP demand of PCR cells in PEP-CK plants is high since PEP-CK uses one molecule of ATP to liberate CO_2 and thus uses ATP at the rate of carbon fixation. The mitochondrial ATP/ADP carrier mediates the exchange of ATP and ADP across the mitochondrial membrane (Emmermann et al., 1991; Winning et al., 1991). Alternatively, ATP may be derived from cyclic electron flow and transported by the plastidic ATP/ADP carrier to the cytosol (Neuhaus et al., 1997; Weber, 2004).

The transport processes in PEP-CK plants are summarized in Table 3.

VI. Transport Processes in Single Cell C_4 Metabolism

Plants which operate the C_4 cycle within one cell do not require intercellular transport through plasmodesmata. In these plants, the initial carbon fixation by PEPC and the subsequent decarboxylation and refixation by Rubisco are spatially separated within one cell, which underscores the importance of intracellular transport processes necessary for enriching CO_2 at the site of Rubisco. Examples include *Bienertia cycloptera*, *Suada aralocaspica* (formerly *Borszczowia aralocaspica*) and *Hydrilla verticillata* (Magnin et al., 1997; Voznesenskaya et al., 2001, 2002). In *B. cycloptera*, the PCA compartment consists of grana-deficient chloroplasts in the cytoplasm around the periphery of the cell which is devoid of mitochondria. This PCA compartment is separated by the vacuole from the central PCR compartment, which contains mitochondria and typical granal chloroplasts. Both compartments are connected by cytoplasmic channels, which cross the vacuole. *B. cycloptera* is a NAD-ME plant (Voznesenskaya et al., 2002) and requires all intracellular transport steps described for typical NAD-ME plants with Kranz anatomy.

Table 3. Summary of transport processes necessary in C_4 photosynthesis in PEP-CK plants; for abbreviations see text.

Tissue	Substrate	Co-substrate	Name	Evidence	References
PCA	PEP	P_i	PPT	Inferred from NADP-ME and NAD-ME plants	—
PCA	OAA	Malate	OAT	Inferred from NADP-ME and NAD-ME plants	—
			DiT	Inferred from NADP-ME and NAD-ME plants	—
PCR	Malate	P_i	DIC	Molecular in C_3 plants	Palmieri et al. 2008
PCR	Pyruvate	?	?	Inferred from localization of soluble proteins	—
PCR	ATP	ADP	NTT (chloroplasts)	Molecular in C_3 plants	Neuhaus et al. 1997
PCR	ATP	ADP	AAC (mitochondria)	Molecular in C_3 plants	Emmermann et al. 1991, Winning et al. 1991
PCA	Pyruvate	H^+	?	Inferred from NADP-ME and NAD-ME plants	—
		Na^+	?	Inferred from NADP-ME and NAD-ME plants	—

In *S. aralocaspica*, the PCA and PCR compartments localize to the proximal and distal ends of the same cell, respectively, and are separated by a large vacuole. *Suada aralocaspica* is also a NAD-ME species (Voznesenskaya et al., 2001) with mitochondria localized to the PCR compartment. *Hydrilla verticillata* is a facultative C_4 species in which C_4 photosynthesis of the NADP-ME type is induced under CO_2 -limiting conditions (Magnin et al., 1997). Although single cell C_4 plants do not need to transfer metabolites symplastically between different cells, they have to move metabolites efficiently from the PCA domain to the PCR domain and back. It is currently not known whether metabolite flow is assisted by specialized structures within the cell.

VII. Future Prospects

A. Discovering the Molecular Identity of C_4 -Adapted-Transport Proteins

For virtually all transport proteins, the molecular identity in C_4 species is unknown, although there are a number of good candidates which can be inferred from proteins characterized in C_3 systems. For example, ever since it was discovered

in *Zea mays* endosperm tissue and characterized from cauliflower, PPT has been assumed to be the exporter of the primary carbon acceptor PEP. A PPT isoform expressed at moderate to high levels in C_4 leaves was recently discovered by proteomic analysis of maize mesophyll chloroplast envelope membranes (Bräutigam et al., 2008a). Where candidate transport proteins have been identified it is far from clear how the high metabolite flow across the envelope is sustained by the transport protein. Possibly, the C_4 isoforms of transport proteins have altered kinetic characteristics compared to C_3 homologues, or the increased flow is simply accomplished by increased amounts of transport proteins, thereby increasing the V_{max} of transport.

Most of the biochemical characterizations of organelle transport proteins have been limited to one C_4 species, *Zea mays*. New tools relying on collecting data from non-model species in a high throughput manner may allow studies with the goal of discovering and characterizing the remaining transport proteins. Transcriptomics approaches, if they involve de novo sequencing of cDNAs, show great promise in both generating sequence information for downstream applications (Bräutigam et al., 2008b) as well as generating quantitative information on transcript

abundance (Weber et al., 2007). After sequence information has been generated, proteomics of organellar membranes and the soluble proteomes can reveal specific adaptations of C_4 organelles to sustain transport capacity (Majeran et al., 2005, 2008; Bräutigam et al., 2008a). The comparison of data from different C_4 species should provide information on differences and similarities in transport properties of specific subtypes of C_4 photosynthesis. Since the different C_4 subtypes employ different enzymes as well as different transport proteins, the identification and characterization of molecular candidate proteins for many of the metabolite fluxes is likely possible. For this, it will be crucial not to limit the analysis to only a single model plant species, such as *Cleome* (Brown et al., 2005), but to include many different C_4 species, such as *Zea mays*, *Flaveria* species and *Cleome* species as well as PEP-CK C_4 photosynthesis plants and single cell C_4 species. Detailed knowledge about the transport proteins and their specific adaptations will assist in engineering C_4 photosynthesis-like metabolism in crop plants, irrespective as to whether the engineering aims to generate single-cell or multiple cell C_4 photosynthesis. The discovery and characterization of novel metabolite transport proteins may also inform studies of metabolite transport in C_3 species. For example, the identification of a pyruvate transporter may help to understand the contributions of pyruvate to fatty acid synthesis during seed filling and to branched chain amino acid biosynthesis, fatty acid biosynthesis, and isoprenoid biosynthesis in leaf tissues.

B. Prospects for Engineering C_4 Photosynthesis into C_3 Crop Species

Metabolite flow across the membrane has been shown to be limiting for metabolic pathways (Häusler et al., 2000; Zhang et al., 2008). For example, antisense repression of TPT in tobacco leads to over-accumulation of transitory starch in chloroplasts during the day because the export of triose-phosphate is compromised (Häusler et al., 2000). A simultaneous increase of glucose-6-phosphate and ATP import into potato tuber amyloplasts by overexpression of both, the glucose-6-phosphate and ATP translocator, increases sink strength and yield in potatoes because carbon can be relocated to the amyloplasts with

greater efficiency (Zhang et al., 2008). Both examples illustrate how metabolite flow through a pathway can be altered by changing metabolite flow across membranes. Numerous studies aiming to engineer or alter metabolic pathways have uncovered substrate availability as one of the major limitations of pathway engineering (reviewed in Kunze et al., 2002). Since metabolite flow across membranes during C_4 photosynthesis exceeds all metabolite flows across membranes known in C_3 plants, increasing membrane transport capacity will play a key role in engineering C_4 photosynthesis in C_3 crops. The over-expression of PPDK alone in C_3 species such as *Arabidopsis*, potato, and rice has minimal physiological impacts, although 40-fold over-expression levels have been achieved (for example, in rice; Fukayama et al., 2001; Matsuoka et al., 2001; Miyao, 2003). It has been discussed that the limited impact of PPDK overexpression is due to free reversibility of the reaction (Burnell and Hatch, 1985) in combination with low activities of inorganic pyrophosphatase and adenylate kinase in C_3 plants (Matsuoka et al., 2001). However, the limitation may well be both the lack of pyruvate import as well as PEP export from the chloroplast, which limit substrate availability and cause product accumulation. To substantially increase the metabolite flow through PPDK both pyruvate import and PEP export need to be increased since both pyruvate and PEP transport are minor activities in C_3 plants and the chloroplast envelope is not adapted to allow major changes without genetic modifications. Recently, single-cell C_4 metabolism has been considered an alternative model system for engineering C_3 plants to perform C_4 photosynthesis since its creation would avoid major alterations to leaf architecture and cell-to-cell connectivity. Intracellular transport, however, is as crucial to single cell C_4 photosynthesis as it is to C_4 photosynthesis in species with Kranz anatomy.

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C₄ Gene Expression in Mesophyll and Bundle Sheath Cells

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Summary	221
I. Introduction and Overview	222
II. C ₄ Gene Expression in Bundle Sheath Cells	225
A. Rubisco	225
1. Rubisco Gene Expression and C ₄ Leaf Development	226
2. Rubisco Gene Expression Patterns in a C ₄ Dicot	226
3. Rubisco Gene Expression Patterns in a C ₄ Monocot	229
4. Rubisco Gene Expression in Different C ₄ Species	230
B. Malic Enzyme Genes	231
1. NAD-Dependent Malic Enzyme	231
2. NADP-Dependent Malic Enzyme	232
C. Genes Encoding Other BS Cell-Specific Proteins	234
III. C ₄ Gene Expression in Mesophyll Cells	235
A. PEPC and PPdK	235
1. Amaranth PEPC and PPdK Gene Expression	235
2. Flaveria PEPC and PPdK Gene Expression	236
3. Maize PEPC and PPdK Gene Expression	238
B. Genes Encoding Other MP Cell-Specific Proteins	238
IV. C ₄ Gene Expression in Organelles	240
V. Factors Affecting C ₄ Gene Expression in BS and MP Cells	240
A. Photosynthetic Metabolism	241
B. Cell Position and Lineage	242
C. Other Factors That Affect C ₄ Gene Expression	242
VI. Levels of C ₄ Gene Regulation	243
A. Transcriptional Control of C ₄ Gene Expression	243
B. Post-transcriptional Control C ₄ Gene Expression	245
VII. Conclusions, Future Directions, and Molecular Engineering of C ₄ Capability	248
Acknowledgments	249
References	250

Summary

A distinguishing characteristic of C₄ plants that possess Kranz-type leaf anatomy is the presence of two photosynthetic cell types, bundle sheath (BS) and mesophyll (MP) cells, which occur in leaves and other photosynthetic organs. In mature C₄ leaves, these two cell types differentially express many genes that are required for the function of this pathway. Well-known examples include the BS cell-specific genes encoding Rubisco (plastid *rbcL* and nuclear *RbcS*) and decarboxylating malic enzymes (*Me* genes), and

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MP cell-specific genes encoding PEPC and PPdK (nuclear *Ppc* and *Pdk* genes). Recent studies have identified several other genes that show specificity to MP and BS cells. Many of the C_4 genes originated from non-photosynthetic ancestors, acquiring several new regulatory properties during their evolution to C_4 function. Within each cell type, the C_4 genes are regulated by many factors, including development, light, and photosynthetic metabolism. Genes encoding the various C_4 proteins often show changes in their patterns of expression during the development of leaves and other organs, and these patterns can vary between different genes and between different species. Such observations indicate a lack of correlation in the expression of different MP and BS-cell specific genes, supporting the conclusion that for the most part these are independently regulated. The complexity of C_4 gene expression has led investigators to incorporate a wide variety of methods in their efforts to understand this regulation at the whole plant level; these include in situ hybridization, immunolocalization, transient expression and transgenic plants, and analysis of expression at the levels of transcription, translation, and RNA stability. These investigations have provided evidence that for some C_4 genes, multiple regulatory mechanisms have become incorporated and function together in order to achieve full BS and MP cell-specificity required for the function of this highly efficient CO_2 assimilation pathway.

I. Introduction and Overview

C_4 photosynthesis typically functions within a structure consisting of two morphologically distinct photosynthetic cell types, bundle sheath (BS) and mesophyll (MP) that occur within the primary photosynthetic organs of the plant (Hatch and Slack, 1970; Hatch, 1987; Berry et al., 1997; Edwards et al., 2001; Ueno, 2001; von Caemmerer and Furbank, 2003). This configuration of two cell types is most commonly present in the leaves (well known examples include maize, *Amaranthus hypochondriacus*, *Flaveria bidentis*, and numerous other monocot and dicot C_4 plants), but also is present in stems, culms and cotyledons of most C_4 species. Within this structural framework, the cell-specific accumulation of key photosynthetic enzymes leads to the compartmentalization and functional separation of the two sets of carboxylation and decarboxylation reactions that are required for C_4 photosynthetic function. The two cell types accumulate distinct enzymes encoded by organellar as well as nuclear genes, which catalyze the cell type-specific biochemical reactions. In most C_4 species, morphological as well as functional dimorphisms are apparent

for organelles present within the two cell types, demonstrating that regulation of processes associated with multiple cellular compartments are required for the characteristic CO_2 fixation capabilities of these plants (Gutierrez et al., 1974; Walbot, 1977; Berry et al., 1997; Sheen, 1999; Majeran et al., 2005). An intriguing variation on the archetypical two-cell compartmentalization is the less common single cell C_4 photosynthesis (described in *Bienertia* and *Suaeda* species). In these plants, cytoplasmic and organellar enzymes that would normally be localized separately to BS or MP cells in a typical C_4 leaf accumulate within a single chlorenchyma cell type, becoming spatially localized to positions at different regions within a single cell (Edwards et al., 2004).

In plants that possess the two typical Kranz-associated cell types, the synthesis and accumulation of different sets of cell-type specific photosynthetic enzymes to BS or MP relies on the differential expression of several nuclear- and organelle encoded genes. The earliest studies to investigate cell type-specific C_4 gene expression used in situ hybridization as well as BS/MP cell-separation techniques with leaves from representative monocot or dicot species such as maize and amaranth (*A. hypochondriacus*). In leaves possessing fully developed Kranz anatomy, mRNAs encoding some C_4 enzymes (such as the BS-specific Rubisco or MP-specific PEPC) were shown to accumulate in only one of the two leaf cell types (Broglie et al., 1984; Martineau and Taylor, 1986; Sheen and Bogorad, 1985, 1986b, 1987a; Wang et al., 1992). This pattern corresponded with the known cell

Abbreviations: AOX – Alternative oxidase; BS – Bundle sheath; CA – Carbonic anhydrase; GDC – Glycine decarboxylase; LSU – Rubisco large subunit; ME – Malic enzyme; MP – Mesophyll; PEP – Phosphoenolpyruvate; PEPC – Phosphoenolpyruvate carboxylase; PPdK – Pyruvate orthophosphate dikinase; PS – Photosystem; Rubisco – Ribulose 1,5 bisphosphate carboxylase; SSU – Rubisco small subunit; UTR – Untranslated region of mRNA

type-specific localization of their corresponding proteins, as established by cell separation, enzymatic assays, and immunolocalization (Kanai and Edwards, 1973; Hattersley et al., 1977; Perrot-Rechenmann et al., 1982; Aoyagi and Bassham, 1986; Sheen and Bogorad, 1987b; Nelson and Langdale, 1989). Taken together, these early studies provided solid evidence that the characteristic BS and MP accumulation patterns of the various C₄ enzymes was dependent on the cell type-specific localization of their corresponding mRNAs. Observed accumulation patterns for the various C₄ transcripts indicated that cell-type expression resulted from differential regulation of transcription, or possibly post-transcriptional control of mRNA stability in BS and MP cells and organelles.

The very strict and selective expression of specific C₄ pathway genes in one cell type but not another, adjacent cell type within a single C₄ leaf provides a unique, clearly defined system in which processes responsible for the development and function of two distinct cell types can be identified and characterized at the molecular level in plants. However, molecular mechanisms involved in the specific recognition and regulation of C₄ genes and transcripts, such as trans-acting factors and interacting sequences on DNA or RNA that determine the characteristic gene expression patterns, are still not well understood. This may be due in part to the complex processes that appear to regulate many of the genes involved in this system. This chapter will review evidence that for some photosynthetic genes, multiple levels of regulation have become integrated together in order to achieve full C₄ function. For Rubisco, plastid genes encoding the large subunit (*rbcL*) and nuclear genes encoding the small subunits (*RbcS*) are expressed at very high levels in all plants. While the two subunits are synthesized in separate cellular compartments, regulatory interactions between the nucleus and chloroplasts ensure that they are expressed coordinately throughout development, and in response to environmental or metabolic influences (Taylor, 1989; Furbank and Taylor, 1995; Berry et al., 1997; Sheen, 1999; Rodermeier, 1999). In C₄ species, both genes become down-regulated in MP cells, while continuing to be expressed at high levels in BS cells, thereby becoming restricted to this one cell type. The C₄ forms of some of the other enzymes, such as

NAD-ME, PPdK, and PEPC, have origins as non-photosynthetic enzymes, and have been recruited to serve a photosynthetic function in C₄ plants (Hatch, 1987; Furbank and Taylor, 1995; Sheen, 1999; Monson, 1999; Westhoff and Gowik, 2004; Sage, 2004). For genes that encode C₄ enzymes derived from non-photosynthetic ancestors, two significant regulatory events must occur. First, their expression levels must become greatly enhanced, so that they can accumulate in leaves at the abundant levels required for their new role in photosynthesis. Second, the expression of these new photosynthetic genes has to become selectively restricted, so that the enzymes they encode become localized to only one of the two leaf cell types. In both BS and MP cells, C₄ genes derived from non-photosynthetic ancestors have acquired many attributes that are shared with Rubisco genes, such as expression that is confined to leaves and other photosynthetic tissues, cell type-specificity, as well as regulation by light and photosynthetic metabolism (Berry et al., 1997; Furbank and Taylor, 1995; Sheen, 1999). However, these genes also display independent patterns of expression, and diverse regulatory processes have been implicated the synthesis and accumulation of their encoded enzymes (Berry et al., 1997; Sheen, 1999; Gowik et al., 2004).

A listing of many of the cell type-specific C₄ proteins and their corresponding genes, as covered within this chapter, is provided in Table 1. An ultimate goal of studies on C₄ gene expression is to identify and understand molecular processes unique to C₄ plant species that mediate their characteristic cell-type specific expression patterns. Ultimately, such processes underlie their enhanced photosynthetic capabilities under most conditions, allowing them to thrive under marginal conditions that restrict the growth of many C₃ species (Hatch, 1987; von Caemmerer and Furbank, 2003; Sage, 2004). It is likely that many regulatory sequences and trans-acting regulatory factors responsible for C₄ expression patterns actually occur in both C₃ and C₄ plants. At some level, regulatory systems must have diverged between these two groups, either through the functional modification of pre-existing regulatory factors, or through the acquisition of completely novel processes not present in C₃ species. This review will focus on studies that provide clues about how some of the major photosynthetic genes are regulated in the BS and MP

Table 1. Characteristics of several proteins and genes that show specificity to either bundle sheath or mesophyll cells of C₄ plants. In some cases, characteristics of non-photosynthetic orthologs are also given. Additional information about most of the genes listed can be found in the text. The column labeled “Model plant” refers to C₄ plant systems in which these proteins or genes have been most extensively studied; some of these may have also been investigated in other plant systems. Initial or primary references are given in the table; additional references related to some of the genes listed can be found in the text.

Nuclear encoded genes						
Protein/enzyme	Gene	Function	Protein location	Cell type	Model plant	References
Rubisco (small subunit)	<i>RbcS</i>	Photosynthetic carbon fixation	Chloroplastic	BS cells	Amaranth, maize, <i>Flaveria</i>	Patel and Berry, 2008
Rubisco Activase	<i>Rca</i>	Carbamylation of a specific lysine residue to convert rubisco from an inactive to an active state	Chloroplastic	BS cells	Amaranth, maize, <i>Atriplex</i>	Salvucci and Ogren, 1995
NAD-MEL	<i>Mel1</i>	Biosynthetic pathways/photosynthetic CO ₂ assimilation	Mitochondrial	BS cells	Amaranth	Long and Berry, 1996
AOX	<i>Aox1</i>	Respiratory electron transport	Mitochondrial	BS cells	Amaranth	Agostino et al., 1996
NADP-ME	<i>ChlMe1</i> , <i>ZnChlMe1</i> <i>ChlMe2</i> , <i>ZnChlMe2</i> <i>CytMe</i>	Biosynthetic pathways/photosynthetic CO ₂ assimilation	Chloroplastic	BS cells	<i>Flaveria</i> / Maize	Lai et al., 2002a
Glycine Decarboxylase (P-subunit)	<i>GLDP</i>	Glycine shuttling in photorespiration	Cytosolic	Leaves and other tissues		
PPDK	<i>Ppdk</i>	Production of phosphoenolpyruvate for initial CO ₂ acceptance	Mitochondrial	BS cells	<i>Flaveria</i>	Englemann et al., 2008
PEPCase	<i>PpcA</i> , <i>PpcZm1</i> <i>PpcB</i> , <i>PpcZm2</i> <i>PpcC</i> , <i>PpcZm3</i>	Initial fixation of CO ₂ into oxaloacetate	Chloroplastic	MP cells	Amaranth, maize, <i>Flaveria</i>	Sheen, 1999
Carbonic anhydrase	<i>CA1</i> <i>CA2</i> <i>CA3</i>	Produces the bicarbonate substrate for PEPCase from CO ₂	Cytosolic	MP cells	<i>Flaveria</i> / Maize	Westhoff and Gowik, 2004
Cholorophyll A/B binding protein	<i>Cab</i>	Binding of chlorophylls A/B in C ₄ plants lacking photosynthesis II activity in BS cells	Chloroplastic	Leaves and other tissues		Tetu et al., 2007
Extrinsic components of the water splitting complex	Genes encoding the 10, 16, and 23 kDa polypeptides	PSII activity/electron transport	Cytosolic	Leaves and other tissues	<i>Flaveria</i>	Sheen, 1999
Some nuclear encoded PSI/II components	<i>PsaD</i> , <i>PsbT</i> , <i>PsbR</i> , <i>PsbO</i> , <i>PsaK</i> , etc.	Electron transport	Cytosolic	MP cells	Maize	Höfer et al., 1991
Chloroplast encoded genes			Chloroplastic	More abundant in MP cells		Furumoto et al., 2000
Protein/enzyme	Gene	Function	Protein location	Cell type	Model plant	References
Rubisco (large subunit)	<i>rbcL</i>	Photosynthetic carbon fixation	Chloroplastic	BS cells	Amaranth, maize, <i>Flaveria</i>	Patel and Berry 2008
Some plastid encoded PSII components	<i>PsbA-F</i>	Electron transport	Chloroplastic	More abundant in MP cells	Maize	Sheen and Bogorad, 1988

cells of C₄ plants. Additional information about the origin, evolution, and regulation of some of these C₄ genes can also be found elsewhere in this volume. The studies reviewed here present an emerging picture of a complex metabolic system made up of numerous interacting proteins and enzymes that are produced from independently regulated genes. These components ultimately function together within and between the different leaf cell types, thereby enabling the characteristic capabilities in plant species that utilize this efficient and highly specialized photosynthetic pathway.

II. C₄ Gene Expression in Bundle Sheath Cells

A. *Rubisco*

Although Rubisco is highly specific to leaf BS cells in C₄ plants, this enzyme serves as a photosynthetic enzyme in all plants, and many aspects of Rubisco gene expression are common to both C₃ and C₄ species. In all plants, Rubisco and its corresponding transcripts are extremely abundant; this enzyme can accumulate to as much as 50% of the total soluble protein in some cells (Ellis, 1979; Mizioroko and Lorimer, 1983; Taylor, 1989; Spreitzer, 1993; Furbank and Taylor, 1995). At the whole plant level, C₄ plants require and accumulate less Rubisco than C₃ plants (approximately 50–80% lower relative to C₃ plants), due to BS cell-specificity and increased CO₂ assimilation efficiency (Hatch, 1987; Furbank and Taylor, 1995; Sage, 2002). The holoenzyme is located within the chloroplasts, where it functions as the principal enzyme of photosynthetic carbon fixation. It is composed of eight large (LSU; 51–58 kDa) and eight small (SSU; 12–18 kDa) subunits (Mizioroko and Lorimer, 1983; Spreitzer and Salvucci, 2002). The LSU, encoded by the plastid *rbcL* gene, is translated on chloroplast ribosomes. Although the *rbcL* gene is present in one copy per molecule of chloroplast DNA, there are many copies of this molecule in each chloroplasts, so that there may be up to several thousand copies of this DNA per cell (Berry et al., 1985). The SSU, encoded by nuclear *RbcS* genes, is translated on cytoplasmic ribosomes as a 20-kDa precursor

that is transported into the plastids via a transit sequence to yield a 12–14 kDa processed protein. *RbcS* genes in most plants occur as a multigene family, with between 2 and 22 *RbcS* genes occurring in a given species (Dean et al., 1989; Wannuer and Gruissem, 1991; Spreitzer, 1993; Ewing et al., 1998; Corey et al., 1999; Sasanuma, 2001; Patel and Berry, 2008). Despite this disparity in the copy number of *rbcL* and *RbcS* genes, similar amounts of each subunit accumulate within a plant cell, and the synthesis of the two Rubisco subunits in the separate cellular compartments is highly coordinated (Berry et al., 1985, 1986; Taylor, 1989; Wang et al., 1993b; Rodermel et al., 1996; Rodermel, 1999; Patel and Berry, 2008). In C₄ and C₃ plants, the expression of both *rbcL* and *RbcS* genes is restricted primarily to leaves and other photosynthetic tissues, although expression of individual members of an *RbcS* gene family can vary within a given tissue, and between different tissues and organs of a single plant (Sugita and Gruissem, 1987; Gruissem et al., 1988; Dean et al., 1989; Wannuer and Gruissem, 1991; Manen et al., 1994; Ewing et al., 1998; Schuster et al., 1999).

Rubisco gene expression is typically highly regulated, and has been shown to be responsive to a variety of influences. In most plants, *rbcL* and *RbcS* mRNA and protein synthesis levels are strongly regulated by light (Berry et al., 1985; Gilmartin et al., 1990; Wang et al., 1993a; Shiina et al., 1998; Sheen, 1999; Zhou et al., 2001), development (Wannuer and Gruissem, 1991; Nelson and Langdale, 1992; Hensel et al., 1993; Jiang et al., 1993; Ramsperger et al., 1996), cell type (Boinski et al., 1993; Furbank and Taylor, 1995; Berry et al., 1997; Kubicki et al., 1994; Sheen, 1999; Majeran et al., 2005; Patel et al., 2004, 2006), photosynthetic metabolism (Sheen, 1990; Jiang et al., 1993; Krapp et al., 1993; Wang et al., 1993b; McCormac et al., 1997; Urwin and Jenkins, 1997; Acevedo-Hernandez et al., 2005), hormones, nitrogen and other nutrients (Furbank and Taylor, 1995; Sheen, 1999; Ookawa et al., 2004; Imai et al., 2005), and even pathogen infection (Bergera et al., 2004). Regulation at the level of transcription has been implicated in all these processes (Nelson et al., 1984; Mullet, 1988; Thompson and Meagher, 1990; Wannuer and Gruissem, 1991; Bansal et al., 1992; Furbank and Taylor, 1995; Urwin and Jenkins, 1997; Cheng et al., 1998; Sheen, 1999; Bergera et al., 2004; Acevedo-Hernandez et al., 2005).

However, it is also clear that many aspects of *rbcL* and *RbcS* gene expression are mediated and coordinated at post-transcriptional levels (Silverthorne and Tobin, 1990; Thompson and Meagher, 1990; Wannuer and Gruissem, 1991; Boinski et al., 1993; Gallie, 1993; Gillham et al., 1994; Rodermel et al., 1996; Roth et al., 1996; Berry et al., 1997; Shiina et al., 1998; Brutnell et al., 1999; Schuster et al., 1999; McCormac et al., 2001; Sinha et al., 2002; Patel et al., 2004, 2006), often through mRNA processing (degradation or stabilization of transcripts) and regulation of translation. Since many of these regulatory aspects of Rubisco gene expression have been demonstrated in both C_4 and C_3 species, they likely represent conserved processes that have been retained during the molecular evolution and development of C_4 photosynthesis. Thus, in C_4 plants, as in C_3 plants, Rubisco gene expression occurs mostly in leaves, requires illumination for continuous production of the *rbcL* and *RbcS* transcripts and proteins, and is highly responsive to internal and external signals. The distinguishing aspect of Rubisco genes in C_4 leaves is the additional restriction in their expression that results in the BS cell-specific localization of this enzyme.

1. Rubisco Gene Expression and C_4 Leaf Development

In many C_4 plants, Rubisco gene expression occurs initially in a C_3 -like pattern, so that *rbcL* and *RbcS* mRNAs and their corresponding subunits are present in both BS and MP cells, which are morphologically distinguishable before the establishment of full cell-type specificity (Wang et al., 1992, 1993b; Berry et al., 1997; Nelson and Langdale, 1989, 1992; Dengler and Nelson, 1999). This early non cell-type specific stage in Rubisco gene expression is considered to be a “default” state, which is later modified to the more specialized “ C_4 -type” pattern. Rubisco gene expression becomes BS cell-specific during leaf maturation, so that as growth proceeds toward full expansion, the Rubisco mRNAs and subunits become localized exclusively to only this one leaf cell type. The progression of the “ C_3 -to- C_4 transition” in Rubisco gene expression has been determined from investigations of early photosynthetic gene expression in two dissimilar C_4 plants, the dicot amaranth and the monocot maize. A comparative review of these

studies (summarized in Table 2) reveals that, in addition to basic differences in dicot and monocot leaf development, these plants also differ in many aspects of early Rubisco gene expression, including patterning and signaling processes involved in the establishment of BS cell-specificity. A comparison of these expression patterns also suggests underlying similarities in the regulation of Rubisco genes in these two C_4 species.

2. Rubisco Gene Expression Patterns in a C_4 Dicot

Rubisco gene expression in amaranth and other C_4 dicots must become established within the framework of normal dicot leaf development. Dicot leaves do not possess a strict developmental gradient along their length (Steeves and Sussex, 1989). These leaves are typically made up of multiple cell lineages that form a loose gradient from the base of the leaf outward towards the edge, with only limited polarity in terms of cell age and development. At any stage of development, nonsynchronous periclinal and anticlinal cellular divisions and expansions generate a mosaic of cells with considerable variations in volume and developmental age throughout the entire leaf. Within this developmental context, expression from both *RbcS* and *rbcL* genes can be observed from the earliest stages of leaf formation. Expression occurs in the apical meristem, in emerging leaf primordia, and in very early leaves when vascular centers are still differentiating (Ramsperger et al., 1996). As the dicot leaf expands, patterns of Rubisco mRNA and protein accumulation undergo significant changes (Wang et al., 1992, and Fig. 1a–d). Following these changes using in situ hybridization and immunolocalization analyses has provided clues about levels of regulation and other processes that determine where Rubisco gene expression occurs in C_4 leaves.

What is most notable about the very early stages is that while *rbcL* and *RbcS* mRNAs accumulate in many tissues and cell types, in some of these locations they are apparently not utilized for synthesis of the LSU and SSU proteins (Ramsperger et al., 1996). For example, the Rubisco mRNAs were found to be very abundant in the apical meristem dome and in all cells of the leaf primordia, whereas Rubisco polypeptides show a more localized pattern of accumulation, appearing only

Table 2. Cell type-specific regulation of key C₄ enzymes in representative dicot (amaranth) and monocot (maize) species. Basic information about the C₄ regulation of these genes is given in the table for comparative purposes. More detailed information about the regulation of the C₄ genes listed, and any non-photosynthetic orthologs, can be found in the text. Initial or primary references are given in the table; additional references related to some of the genes listed can be found in the text.

Gene	Plant	Regulation signal	Cell-type specific regulation	C ₄ regulation pattern	References
Rubisco (<i>rbcL</i> and <i>RbcS</i>)	Amaranth	Regulation by light, developmental, and photosynthetic signals	Transcriptional and post-transcriptional mRNA repression in MP cells or activation in BS cells	Initially equal in BS and MP cells (C ₃ -like default pattern), becomes BS cell-specific in response to developmental signals	Patel and Berry, 2008
	Maize			Initially C ₃ -like default pattern, becomes BS cell-specific in response to light	
PEPCase (<i>Ppc</i>)	Amaranth	Regulation of expression by light and developmental signals	Transcriptional or post-transcriptional control of PEPCase mRNA accumulation	Transcript levels equal in BS and MP cells precursors early in development, polypeptide is MP cell type-specific throughout development	Berry et al., 1997
	Maize			Accumulation of mRNA and protein in MP cells lags behind Rubisco along the developmental leaf gradient	Sheen, 1999
<i>Ppdk</i>	Amaranth	Developmental and light-inducible regulation of expression	Transcriptional regulation; repression of <i>Ppdk</i> mRNA accumulation in BS cells	Initially equal transcript levels in BS and MP cells, becomes MP cell-specific during leaf expansion; protein MP cell type-specific throughout development	Berry et al., 1997
	Maize			Accumulation of mRNA and protein in MP cells lags behind Rubisco along the developmental leaf gradient	Sheen, 1999
NAD-ME	Amaranth	Tissue, cell type, and light mediated expression signals	Transcriptional or post-transcriptional regulation of <i>Ppdk</i> mRNA accumulation	mRNA and protein are specific to BS cells throughout development	Long and Berry, 1996
NADP-ME	Maize	Light regulated cell type-specific expression	Transcriptional or post-transcriptional control of mRNA accumulation	Expressed at low levels in embryos, light causes BS cell type-specific expression in older maize leaves	Tausta et al., 2002

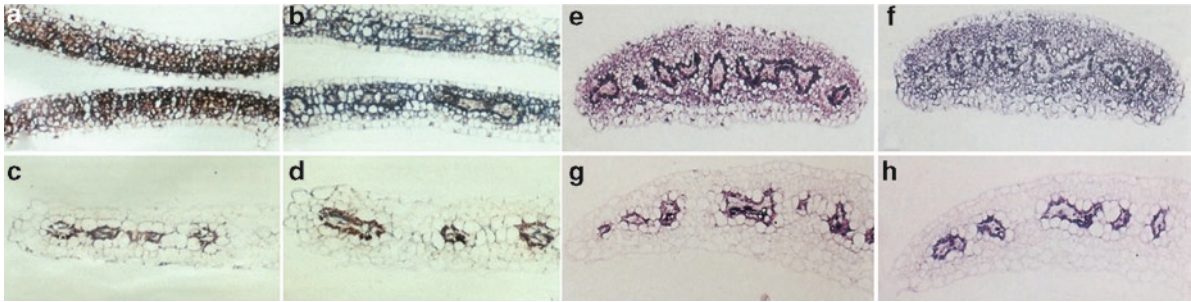


Fig. 1. Developmental C_3 -to- C_4 transition in Rubisco gene expression. Sections from developing amaranth leaves (Panels a–d, on left) or cotyledons (Panels e–h, on right) were hybridized with antisense *RbcS* and *rbcL* probes for in situ hybridization. Panels (a) and (b) show *RbcS* and *rbcL* mRNA, respectively, present in both BS and MP cells of early 5 mm long leaves. Panels (c) and (d) show the *RbcS* and *rbcL* transcripts become specific to BS cells in older 10 mm leaves. Panels (e) and (f) show *rbcL* and *RbcS* transcripts, respectively, present in BS and MP cells of 2 day-old cotyledons. In the 5 day-old cotyledons shown in panels (g) and (h), transcripts for *rbcL* and *RbcS*, respectively, have become specifically localized to BS cells (Modified from Wang et al., 1992, 1993a).

in ground meristem cells within the primordia, and not at all in the apical dome or in the prevascular procambium. The lack of correlation between the accumulation patterns of the transcripts and their corresponding polypeptides is indicative of post-transcriptional regulation, most likely at the level of translation. The Rubisco transcripts continue to show very little cell-type specificity as leaf expansion continued to 2 mm in length. At this stage some differentiating structures (such as vascular centers) are starting to become recognizable, although BS and MP cells are not yet morphologically distinguishable. *rbcL* and *RbcS* mRNAs accumulate in both MP and BS precursor cells within these very young leaves, while LSU and SSU polypeptides accumulate only in BS cell precursors. It is likely that selective post-transcriptional repression of the Rubisco mRNAs in the MP cell precursors, or possibly their selective activation in BS cell precursors, is already established in the 2 mm amaranth leaves even before differentiation of the two Kranz cell types is completed.

The next major step in the Rubisco developmental sequence is apparent when the developing C_4 dicot leaves are approximately 5 mm in length (Wang et al., 1992, 1993b). This stage shows leaves with fully developed Kranz anatomy, and Rubisco mRNAs equally abundant in both BS and MP cells in the “ C_3 -like” default pattern (Fig. 1a and b). Unlike in the MP precursors of the younger 2 mm leaves, it is clear that some Rubisco polypeptides have accumulated in the morphologically differentiated MP cells of these

older leaves, although at relatively lower amounts than in the BS cells. We speculate that the abundant Rubisco mRNAs present in the MP cells of 5 mm leaves might allow a portion of them to overcome the translational repression or lack of activation occurring in the younger leaves. This would lead to the presence of Rubisco polypeptides in both C_4 cell types at this developmental stage.

The C_4 Rubisco developmental sequence in amaranth is completed during the period when the leaves expand from 5 to 10 mm in length (Wang et al., 1992, 1993b). At this time the leaves simultaneously undergo two developmental transitions, a C_3 -to- C_4 transition in which the Rubisco polypeptides and mRNAs completely disappear from the MP cells, thereby becoming specific to BS cells, and a sink-to-source transition in carbon transport. These two developmental transitions initiate at the leaf tip and progress rapidly and coordinately in the basipetal (apex to base) direction. The tight correlation between the two transitions is striking, suggesting a link between the establishment of BS cell-specific Rubisco gene expression and the onset of photosynthetic activity and vascular transport in these C_4 dicot leaves. Both transitions are completed by the time the leaves have reached 10 mm in length, at approximately one-tenth of their final expansion (Fig. 1c and d). The leaves then remain in the C_4 state with regard to Rubisco polypeptide and mRNA localization throughout maturity (Wang et al., 1992, 1993b; Boinski et al., 1993).

Post-embryonic amaranth cotyledons also possess characteristic Kranz-type anatomy and undergo a C₃-to-C₄ transition in Rubisco gene expression that is nearly identical to true leaves. (Fig. 1e–h). Morphologically distinguishable BS and MP cells are already present in cotyledons when they first emerge from the seed coat, about 2 days after planting. Rubisco mRNAs and proteins are also present in the cotyledons at germination, initially accumulating in both BS and MP cells in a C₃-like pattern. These became progressively more localized to BS cells in the typical C₄-type pattern as the cotyledons expand over 2–7 days, becoming fully BS specific by 7 days after planting (Wang et al., 1993a). Unlike leaves, cotyledons do not undergo a basipetal sink-to-source transition. The cotyledons serve as a storage organ during early seed germination, and transition to serve as photosynthetic organs during the period between seed germination and the complete photosynthetic development of the first true leaf. It is during the transition to photosynthetic function that the Rubisco mRNAs and proteins become specific to BS cells, and this occurs uniformly throughout the cotyledon (Wang et al., 1993a). It is possible that the C₃-to-C₄ transition occurs in amaranth cotyledons because C₄ photosynthetic capacity is required during the time when cotyledons are functioning as the only photosynthetic organs of the young seedlings. As with leaves, this transition in Rubisco gene expression is not correlated with a developmental gradient in terms of cell age; in dicots, the cotyledons form from cell divisions that take place during embryogenesis in developing seeds (Scott and Possingham, 1982; Meinke, 1992). Cotyledon development stops at seed desiccation, and resumes during germination. Post-embryonic growth and development of cotyledons results from cell expansion that occurs in the absence of cell division.

Light affects many aspects of leaf development and Rubisco gene expression in all plants (Berry et al., 1985; Gilmartin et al., 1990; Fankhauser and Chory, 1997; Shiina et al., 1998; Sheen, 1999; McCormac et al., 2001; Zhou et al., 2001; Tang et al., 2003). Because true leaves do not develop in dark-grown (etiolated) amaranth plants, the role of light in determining BS-specificity for *rbcL* and *RbcS* gene expression was investigated in the cotyledons. Kranz anatomy still develops in etiolated cotyledons, and the Rubisco mRNAs

and subunits initially accumulate in both MP and BS cells in the default “C₃-like” pattern, but at reduced levels relative to cotyledons of light-grown seedlings (Berry et al., 1985; Wang et al., 1993a). In the absence of any illumination, these less abundant Rubisco mRNAs and proteins still undergo a C₃-to-C₄ transition in cell specificity, becoming fully localized to the BS cells by 7 days after planting. It is apparent that the leaves and postembryonic cotyledons of amaranth share a common developmental program of C₄ Rubisco gene expression during maturation. Moreover, light is not a requirement for the establishment of BS cell-specificity for genes encoding the two Rubisco subunits in this C₄ dicot.

3. Rubisco Gene Expression Patterns in a C₄ Monocot

Comprehensive studies of expression patterns for genes encoding Rubisco and other photosynthetic enzymes within the framework of maize C₄ leaf development have been discussed in several previous reviews (Nelson and Langdale, 1989, 1992; Langdale and Nelson, 1991; Dengler and Nelson, 1999). Here we present a summary of Rubisco gene expression patterns in developing maize leaves, focusing on changes in expression that occur during the process of monocot leaf development.

Leaf development in the C₄ monocot maize follows a pattern that is typical of most grasses, with cells originating primarily from divisions occurring at an intercalary meristem located at the leaf base, although some divisions also occur throughout the leaf itself during early expansion. Cell divisions gradually become more restricted to the basal meristem as leaf expansion progresses, and are entirely basal by the time the leaf has reached its full expansion (Steeves and Sussex, 1989; Sylvester et al., 1990; Nelson and Langdale, 1992). Taken as a whole, a C₄ monocot leaf is comprised of a mostly linear developmental gradient of cells, with younger cells occurring at the base and older cells at the tip, with much more polarization and uniformity than a C₄ dicot leaf.

In maize plants grown under normal illumination, *rbcL* and *RbcS* mRNAs are first detectable in the leaf primordium before the morphological differentiation of BS cells, and before chloroplasts are discernable. These initial Rubisco transcripts

occur in a ring of BS precursor cells that surround the provascular centers, suggesting that processes affecting the cell-type specific expression of the maize *rbcL* and *RbcS* genes are already active at this early stage, preceding vascular differentiation and BS cell morphogenesis. The accumulation of the Rubisco transcripts in the BS cell precursors of the leaf primordia, and in new cells near the basal meristem, occurs before the appearance of their corresponding proteins, so that the transcripts are not immediately utilized for synthesis of the LSU and SSU proteins (Nelson et al., 1984; Nelson and Langdale, 1992). Non-synergistic accumulation of the Rubisco mRNAs and proteins continues along the length of the maize leaf developmental gradient; these mRNAs show maximum accumulation in regions near the leaf base, while their encoded proteins reach maximum levels at the tip regions. As leaf development progresses in illuminated maize plants, Rubisco proteins and mRNAs remain highly cell-type specific, accumulating only in the BS precursors and cells, with no accumulation detected in the MP precursors or cells.

Although monocot leaves do not undergo a sink-to-source transition like that of dicot leaves, maize leaves still can undergo a similar C_3 -to- C_4 transition in Rubisco gene expression. Leaves will develop on etiolated maize seedlings, and Rubisco mRNAs and proteins accumulate, but at greatly reduced levels relative to light grown plants (Sheen and Bogorad, 1985, 1986b). Unlike light-grown maize leaves, *rbcL* and *RbcS* genes in the etiolated leaves do not show any C_4 cell type-specific expression; the low levels of mRNAs and proteins within etiolated leaves are present in both BS and MP cells (Sheen and Bogorad, 1985, 1986b, 1987a, b; Langdale et al., 1988; Nelson and Langdale, 1992). When etiolated maize plants are transferred to light, the expression of *rbcL* and *RbcS* genes become greatly enhanced in BS cells and suppressed in MP cells, thereby establishing C_4 patterns of Rubisco mRNA and protein accumulation within 12 h of illumination (Nelson et al., 1984; Sheen and Bogorad, 1987a, b). Furthermore, transient expression analysis using a maize *RbcS-m3* reporter gene construct has shown that for at least one member of the maize *RbcS* gene family, the light-mediated C_3 -to- C_4 transition in Rubisco gene expression is mediated by two specific photoreceptors. One of these is phytochrome, which stimulates expression in BS

cells, and the other is blue light, which suppresses expression in MP cells (Purcell et al., 1995). Thus for maize leaves, illumination is a primary determining factor in the shift from the initial " C_3 -like" default state to the more specialized " C_4 type" expression pattern. This implies that specific photoreceptors, or possibly light-associated photosynthetic processes, are involved in the establishment of cell-type specific gene expression in leaves of this C_4 monocot.

4. Rubisco Gene Expression in Different C_4 Species

The divergent C_4 patterns of Rubisco gene expression observed during leaf development in amaranth and maize are not necessarily representative of all monocot and dicot C_4 species. While some plants undergo a C_3 -to- C_4 transition, species-specific variations in the timing and establishment of C_4 specificity have been reported. For example, in cotyledons of the C_4 dicot *F. trinervia*, the overall timing in the C_3 -to- C_4 transition for *RbcS* mRNA accumulation is very similar to that observed in amaranth, with full BS specificity observed by 7 days after planting (Shu et al., 1999). However, in etiolated *F. trinervia*, differentiation of the BS and MP cells is arrested at 4 days post-planting, and *RbcS* mRNAs remain localized to both cell types through day 7. Thus for this C_4 dicot, illumination is required for full differentiation of the two C_4 cell types, and for the establishment of BS cell-specific *RbcS* gene expression. This light-associated developmental pattern is more reminiscent of maize than amaranth. Such variation likely results from differences in embryonic and post-embryonic cotyledon development between the two dicot species, as well as differences in the timing of photosynthetic development (Shu et al., 1999). During leaf development in *Atriplex rosea*, another C_4 dicot, Rubisco protein accumulates in the ground meristem of the leaf primordia, as it does in amaranth leaf primordia (Dengler et al., 1995). However, this accumulation appears to be only transitory, and does not carry over into later stages of leaf development. As morphogenesis progresses, the next detectable accumulation of the Rubisco protein is highly specific to leaf BS cells, coinciding with the structural differentiation of these cells, so that in this plant accumulation of the Rubisco enzyme does not go through an

early C₃-like stage, remaining highly specific to BS cells throughout development.

A dramatic change in C₃ and C₄ states with regards to Rubisco localization is found in the monocot sedge *Eleocharis vivipara*. Within the mature internodal region of the culm (the photosynthetic organs of this plant), this semi-aquatic species shows C₄-type BS cell-specific Rubisco accumulation in its terrestrial form, and C₃-like accumulation in a submerged form (Uchino et al., 1998; Ueno, 2001). In the submerged form, *RbcS* mRNA and its corresponding protein were found to accumulate initially in both BS and MP cells, and this C₃-like pattern was maintained throughout the development and maturation of these tissues. In the terrestrial form, this same C₃-like pattern was observed during early developmental stages in the immature internodal regions; the C₄-type *RbcS* expression pattern becomes established in coordination with the morphological development of BS cells during maturation of the terrestrial internodes. However, when the terrestrial form is submerged, a transition region is formed in newly emerging culms. Within this region, C₄ cell type-specific *RbcS* expression (as well as cell type-specific expression of genes encoding the MP cell-specific PEPC) is still maintained, even though the development of BS cells is repressed (Uchino et al., 1998). These findings suggest that the cell-type specific gene expression of Rubisco and other photosynthetic enzymes is separable from the formation of Kranz anatomy in this unique C₄ species. Patterns of C₄ mRNA and protein accumulation have been found to correlate in the two forms of this plant, although the molecular processes involved in this transition are not yet known (Ueno, 2001).

Most likely there are many such variations in the establishment of BS-specific Rubisco localization within the C₄ dicot and monocot groups (Dengler et al., 1995; Berry et al., 1997; Shu et al., 1999). Variations in the establishment of BS cell-specific *rbcL* and *RbcS* gene expression could be due the activities of multiple independent molecular recognition and regulatory processes that have arisen during the evolution of the C₄ pathway in different species. Alternatively, these differences may represent diversity in the manifestations of the same processes within the framework of each species' distinct developmental programs, or within the context of each species' environmental parameters.

B. Malic Enzyme Genes

Malic enzymes catalyze the conversion of malate into pyruvate, which results in the release of CO₂. These enzymes occur in all organisms and serve in a variety of metabolic pathways and house-keeping functions. Three classes of malic enzyme have been identified, two of which occur in plants (Gutierrez et al., 1974; Artus and Edwards, 1985; Wedding, 1989; Edwards and Andreo, 1992; Drincovich et al., 1998, 2001); one of the plant groups, NAD-ME (EC 1.1.1.39), utilizes NAD as a cofactor and is localized to the mitochondria. The other group, NADP-ME (EC 1.1.1.40) uses NADP as a cofactor and has both cytosolic and chloroplastic forms. Malic enzymes can occur throughout all tissues of the plant, where they have become incorporated into a broad range of cellular processes. Some examples include providing supplemental pyruvate for the tricarboxylic acid cycle and other biosynthetic pathways, maintenance of cellular pH and ionic balance, inducing osmotic changes that cause movement of guard cells at stomatal openings, and generation of metabolites required for pathogen defense (Artus and Edwards, 1985; Wedding, 1989; Edwards and Andreo, 1992; Drincovich et al., 2001). In addition to their roles as metabolic enzymes, malic enzymes have been recruited in some plants to function in certain specialized processes, such as thermogenesis in species of the Araceae (Chivasa et al., 1999). The best-known specialization of malic enzyme function occurs in C₄ plants, in which NADP-ME or NAD-ME forms of malic enzyme have acquired a principle role in photosynthetic CO₂ assimilation. Within the organelles of BS cells, photosynthetic malic enzymes decarboxylate the C₄ acid malate to pyruvate, thereby releasing CO₂ for re-fixation by Rubisco. In accordance with this C₄ specialization, genes encoding these malic enzymes are expressed primarily in leaves and cotyledons, where they are highly BS cell-specific, and much more abundant than their non-photosynthetic counterparts.

1. NAD-Dependent Malic Enzyme

The C₄ NAD-ME of amaranth is composed of two 65 kDa subunits (NAD-MEL) and two 60 kDa subunits (NAD-MES) (Long et al., 1994). Both subunits are encoded in the nucleus and

targeted to the mitochondria. Southern analysis indicates that a gene encoding the NAD-MEL subunit (*Mel1*) is present as a single copy in the amaranth genome (Rydzik et al., 1996). Although the Rubisco SSU and MEL proteins are targeted to different compartments within leaf BS cells, these enzymes catalyze consecutive decarboxylation and CO₂ fixation steps of the amaranth C₄ pathway. Thus, it might be expected that the regulation of the nuclear *Mel1* and *RbcS* genes would be highly coordinated during leaf development. In fact, even though the C₄ NAD-ME of amaranth leaves most likely evolved from a non-photosynthetic form of the enzyme, the *Mel1* gene has acquired many tissue-specific, cell-type specific, and light-mediated expression patterns that enable its function in the C₄ pathway (Long et al., 1994; Long and Berry, 1996). Like *RbcS*, *Mel1* expression is most abundant in photosynthetically active leaves and cotyledons, and much less abundant (but still detectable) in roots, stems, petioles, and flowers, which have little or no photosynthetic activity. In addition, expression levels of genes encoding both of these BS cell-specific enzymes is strongly regulated by light; *Mel1* transcripts cannot be detected in etiolated amaranth cotyledons. Within light-grown leaves and cotyledons, the abundant *Mel1* mRNAs and protein exhibit full BS cell-specificity. However, during early leaf development, the initial accumulation of *Mel1* mRNA and protein occurs later than *RbcS* mRNAs and protein (which are present at all stages of early leaf development), and is highly specific to BS cells from their earliest detection (Berry et al., 1985; Long and Berry, 1996). Most notably, *Mel1* gene expression does not correlate with the dicot leaf carbon sink-to-source transition, and does not undergo a developmental C₃-to-C₄ transition like that observed for Rubisco. Such findings indicate that, while nuclear genes encoding these two BS cell-specific C₄ enzymes share some regulatory characteristics, it is likely that independent processes are involved in the establishment of their characteristic cell-type specificity during leaf development.

Within mature amaranth leaves, C₄ regulation of *Mel1* expression leads to very tight BS-specificity for the NAD-ME enzyme; no immunofluorescence or immunogold labeling was found outside of the BS cell mitochondria (Long et al., 1994). This is somewhat surprising, considering the

ubiquitous role of this enzyme in many basic cellular processes. Lower levels of *Mel1* expression in other tissues, such as stems and petioles, may be required to support low levels of photosynthetic activity in these tissues. However, this explanation does not account for the small amount of transcript detected in roots and flowers. It is not yet known if low abundant *Mel1* mRNAs in non-leaf/cotyledon tissues results from basal levels of expression from the same gene used in C₄ photosynthesis, or possibly normal levels of a related non-photosynthetic NAD-ME gene (ancestral, not detected in previous Southern analysis) that is required for metabolic activity within these tissues. To date, expression of genes encoding the ME small subunit has not been examined.

2. NADP-Dependent Malic Enzyme

There are three nuclear-encoded isoforms of NADP-ME in C₄ dicot *Flaveria* species, distinguishable by their molecular masses of 62, 64, and 72 kDa (Drincovich et al., 1998; Lai et al., 2002a, b). The plastidic C₄ isoform is encoded by *ChlMe1*, while another non-C₄ plastidic isoform is encoded by *ChlMe2* (Marshall et al., 1996; Lai et al., 2002b). The third isoform is cytosolic and encoded by a small *CytME* gene family (Lai et al., 2002a). It has not yet been firmly established which gene encodes which isoform, although a likely arrangement is that the 62, 64, and 72 kDa polypeptides are encoded by *ChlMe1*, *ChlMe2*, and *CytMe*, respectively (Lai et al., 2002a). The three isoforms are also found in C₃ and C₃-C₄ intermediate *Flaveria* species, indicating that all three NADP-ME gene classes are expressed in these plants (Marshall et al., 1996, 1997; Drincovich et al., 1998; Lai et al., 2002a, b).

A series of studies (Marshall et al., 1996, 1997; Lai et al., 2002a, b) have provided insights into the origins of the three NADP-ME isoforms, and how their regulation patterns differ in C₄, C₃, and C₃-C₄ intermediate species of *Flaveria*. These studies used quantitative RT-PCR to measure overall mRNA levels, as well as analysis of reporter gene expression driven by *F. trinervia*-derived promoters of the three NADP-ME genes expressed in transgenic *F. bidentis* (these are both C₄ species). It was shown that the genes encoding the three forms have distinct expression patterns that are reflective of their individual functions

(Lai et al., 2002a, b). During leaf development in C₄ *Flaveria*, expression of the C₄ *ChlMe1* gene increased in coordination with the morphological differentiation of BS cells and the beginning of C₄ competency. There is an early transient peak of *ChlMe1* mRNA accumulation in very young C₄ leaves, followed by a much greater increase in accumulation at later stages of leaf development. In the transgenic C₄ plants it was observed that near the base of the leaf, where *ChlMe1* expression was lower, some reporter gene product was present in MP cells, although much less than in BS cells. Overall levels of expression, and specificity to BS cells, increased basipetally in coordination with maturation of BS cells, with both reaching their highest at the leaf apex. Studies of light-mediated expression, using cotyledons of transgenic *F. bidentis*, indicated low levels of *ChlMe1* expression in the absence of light, occurring preferentially in BS cells, but also with reduced amounts in MP cells. Upon illumination, overall levels of expression from the *ChlMe1* reporter gene construct increased, as well as specificity of expression to the cotyledon BS cells. Thus, it appears light has a role in the establishment of full BS cell-specificity for *ChlMe1* expression, possibly working in cooperation with other developmental processes (Lai et al., 2002b).

ChlMe2, encoding the non-C₄ plastidic isoform, is also expressed in C₄ leaves and cotyledons, but in different pattern than *ChlMe1* (Lai et al., 2002b). Expression from the *ChlMe2* promoter is highest in cells with the greatest number of chloroplasts, but was not specific to BS cells at any time during leaf or cotyledon development. In very young expanding leaves from transgenic *F. bidentis* plants, overall levels of expression from a reporter gene construct driven from the *F. trinervia* *ChlMe2* promoter were found to be highest at the base of the leaf, and then decrease basipetally to be lowest near the apex, a pattern opposite that observed from the *ChlMe1* promoter. As the leaves matured, there was an overall reduction in *ChlMe2* expression, which also became more uniform throughout the leaf. The differences in expression patterns for genes encoding these two isoforms suggests that they have separate, non-overlapping functions in the leaves of C₄ *Flaveria* species, with *ChlMe1* serving exclusively as a photosynthetic enzyme in BS cells, and *ChlMe2* performing non-photosynthetic functions related

to more basic leaf processes (Marshall et al., 1996; Lai et al., 2002b).

Genes encoding all three NADP-Me isoforms are expressed in C₃ species of *Flaveria* (Lai et al., 2002a, b). In the C₃ species, *ChlMe1* expression occurs only briefly during early leaf development, possibly serving a role in early, pre-photosynthetic chloroplast respiration (Lai et al., 2002b). Interestingly, the early transient peak of *ChlMe1* expression in C₄ *Flaveria* leaves provides evidence that this same enzyme may also perform this same pre-photosynthesis role before its expression becomes enhanced and BS cell-specific. The three classes of NADP-ME genes are expressed in non-leaf tissues as well. Transcripts for the cytoplasmic *CytMe* are found in all tissues of the plants, while *ChlMe1* and *ChlMe2* have distinct expression patterns in meristems and in non-photosynthetic tissues. These expression patterns are likely reflective of the individual functions of each isoform within these tissues. Overall, expression patterns of *ChlMe2* and *CytMe* genes do not differ much between the C₄ and C₃ species, indicating that they have been retained to perform their basic metabolic roles in the C₄ *Flaveria*. In leaves, *ChlMe2* likely has a role in regulation of malate metabolism during plastid development, while *CytMe* appears to have multiple functions, including response to wounding and maintaining cellular pH. The presence of multiple transcripts for *CytMe* appears to correlate with its different metabolic functions in both C₃ and C₄ *Flaveria* species.

Expression of the nuclear-encoded NADP-ME genes in maize also shows BS cell-specific, light-regulated expression (Sheen and Bogorad, 1987b; Tausta et al., 2002). Like C₄ *Flaveria*, maize expresses two classes of plastidic NADP-ME genes; *ZmChlMe1* encodes a C₄ isoform, and *ZmChlMe2* encodes a non-photosynthetic form (Tausta et al., 2002). *ZmChlMe1* is expressed very abundantly in leaves, and is specific to BS cells. This C₄ gene is not expressed in non-photosynthetic tissues such as roots or endosperm, but is expressed at low levels in embryos. Like most C₄ genes, *ZmChlMe1* is regulated by light, but only in older maize leaves. Light does not increase expression in very young maize leaves, or in illuminated roots. *ZmChlMe2* is expressed in nearly all tissues and at all developmental stages, except for leaf BS cells. As proposed for the non-photosynthetic

plastid NADP-ME isoform in *Flaveria*, *ZmCIME2* is likely to have roles in several cellular processes that involve the metabolism of malate, while the photosynthetic form serves the specialized function of decarboxylation of malate in BS cell chloroplasts for refixation by Rubisco.

The presence of genes encoding multiple NADP-ME isoforms in *Flaveria* and maize has provided clues as to the origin of C_4 photosynthetic function for this enzyme (Marshall et al., 1996; Drincovich et al., 1998, 2001; Lai et al., 2002a, b; Tausta et al., 2002). The proteins encoded by these genes are all very similar, so that NADP-MEs within the genus *Flaveria* share approximately 76% identity at the amino acid level (Lai et al., 2002a, b). The major difference is that *CytMe* encoded proteins lack the plastid transit sequence. It is proposed that the cytosolic NADP-ME is the ancestral isoform, which has been retained with mostly conserved metabolic functions in all plants. According to this model, a plastidic form of this enzyme was produced as a result of duplication of the cytosolic NADP-ME gene, and subsequent acquisition of a plastid transit sequence, possibly by recombination. This plastid form has also been retained as a basic metabolic enzyme in all plants. A second duplication of the plastidic NADP-ME appears to have occurred in C_4 plants, allowing a copy of this gene to attain new C_4 regulatory elements, leading to the C_4 photosynthesis-associated properties of enhanced expression, light regulation, and specificity of expression to leaf BS cells. In addition to attaining novel molecular regulatory properties, the coding region of this C_4 NADP-ME also appears to have been altered, producing an enzyme with new properties, the most notable being its higher specific activity (Drincovich et al., 1998, 2001). It is interesting to note that genes encoding the ancestral cytosolic NADP-ME in some C_3 species exist as multigene families (for example, in C_3 *Flaveria* and *Arabidopsis*), while in C_4 *Flaveria* and maize there is only one copy of this gene (Lai et al., 2002a, b; Tausta et al., 2002). One possible reason for a reduction in copy number for the ancestral gene during the evolution of C_4 capability is that this might simplify the regulation of multiple NADP-ME genes in the presence of more complex malate metabolism associated with the C_4 pathway (Lai et al., 2002a).

C. Genes Encoding Other BS Cell-Specific Proteins

It is important to consider that, in addition to the abundantly expressed BS-cell specific photosynthesis genes reviewed above, there are other less-well characterized genes encoding protein products that accumulate specifically or preferentially in BS cells. In terms of overall gene expression, proteomic, cDNA, and microarray analyses of proteins or mRNAs unique to C_4 BS or MP cells have indicated there is more commonality than diversity in gene expression for the two cell types, and this can vary significantly between different C_4 plants (Harrison and Black, 1982; Wyrich et al., 1998; Furumoto et al., 2000; Sugiharto et al., 2002; Majeran et al., 2005). Recent microarray analysis has indicated that approximately 18% of maize genes may be differentially expressed in the two cell types (Sawers et al., 2007). In general, it appears that there are fewer BS-specific proteins than MP-specific proteins accumulating in C_4 leaves.

A recent study has shown that the P-subunit of glycine decarboxylase (GDC) is specific to BS cells and vascular bundles of *F. bidentis* leaves (Engelmann et al., 2008). This protein is an essential component of the four-subunit photorespiratory GDC, and serves as the actual decarboxylating subunit. GDC activity is present in C_4 plants and is highly specific to BS cells. This enzyme functions in glycine shuttling, so that CO_2 released by the oxidation of glycine in BS cells can be reutilized within these cells. A characteristic of C_3 - C_4 intermediate plant species is that they also have GDC activity confined to BS cells, whereas in C_3 plants this enzyme functions in all photosynthetic cells (Engelmann et al., 2008). It is thought that the establishment of BS-specificity for GDC, as occurs in the C_3 - C_4 intermediate species, may be a common initial step in the multi-lineage evolution of the C_4 pathway. However, it is not universally accepted that the localization of GDC to BS cells is actually an essential first step in C_4 evolution; it is possible that this is a secondary effect of the process (Edwards et al., 2001; Sage, 2004; Engelmann et al., 2008). In either case, the acquisition of BS specificity for the P-subunit is likely the first step in the relocation of GDC activity to BS cells in *F. bidentis*, mediated by

promoter elements that repress expression in MP cells and promote expression in BS cells (Engelmann et al., 2008).

As expected, proteins directly related to Rubisco function, such as Rubisco activase, localize to the BS chloroplasts (Salvucci and Ogren, 1995). Many other proteins showing specificity to BS cells are also localized to chloroplasts, and are related to pathogen defense, redox regulation, starch synthesis, transport of metabolites, and many other functions. In the C₄ monocots maize, sorghum, and sugarcane, many stress-response genes were found to be expressed only in BS cells, most notably genes involved in oxidative or drought stress (Wyrich et al., 1998; Furumoto et al., 2000; Sugiharto et al., 2002; Majeran et al., 2005), leading to the conclusion that BS cells are the major site of stress response in C₄ leaves. In C₄ plants such as amaranth, the NAD-ME colocalizes with alternative oxidase (AOX) within the mitochondria of leaf BS cells (Agostino et al., 1996). In C₄ plants, photosynthesis-related malate decarboxylation occurs at levels in excess of what is required for ATP synthesis, and the alternative respiration pathway allows decarboxylation to continue without limitations that would result if respiration was entirely dependent on electron flow through the cytochrome chain (Chivasa et al., 1999). Thus the coupling of AOX and NAD-ME in BS mitochondria is essential for the function of this type of C₄ pathway, and it might be expected that the expression of genes encoding C₄-related forms of these two enzymes would be highly coordinated. An interesting example of protein isoforms with specificity to both BS and MP cells is the α -glucan phosphorylases of maize (Mateyka and Schnarrenberger, 1988). Isoform II is found exclusively in the chloroplasts of BS cells, while the other cytosolic form I is present only in MP cells. An examination of the regulatory properties of genes encoding these two isoforms may provide insights about how opposing regulatory attributes are distributed to different but related genes to achieve specificity to each leaf cell type.

These additional examples of BS cell-specificity are indicative of a diversity of gene expression that ultimately has allowed for a select group of proteins to become localized specifically to this one leaf cell type, thereby ensuring their C₄-associated functions.

III. C₄ Gene Expression in Mesophyll Cells

A. PEPC and PPdK

The photosynthetic forms of phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPdK) are specific to the MP cells of C₄ plants. Both serve key functions in the CO₂ assimilation pathways of all known C₄ species, and have been extensively studied in both monocot and dicot species. PEPC is the first enzyme to fix CO₂ into an organic form within the leaf MP cells, combining solubilized CO₂ (in the form of HCO₃⁻ produced from carbonic anhydrase) with phosphoenolpyruvate (PEP) to form the C₄ acid oxaloacetate. PEPC is composed of four identical subunits approximately 110 kDa in size that are encoded in the nucleus and located in the cytoplasm (Hudspeth and Grula, 1989; Nelson and Langdale, 1992). PPdK produces PEP, which serves as the initial CO₂ acceptor molecule in C₄ plants. This nuclear-encoded C₄ enzyme is composed of four subunits approximately 100 kDa in size, and is located in the MP chloroplasts (Matsuoka et al., 1988; Nelson and Langdale, 1992). Much more information about these enzymes can be found in other chapters of this volume, here we will review current knowledge about how their genes are regulated.

1. Amaranth PEPC and PPdK Gene Expression

In the C₄ dicot amaranth, mRNAs for PEPC and PPdK are present in young seedlings from the earliest stages of leaf development (Ramsperger et al., 1996). Transcripts encoding these MP-specific enzymes are very abundant in apical meristems and leaf primordia. However, the PEPC and PPdK enzymes cannot be detected in these same young tissues. The production of these two proteins lags behind the accumulation of their transcripts, and there are some differences in their initial patterns of accumulation. The PEPC and PPdK polypeptides are first observed at approximately the same time as morphological differentiation of the vascular system becomes evident, when leaves have reached 2 mm in length, but prior to the morphological differentiation of MP and BS cells. At this

stage there is a dramatic increase in PEPC peptide accumulation in the basal regions of 2-mm long leaves, and this occurs specifically in the MP cell precursors. This is accompanied by an equally robust accumulation of PPdK, which does not show any cell type-specificity at this early stage. In the slightly more developmentally advanced midregion of 2-mm leaves, PPdK polypeptides decrease in abundance and become specific to the pre-MP cells, while PEPC polypeptide levels retain their same abundance and cell-specific pattern as in the basal regions. It is interesting that PPdK initially shows higher accumulation levels within MP precursor cells that are adjacent to the lower epidermal cells than those adjacent to the upper epidermis. It is possible that in this plant, pre-spongy MP cells that are located at the lower surface of the leaf develop slightly ahead of the pre-palisade MP cells that are located near the upper leaf surface. At the same time as these changes in protein accumulation are occurring, the mRNAs that encode these enzymes show no cell type-specificity, with equally abundant accumulation in MP and BS cell precursors.

Unlike genes encoding the Rubisco subunits, but comparable to BS cell-specific *Mell* expression, changes in the spatial expression of C_4 PEPC and PPdK genes do not correlate with the dicot leaf carbon sink-to-source transition. Except for the reduced PPdK accumulation in upper pre-mesophyll cells, the MP-specific patterns of PEPC and PPdK polypeptide accumulation that are first established in midregions of 2-mm-long amaranth leaves remain constant as the leaves continue to expand through 10 mm in length and reach maturity (Wang et al., 1993b). However, patterns of PPdK mRNA accumulation do change during early development. As the leaves expand from 2 to 5 mm in length, PPdK transcripts disappear from BS cells, thereby becoming MP cell-specific. During this same period PEPC transcripts still remain abundant in both cell types, not becoming specific to MP cells until after the leaves have expanded to greater than 10 mm in length (Wang et al., 1992). PEPC is encoded by a large multigene family in amaranth (Rydzik et al., 1996), and it is possible that the presence of PEPC transcripts in BS cells during much of early leaf development is due to expression from different genes encoding photosynthetic and non-photosynthetic isoforms. However, the abundance

of the transcripts in both leaf cell types, and the observation that PEPC protein was not detectable in BS precursors or cells, is indicative of post-transcriptional control of one or more C_4 PEPC genes. Such findings provide evidence that different levels of regulation may work to establish and maintain the cell-type specificity of these two MP cell-specific enzymes during the maturation of amaranth leaves. The lack of correlation between the timing and patterns of accumulation for these enzymes and the mRNAs that encode them provides evidence that, as for Rubisco, there are both transcriptional and post-transcriptional levels of control responsible for full cell type-specificity in the mature leaves. Furthermore, the fact that these two MP cell-specific enzymes show differences in their early patterns of expression indicates that they are independently regulated during the establishment of C_4 capability in developing amaranth leaves.

The amaranth PEPC and PPdK genes are both regulated by light, being very abundant in the MP cells of leaves and cotyledons of light-grown seedlings, but very low or undetectable in cotyledons of etiolated seedlings (Berry et al., 1997). Accumulation of both transcripts increases rapidly upon illumination, reaching the levels of light-grown plants over a period of 4–24 h. However, light does not appear to affect the spatial localization of PEPC or PPdK mRNA or enzymes in cotyledon MP cells (Wang et al., 1993a).

2. *Flaveria* PEPC and PPdK Gene Expression

In C_4 *Flaveria* species, the expression of genes encoding PEPC and PPdK is also strongly regulated by light at the level of mRNA accumulation (Hermans and Westhoff, 1990; Rosche and Westhoff, 1995; Rosche et al., 1998; Shu et al., 1999). In contrast to amaranth cotyledons, light does appear to affect the establishment of full MP specificity for PEPC and PPdK gene expression in *F. trinervia* cotyledons (Shu et al., 1999), again demonstrating that parameters affecting the establishment of cell type-specificity vary between these two C_4 dicots.

In *F. trinervia* there are three classes of genes that encode photosynthetic and non-photosynthetic isoforms PEPC, and these are designated as *PpcA* through *PpcC* (Hermans and Westhoff,

1990; Westhoff and Gowik, 2004). The C₄ isoform is encoded by two members of the *PpcA* class, which are both expressed at very high levels in leaves, and at very low levels in stems and roots. Within the C₄ leaves, *PpcA* gene expression is highly specific to MP cells. The single copy *PpcB* and *PpcC* genes code for non-photosynthetic isoforms that are expressed constitutively at low levels in all tissues of the plant. These three classes also occur in the C₃ species *F. pringlei* where they are all, including *PpcA*, expressed at low levels as non-photosynthetic genes throughout the plant. Like most C₄ enzymes derived from non-photosynthetic ancestors, the *PpcA* genes of *F. trinervia* are very similar to those of *F. pringlei*, so that their encoded proteins are 96% identical at the amino level. These are in fact more similar to each other than they are to the other *Ppc* classes within the same species. The high amount of shared identity for each class between the C₃ and C₄ species, and the differences between each class within a species, indicates that each of the three *Ppc* gene classes were already present before the divergence of these plants from their common ancestor. The very small differences in the amino acid sequence between the C₄ and C₃ *PpcA*-encoded proteins account for the distinctive regulatory and kinetic characteristics associated with each isoform, including increased tolerance to malate and lower affinity for PEP for the C₄ PEPC (Westhoff and Gowik, 2004; Chapter 13, this volume).

In transgenic *F. bidentis* plants, a promoter derived from the *F. trinervia* C₄ *PpcA1* gene mediates full MP-specific expression to a β -glucuronidase (GUS) reporter gene in mature leaves. Expression from this construct could not be detected anywhere in the leaf primordia, but was shown to initiate near the tip of very young leaves and then progress basipetally in coordination with maturation of the leaf MP cells (Stockhaus et al., 1997). This pattern is somewhat reminiscent of the sink-to-source transition associated C₃-to-C₄ transition in Rubisco expression that occurs in developing amaranth leaves (Wang et al., 1993b); however, this process has not yet been investigated in *F. bidentis* leaves. Overall levels of *PpcA1*-GUS expression are low in young emerging leaves, reach a maximum at full expansion, and then decrease, a pattern that mimics steady state levels of endogenous *PpcA* mRNA in the developing

leaves (Stockhaus et al., 1997). Expression levels are highest in MP cells near the upper leaf surface, and lower in MP cells at the underside of the leaf, suggesting differences in photosynthetic capability and function for MP cells located at different positions within the leaf. *PpcA1*-GUS expression is very low or not detected in leaf BS cells, or in non-photosynthetic tissues such as stems and roots.

In contrast to the C₄ form, a promoter derived from the non-photosynthetic *PpcA* ortholog of the C₃ species *F. pringlei* mediates only low levels of reporter gene expression that are mainly confined to vascular tissues and leaf MP cells in transgenic C₄ *F. bidentis* (Westhoff and Gowik, 2004). These findings demonstrate functional differences in the *PpcA* genes from these two *Flaveria* species. It is likely that the *PpcA* gene of *F. trinervia* contains regulatory regions that have been evolutionarily modified to achieve the C₄ characteristics of high-level and MP-specific expression, and this can be observed when expressed from the *F. bidentis* genome. In contrast, the highly similar but more basic *F. pringlei* gene cannot produce this characteristic expression pattern, even when it is expressed in the transgenic C₄ plants (Westhoff and Gowik, 2004).

All *Flaveria* species contain a single gene encoding PPdK, designated *Pdk* (Rosche et al., 1994, 1998; Rosche and Westhoff, 1995). The unique structure of this gene allows it to encode both a chloroplastic form and a cytosolic form from the same open reading frame. Within the single *Pdk* gene a large intron separates exon one, which encodes a plastid transit sequence, from exon two, which encodes the mature polypeptide. A large 3.4 kb transcript is produced from a promoter upstream of the first exon, so that the protein translated from this mRNA contains both the transit sequence and the mature polypeptide sequence. In C₄ *Flaveria* this large transcript is most abundant in leaf MP cells, although low levels are also detected in BS cells and other tissues. The accumulation of this mRNA in MP cells is enhanced by light. A second promoter is located within the large intron, so that 3.0 kb transcripts encoded from this promoter lack the first exon. The resulting protein therefore lacks the plastid transit sequence, and represents a cytoplasmic form of the enzyme. This smaller transcript is present at low levels in roots and in

stems, is not light-regulated, and does not appear to be associated with C_4 function. In C_3 and C_3 - C_4 intermediate *Flaveria* species, the 3.4 kb transcript is present in leaves at low and intermediate levels of abundance, respectively. The abundance of this larger transcript, but not the smaller transcript, correlates with the extent of the C_4 traits in the various *Flaveria* species (Rosche et al., 1994, 1998). When expressed in transgenic *F. bidentis*, the expression patterns of the upstream C_4 *Pdk* promoter of *F. trinervia* (linked to a GUS reporter) were very similar to those conferred by the C_4 *PpcA1* promoter, in terms of timing and levels of expression, including the onset of MP-preferential expression at specific stages of leaf development (Rosche et al., 1998). Thus it is likely that similar processes regulate both genes in *F. bidentis* MP cells.

3. Maize PEPC and PPdK Gene Expression

There are multiple genes encoding PEPC in maize, and these have been categorized into three subgroups (Hudspeth and Grula, 1989; Schäffner and Sheen, 1992). *PpcZm1* encodes the C_4 form, which is expressed only in leaf MP cells, and in the MP-like cells of glumes and husk leaves, but not in roots, stems, or other tissues. *PpcZm2* and *PpcZm3* encode non-photosynthetic forms that are expressed at low levels in all tissues and at all developmental stages. *PpcZm1* expression is light-inducible, while genes encoding the non-photosynthetic forms are not.

There are two PPdK-encoding genes in the maize genome. One encodes a cytosolic form that is expressed in all tissues of the plant, while the other is equivalent to the single-copy dual-function gene of *F. trinervia*, so that both the C_4 chloroplastic form and a non-photosynthetic cytosolic form are encoded from the same open reading frame of this gene (Sheen, 1991, 1999; Matsuoka, 1995). Expression of the chloroplastic form is induced by light and occurs only in leaf MP and the MP-like cells of the leaf-like organs, while the non-photosynthetic form, transcribed from a promoter within the first intron, is not responsive to light and expressed at low levels throughout the plant.

Using *in situ* hybridization and immunolocalization, it was demonstrated that within the developmental gradient of the monocot maize leaf, the

accumulation of C_4 PEPC and PPdK mRNAs and proteins in MP cells lag behind those of Rubisco in BS cells, appearing in coordination with the morphological differentiation of the vascular centers and Kranz cell types (Langdale and Nelson, 1991; Nelson and Langdale, 1989, 1992; Dengler and Nelson, 1999; Sheen, 1999). Furthermore, light enhances the expression of these genes in MP cells, while at the same time reducing the expression of Rubisco genes in these cells (Sheen and Bogorad, 1986b, 1987a, b; Langdale et al., 1988; Sheen, 1999). A more recent investigation, utilizing transgenic maize plants expressing a reporter gene linked to a *PpcZm1* promoter, confirmed the developmental and light-regulated patterns that were observed for the endogenous C_4 PEPC gene in the previous studies (Kausch et al., 2001). In agreement with those studies, *PpcZm1* promoter conferred expression in MP cells of maize leaves, and also in the MP-like cells of glumes and husks. The expression of this reporter gene construct paralleled differentiation of leaf MP cells, and was increased by light within these cells. There was no reporter gene expression from this C_4 PEPC-derived construct observed in roots, stems, or shoot apical meristems.

B. Genes Encoding Other MP Cell-Specific Proteins

Carbonic anhydrases (CA) catalyze a reversible reaction that converts CO_2 and HCO_3^- (Reed and Graham, 1981; Ku et al., 1996; Moroney et al., 2001; Tetu et al., 2007). There are many forms of CA, which occurs in all organisms, and these have been grouped into the α -CA, β -CA, and γ -CA classes. Genes encoding multiple forms of CA have been identified in many plant species, mostly from the β -CA class, and these occur in both cytoplasmic and plastid forms. In C_3 plants, the predominant form of this enzyme is localized to the chloroplasts, and may aid in the diffusion of CO_2 across the plastid membrane, lipid biosynthesis, and antioxidase functions. A separate cytosolic form is also present in C_3 plants, and appears to function in non-photosynthetic reactions, such as providing bicarbonate to non-photosynthetic PEPC (Moroney et al., 2001; Tetu et al., 2007). In C_4 plants, the most abundant form of CA is localized to the cytoplasm of MP cells, where it produces bicarbonate substrate for PEPC

from atmospheric CO₂. The activity of the chloroplastic form is greatly reduced in C₄ leaves, particularly in BS cells. The strict localization and function of cytosolic CA in MP cells of C₄ plants appears to be critical for the efficient function of this pathway, since reducing levels of the MP-specific cytosolic CA by antisense RNA in *F. bidentis* greatly reduces overall rates of carbon assimilation (von Caemmerer et al., 2004). The strong reduction of CA in BS cells may also ensure that all of the CO₂ released by decarboxylation within these cells is available for fixation by Rubisco, rather than having some loss due to conversion to bicarbonate and subsequent leakage via plasmodesmata (Burnell and Hatch, 1988). In *F. bidentis* leaves, developmental studies have shown that mRNAs encoding the MP-specific cytosolic C₄ form of CA, designated CA3, is 50 times more abundant in than mRNAs encoding the other non-C₄ forms, CA1 (chloroplastic form) and CA2 (cytosolic). Southern-blot analysis suggests the presence of these two CA3 genes in the *F. bidentis* genome, reminiscent of the two MP cell-specific *PpcA* genes in *F. trinervia*, although as yet there is no direct evidence for two CA3 genes (Hermans and Westhoff, 1990; Westhoff and Gowik, 2004; Tetu et al., 2007). Genes encoding the CA1 and CA2 transcripts likely represent ancestral forms present in all plants; transcripts encoding these non-C₄ forms are found in all organs of the plant, consistent with a role in housekeeping functions (Tetu et al., 2007).

In NADP-ME type C₄ plants such as maize, which lack photosynthesis II activity in the chloroplasts of their BS cells (see below), the expression of many members of a nuclear gene family encoding chlorophyll A/B binding proteins (*Cab* genes) is enhanced preferentially in MP cells (Sheen and Bogorad, 1986a; Bansal and Bogorad, 1993; Sheen, 1999), and selectively inhibited in BS cells. Other than their MP cell-preferential expression, most of the expression properties of C₄ *Cab* genes, such as high levels of expression, regulation by light and metabolic signaling, and organ specificity, are shared by *Cab* genes of all plants (Sheen, 1999; Tyagi and Gaur, 2003). Thus, only very minor changes in regulation may have been required in order for *Cab* genes to achieve their more specialized cell type-specific expression patterns associated with dimorphic chloroplast function in maize and some other C₄ species.

Surveys of differentially accumulating proteins and transcripts in maize and other NADP-ME type C₄ monocots have identified numerous genes, in addition to those described above, that are specifically or preferentially expressed in MP cells (Harrison and Black, 1982; Wyrich et al., 1998; Furumoto et al., 2000; Sugiharto et al., 2002; Majeran et al., 2005; Sawers et al., 2007). Many MP-preferential or specific genes are associated with functioning of the photosystems (PSI or PSII), such as *PsaD*, *PsaK*, *PsaG*, *PsaN*, *PsbT*, *PsbR*, *PsbO*, ferredoxin, plastocyanin, and NADP-ferredoxin oxidoreductase. The MP-specificity of PSII genes is to be expected, since these plants lack PSII activity in their BS plastids. However, the significance of mRNAs for some PSI being most abundant in or specific to MP cells is worthy of further investigation, since PSI activities of BS and MP chloroplasts are mostly similar (Furumoto et al., 2000). In most NADP-ME species, including the NADP-ME type C₄ dicot *F. bidentis*, it has been found that some extrinsic components of the water splitting complex, the 10-, 16- and 23-kDa polypeptides, are depleted in BS chloroplasts (Oswald et al., 1990; Höfer et al., 1991; Pfündel and Neubohn, 1999). In contrast, proteins of the core complex are present in plastids of both cell types. These findings indicate that the absence of the peripheral proteins in BS cell chloroplasts is largely responsible for the reduced PSII activity in NADP-ME type C₄ plants. Differential accumulation of the extrinsic proteins in sorghum correlates with the cell-type specific accumulation of their nuclear-encoded mRNAs, suggesting regulation at the level of transcription or mRNA stability (Oswald et al., 1990). In *F. bidentis*, this regulation is likely to be post-transcriptional, since mRNAs encoding these components occur in both leaf cell types (Höfer et al., 1991). Some additional proteins or mRNAs found to be MP-specific or preferential include a vacuole transporter, triosephosphate isomerase, as well as several others of unknown function. Fewer MP cell-specific genes would be expected in an NAD-ME type C₄ plant such as amaranth, since differences in the function and morphologies of chloroplasts in the BS and MP cells of these plants are not as numerous (see below) (Gutierrez et al., 1974; Harrison and Black, 1982; Boinski et al., 1993).

IV. C_4 Gene Expression in Organelles

In all Kranz-type C_4 species, the expression of the chloroplastic *rbcL* gene encoding the Rubisco LSU is highly specific to BS cells. The extent to which the expression of other plastid-encoded genes differs between BS and MP cells can vary greatly between different types of C_4 species. This concept is well illustrated by comparing the expression patterns of plastid-encoded genes in amaranth, an NAD-ME dicot, and maize, an NADP-ME type monocot. In amaranth, chloroplasts within BS and MP cells are morphologically very similar (Gutierrez et al., 1974; Boinski et al., 1993). Plastids within each cell type have well developed grana, and display normal PSI and PSII activities. Aside from the BS-specific *rbcL*, genes in BS and MP chloroplasts that were isolated and separated from amaranth leaves appear to be expressed at nearly identical levels in both plastid types, both in terms of mRNA and protein accumulation which is consistent with the similar function and form of these plastids (Boinski et al., 1993). In leaves of light-grown maize plants, which are depleted in PSII activity in BS chloroplasts, the expression of plastidic genes that encode some photosystem II components is at least tenfold more abundant in MP chloroplasts than in BS chloroplasts, both at the levels of mRNA and protein accumulation (Sheen and Bogorad, 1988). As with many of the nuclear-encoded C_4 genes, the expression of *rbcL* and many other plastid genes in both maize and amaranth is highly regulated by light. In both maize and amaranth, *rbcL* mRNAs accumulate in the plastids of dark-grown plants, while LSU protein is present at very low levels, or not detectable. Illumination greatly enhances *rbcL* gene expression, but only in BS cells; this is often a result of positive regulation occurring one or more post-transcriptional levels (Berry et al., 1985, 1986; Langdale et al., 1988; Sheen and Bogorad, 1986b, 1988; Patel and Berry, 2008). Although plastid-encoded genes account for some of the cell type-specific processes associated with differential chloroplasts function in C_4 plants, most are dependant on nuclear encoded proteins (such as the nuclear-encoded water-splitting and PSII components described in the previous section) that are targeted to the plastids (Harrison and Black, 1982; Wyrich et al., 1998; Furumoto et al.,

2000; McCormac et al., 2001; Sugiharto et al., 2002; Majeran et al., 2005).

Like chloroplasts, mitochondria in C_4 plants differ between BS and MP cells in terms of their morphology and functions related to carbon fixation, and these differences are most evident in NAD-ME species which use mitochondrial enzymes to decarboxylate C_4 acids (Ohnishi and Kanai, 1983; Long et al., 1994; Agostino et al., 1996; Taniguchi and Sugiyama, 1997). Cell-type specific expression of nuclear genes that produce metabolic, respiratory, and transport proteins targeted to these organelles can account for known differences in mitochondrial function between the two cell types. The mitochondrial genomes of higher plants code for only a small number of proteins and non-coding RNAs, which are all regulated post-transcriptionally (Gagliardi and Gualberto, 2004). Since steady-state levels of mitochondrial mRNAs do not correlate with changes in gene expression, variations in MP and BS mitochondrial gene activity would be difficult to detect using standard genomic screening procedures. Thus, while it is not known if there are any differences between BS and MP cells at the level of the mitochondrial genome, the involvement of some mitochondrial genes cannot be ruled out.

V. Factors Affecting C_4 Gene Expression in BS and MP Cells

By observing developmental patterns of gene expression in three representative C_4 plants, amaranth, *Flaveria*, and maize, it is apparent that there are both variations and similarities in the expression of orthologs of the various photosynthetic genes between species, and also for different C_4 genes within a species. Such observations lead to the conclusion that individual C_4 genes within each plant are regulated independently in response to a variety of signals (Berry et al., 1997; Sheen, 1999). In amaranth, maize, and likely most other C_4 plants, Rubisco mRNAs and proteins accumulate prior to those of the other major photosynthesis enzymes. This suggests an initial, more basic C_3 -like stage of gene expression, which then becomes modified by the activation of more specialized C_4 regulatory processes. The initial pattern is considered to be the ancestral default state, and is likely common to

all plants. Activation of molecular processes that determine the modified C₄ state are delayed until the leaf becomes photosynthetically competent, either by light or innate developmental processes (Langdale and Nelson, 1991; Nelson and Langdale, 1989, 1992; Berry et al., 1997; Sheen, 1999). Ultimately, for each plant, the same end result is achieved, so that by the time a leaf has reached full photosynthetic capacity C₄ mRNAs and proteins accumulate and function in only one of the two Kranz cell types. Table 2 summarizes and compares developmental patterns of BS and MP gene expression in two representative C₄ plants, the dicot amaranth and the monocot maize. The sections below will discuss some of the factors identified as determinants in the expression of photosynthesis genes before and after the establishment of C₄ capacity.

A. Photosynthetic Metabolism

As discussed previously in this chapter, both amaranth and maize have two levels of Rubisco gene expression, the “C₃-like” default pattern that is likely the ancestral program, and the more specialized “C₄-type” that occurs following a specific developmental transition (summarized in Table 2). In amaranth, this signal is associated with changes in carbon transport status and works independently of light. In maize, this transition is only observed when etiolated plants are used as the starting material, and light is required for BS-specific Rubisco gene expression. Based on these observations, it would appear that entirely different signals are responsible for the C₃-to-C₄ transition in Rubisco gene expression in these two plants. However, this can also be interpreted as commonality in events leading to the C₃-to-C₄ transition in the leaves of both species. Changes in photoassimilate production, accumulation, or transport brought about by the sink-to-source transition (in amaranth) or the initiation of photosynthesis in the light (in maize) could result in similar metabolic signals that directly or indirectly affect *rbcL* and *RbcS* gene expression patterns within the C₄ leaves (Berry et al., 1997; Nelson and Langdale, 1992; Sheen, 1999).

Additional evidence that photosynthetic function is involved in the establishment of BS-specific Rubisco gene expression comes from

studies of *Amaranthus tricolor* leaves (McCormac et al., 1997). Prior to flowering in *A. tricolor*, three-colored leaves emerge that have both photosynthetic (green) and non-photosynthetic (red and yellow) regions. The green regions of the leaf show normal C₄ expression patterns for both Rubisco genes, with mRNAs and proteins accumulating only in BS cells. The absence of photosynthetic activity in the yellow and red sectors of the leaf is associated with a loss of cell-specificity for the *rbcL* and *RbcS* mRNAs, which accumulate in both BS and MP cells of those regions. Interestingly, the lack of photosynthetic activity did not affect either of the Rubisco proteins, which remain BS-cell specific. This breakdown of correlation, in which *rbcL* and *RbcS* transcripts accumulate in both cell types while their corresponding proteins accumulate only in the BS cells, is identical to that observed in the very early 3 mm amaranth leaves (Ramsperger et al., 1996). Thus, the non-photosynthetic regions of the mature *A. tricolor* leaves appear to imitate the regulatory events of early leaf development that occur prior the C₃-to-C₄ transition in Rubisco gene expression.

Metabolic regulation of maize C₄ photosynthesis genes has been well characterized in a transient expression system with protoplast of MP cells, and in transgenic plants. Promoters of several maize genes, including C₄ *Ppdk*, *Ppc*, *RbcS*, and *Cab*, are all strongly regulated by sugars, acetate, and other carbon metabolites (Sheen, 1990, 1999; Schäffner and Sheen, 1992; Jang and Sheen, 1997; Kausch et al., 2001). The *PpcZm1* gene of maize is activated under illumination but does not respond directly to light itself, but rather indirectly to signals associated with light-induced development, including chloroplasts biogenesis (Schäffner and Sheen, 1992; Sheen, 1999; Kausch et al., 2001). Control of photosynthesis genes by carbon metabolism has been documented in other systems as well, and is likely a process common to all higher plants (Jiang et al., 1993; Krapp et al., 1993; Urwin and Jenkins, 1997; Acevedo-Hernandez et al., 2005). Metabolic regulation can override other control signals, such as development and light, and may be associated with changes brought about by activation of photosynthesis or the carbon sink-to-source transition, processes associated with C₃-C₄ transitions in Rubisco gene expression in maize and amaranth. Taken together, these studies provide strong evidence that signals arising from

photosynthetic carbon assimilation have a strong regulatory influence on the expression of all of the major C_4 pathway genes.

B. Cell Position and Lineage

In addition to light, the location of a cell relative to a vascular center provides positional information that determines the differentiation of BS and MP cells within a C_4 leaf, and the associated expression of cell type-specific photosynthetic enzymes (Dengler and Nelson, 1999; Chapter 9, this volume). In the foliar leaves of light-grown maize plants, the organization of BS and MP cells normally occurs no more than four cells distant from a vascular center; cells within this proximity accumulate C_4 photosynthetic transcripts and proteins in the appropriate cell types. In leaf sheaths and husk leaves, which have wider spacing between veins (up to 20 cells), cells that are more proximal to the veins develop and express BS and MP cell-specific genes characteristic of normal C_4 plants (Langdale et al., 1988; Nelson and Langdale, 1989, 1992; Dengler and Nelson, 1999). However, cells that are more distant from the vascular centers develop the characteristics of C_3 MP cells, expressing genes such as *RbcS* and *Cab*, but not genes for PEPC or other C_4 MP cell-specific enzymes. These observations suggest that factors determining C_4 development, at least for the MP cells, can only function in close proximity to a vein (Langdale et al., 1988; Jankovsky et al., 2001). There is some evidence for this phenomenon in dicots as well, since in some C_3 - C_4 intermediates (such as *Flaveria brownii* and *F. ramosissima*) C_4 -type photosynthetic activity is detected only in MP cells that are proximal to a vein (Cheng et al., 1988; Moore et al., 1988; Jankovsky et al., 2001).

In maize, the expression pattern in husks leaves can be modified by light intensity. Under very low light, genes encoding Rubisco are expressed in MP and BS cells, while expression from genes encoding other C_4 enzymes such as PEPC, PPdK, and NADP-MDH is not detected in these tissues (Langdale et al., 1988; Nelson and Langdale, 1992). In high light, the C_4 genes are expressed in their appropriate cell type, but only in cells that are close to a vein. Studies with the maize *argentina* mutant, which delays photosynthetic development relative to vein development, indicate that

timing of C_4 gene expression is tightly correlated with the maturation of individual vascular centers (Nelson and Langdale, 1989, 1992). This suggests that BS and MP cell-specific genes are responsive to signals produced locally from a nearby vein, rather than from systemic signals throughout the leaf. These findings support a model that light and/or photosynthetic processes induce one or more signaling factors that are transported through and diffusible from the veins, with diffusion limited to only a few cell volumes. As yet no particular substance has been identified, although several lines of evidence suggest that one or more transportable products or by-products of photosynthesis may be involved in the positional signaling process (Chapter 9, this volume).

Cell position may be less important for the differentiation of maize leaf BS cells than for MP cells. In the maize tangled (*tan1*) mutation, abnormal cell divisions lead to the production of cells with BS-like characteristics in abnormal locations that are more distant from a vein (Jankovsky et al., 2001). Like normal BS cells located adjacent to a vein, these cells express genes encoding NADP-ME but not PEPC genes. Since the BS-like cells are clonally derived from the normally-positioned BS cells, the acquisition of BS-like characteristics in the more distant cells must rely more on lineage than position relative to a vein. Lineage-dependent developmental commitment such as this is very uncommon in plants, and is more typical of animal development. The implication is that very different developmental processes are responsible for the differentiation of BS and MP cells, and the corresponding cell type-specific expression of the various photosynthetic genes. This further supports the concept that molecular regulatory processes must become integrated to achieve expression patterns that are associated with full C_4 function.

C. Other Factors That Affect C_4 Gene Expression

Photosynthesis in all higher plants is responsive to a wide range of environmental factors. It is likely that all of the environmental and stress factors that affect the expression of Rubisco genes will also be found to directly or indirectly affect other C_4 photosynthesis genes expressed in BS or MP cells. As an example, it is known that stress

and abscisic acid (ABA, a plant stress hormone) can repress both *RbcS* and *C4Pdk* gene expression in maize MP protoplasts (Sheen, 1998, 1999). Of particular note are the roles of nitrogen and cytokinin as environmental determinants of C₄ gene expression. Nitrogen availability and uptake are major factors that influence photosynthetic capacity in all plants, and C₄ plants are highly efficient in nitrogen utilization (Moore and Black, 1979; Ku et al., 1996; Chapters 7, and 8, this volume). Nitrogen metabolism is mediated by a set of ubiquitous root and leaf transporters, as well as numerous enzymes and molecules involved in nitrogen assimilation, metabolism, and signaling (Crawford and Glass, 1998; Takei et al., 2002; Yin et al., 2007). Most of the effects of nitrogen on photosynthesis and other processes can be mimicked by cytokinin, which is tightly associated with nitrogen signaling in plants. In maize, several nitrogen sources, including nitrate, ammonium, and glutamine, as well as cytokinin, can affect the expression of the MP-specific *Ppc* and *CA* genes (Sugiharto and Sugiyama, 1992; Sugiharto et al., 1992; Suzuki et al., 1994). Of the many factors affecting photosynthetic gene expression in BS and MP cells, it is probable that most will have overlapping and/or cumulative effects, possibly similar to those that occur in the integrative plant signaling processes of sugars and hormones (Leon and Sheen, 2003).

VI. Levels of C₄ Gene Regulation

As described in previous sections, there is evidence that transcriptional as well as post-transcriptional levels of regulation contribute to BS- or MP-specific patterns of expression in C₄ leaves. It becoming increasingly apparent that multiple levels of regulation are utilized to achieve the cell-specific gene expression patterns required for full CO₂ fixation capacity in the mature leaves of C₄ plants. The establishment of full cell type-specific expression for *rbcL*, *RbcS*, and other C₄ genes may require coordination of many separate regulatory mechanisms, with the involvement of both organelle- and nuclear- encoded genes. Each mechanism could act independently, or in synergy, ultimately leading to full cell type-specific localization of the C₄ enzymes. Close interaction between different phases of transcriptional and post-transcriptional

regulation appears to be a routine process in eukaryotic gene expression (Orphanides and Reinberg, 2002). Such redundancy and overlap for mechanisms determining BS-specificity would ensure that full C₄ capability occurs in the leaves of these plants, at all stages of development and under varying physiological or environmental conditions.

A. Transcriptional Control of C₄ Gene Expression

Several studies have implicated transcriptional control as the primary determinant in the BS or MP cell-specific expression of some C₄ genes (Langdale et al., 1991; Sheen, 1991, 1999; Bansal, et al., 1992; Viret et al., 1994; Furbank, and Taylor, 1995; Long and Berry, 1996; Taniguchi et al., 2000a; Ali and Taylor, 2001a, b; Kausch, et al., 2001; Lai et al., 2002b; Gowik et al., 2004). Many of these studies have utilized expression vectors that combine promoter/enhancer segments derived from MP or BS cell-specific genes that have been linked to reporter genes, with assays involving transient expression or transgenic plants. Most of these vectors reproduce cell-type specific expression patterns when introduced into in C₄ plants, and cis-acting enhancer/promoter regulatory regions involved in cell type-specific regulation have been identified. Some examples of cell type-specific gene expression determined by C₄ promoter/enhancers are described below.

Cis-acting elements within C₄ enhancer/promoter regions have been most extensively characterized in *Flaveria*, where the ability to produce transgenic plants has facilitated the characterization of these regions. Using transgenic *F. bidentis*, Westhoff and coworkers (reviewed in Westhoff and Gowik, 2004; Chapter 13, this volume) have identified two elements within the upstream region of the *F. trinervia* C₄ *PpcA1* gene that greatly enhance reporter gene expression in MP cells. The more distal element (designated DR) is located between -1566 and -2141, and the more proximal element (designated PR) extends to -570 bp upstream of the translation start codon (Gowik et al., 2004). Enhanced MP-specific expression of the *F. trinervia* C₄ *PpcA1* gene requires both the DR and PR elements in the C₄ *PpcA1* promoter/enhancer. These regions did not cause enhanced expression when they were inserted into a *PpcA1*

promoter derived from *C₃ F. pringlei*, although the DR was able to confer low levels of MP-specific expression to this *C₃* promoter. The DR element can function in forward as well reverse orientations (characteristic of eukaryotic enhancers), and contains a specific element, designated MEM-1 (mesophyll expression module 1) that in combination with the PR is sufficient for *C₄ PpcA1* expression. Interestingly, a sequence similar to MEM-1 has also been found in the upstream region of the *C₄ CA3* gene of *F. bidentis*, but not in the upstream region of its ortholog from the *C₃ F. pringlei* (M. Ludwig, personal communication, 2008). The presence of similar upstream sequences in two MP cell-specific *C₄* genes is intriguing, and the possible involvement of this sequence in *C₄* CA expression is currently under investigation (M. Ludwig, personal communication, 2008).

The evolutionary acquisition of *C₄ PpcA1* characteristics from the ancestral *C₃ PpcA1* gene is thought to have involved three processes (not necessarily in order or occurrence). First, the inactivation of promoter/enhancer elements that allowed for expression in all cell types; second, the development of novel MP-specificity elements; and third, the creation of quantitative elements that allowed for enhanced expression in MP cells. Analyses of nucleotide differences in the DR and PR regions within the promoter/enhancers of *C₃* and *C₄ PpcA1* genes are providing clues about how ancestral *C₃* genes have been evolutionarily modified to function in the *C₄* photosynthetic pathway.

A similar experimental approach has demonstrated that a *C₄ ChlMe1* -1,758 to +305, promoter/enhancer region derived from *F. trinervia* is sufficient to confer high-level, light-dependent, and BS cell-preferential reporter gene expression in transgenic *F. bidentis* (Lai et al., 2002b). These characteristics were not conferred by a promoter/enhancer derived from a *C₃ ChlMe2* gene, suggesting that there are regulatory elements on the *C₄* promoter that are lacking on the *C₃* promoter. In earlier studies, some sequences determining high level expression from a construct derived from the endogenous *F. bidentis ChlMe1* gene were found to occur within the 3' flanking region (Marshall et al., 1997; Ali and Taylor, 2001a, b). When similar 3' flanking regions derived from the *F. trinervia ChlMe1* were used in an expression construct, this enhancement effect did not

occur, possibly due to the heterologous versus endogenous origins of the 3' sequences, or to differences in 3' regions included in the constructs (Lai et al., 2002b). The potential role of regions outside of the promoter in determining *C₄ ChlMe1* expression warrants further investigation.

Studies using transgenic maize have shown that the promoter/enhancer of the maize *C₄ Pdk* gene can confer high levels of MP cell-specific reporter gene expression (Nomura et al., 2000; Taniguchi et al., 2000a). A short element within this region, from -330 to -76, has been implicated in this regulation. The promoter/enhancer from a *C₃ Pdk* ortholog from rice did not confer cell-type specific expression in transgenic maize plants, providing additional evidence that the *C₄* gene has acquired new cis-acting elements that allow for cell-type specific transcriptional regulation. In *Flaveria*, a 1.5 kb 5' flanking region from the *C₄ F. trinervia Pdk* gene, which included the entire 5' untranslated region, produced high levels of GUS activity in MP cells when expressed in transgenic *F. bidentis* (Rosche et al., 1998). Low levels of GUS activity were also detected in BS cells, stems, and roots of these plants. Since the ancestral non-photosynthetic form and the photosynthetic form of this enzyme are both encoded by a single gene, it was concluded that cis-acting DNA sequences controlling the expression of both forms are both contained within this same fragment of the *F. trinervia* gene (Rosche et al., 1998). These findings suggest that new *C₄* cis-acting sequences may have been added to the ancestral Pdk promoter, which was already present and functional in a wide range of cells, without significantly changing the activity of that promoter. Similarly, a 1.7 kb 5' flanking region from the maize *C₄ Ppc* gene, which confers light-mediated and MP cell-specific reporter gene expression in transgenic maize plants, has likely acquired new cis-acting sequences enabling its photosynthetic function (Kausch et al., 2001).

Promoters from *C₄* genes showing cell-specific expression in several species (maize, *Flaveria*, and recently *Urochloa panicoides*) have been shown to confer some cell- or tissue-specificity to reporter genes when transformed into the *C₃* plants tobacco or rice (Matsuoka et al., 1994; Stockhaus et al., 1997; Nomura et al., 2000; Suzuki and Burnell, 2003), but in themselves are not sufficient to induce full *C₄*-like gene expression patterns under these conditions. Such findings

suggest that C₃ species may lack trans-acting factors (such as proteins that interact with enhancer/promoter DNA) or specific DNA modification processes that are responsible for the MP or BS cell-specific transcription patterns of C₄ plants.

Differences in DNA methylation and histone acetylation at promoter/enhancer regions have been associated with changes in the expression of the maize C₄ *Ppc*, and some other C₄ genes (Ngernprasirtsiri et al., 1989; Langdale et al., 1991; Offermann et al., 2006). However, the relationship between chromatin modifications such as these and the establishment of MP specific gene expression is not yet clearly understood. For example, the 1.7 flanking region of the C₄ *PpcZm1* gene that was sufficient in itself to confer MP cell-specific expression in transgenic maize plants did not include a methylation site (located 3 kb upstream) that was correlated with *Ppc* expression in an earlier study (Langdale et al., 1991). Thus, the methylation occurring at the more upstream site may not be involved in MP cell-specific expression. In addition, acetylation of histones at the C₄ *Ppc* promoter occurred in both BS and MP cells of maize in response to illumination, even though light-induced transcription from this gene occurred only in MP cells (Offermann et al., 2006).

In maize, two Dof-domain (DNA-binding with one finger) transcription factors have been implicated in the regulation of C₄ *Ppc* gene expression, through interactions with the upstream enhancer/promoter of this gene (Yanagisawa and Sheen, 1998; Yanagisawa, 2000, 2004). Dof transcription factors are ubiquitous in plants, and associated with many plant-specific biological processes. In maize, both Dof1 and Dof2 have been shown to bind to an AAAAGG motif found in the 5' upstream enhancer region of the C₄ *Ppc* gene. Transiently expressed Dof1 protein activated transcription from a synthetic C₄ *Ppc* promoter in maize protoplasts, while expression of antisense RNA specific to the *Dof1* transcript reduced the transcriptional activity of this promoter. These studies have implicated Dof1 as a positive regulator of C₄ *Ppc* expression, with roles in tissue-specific and light-induced expression. In contrast, the related Dof2 protein was shown to block transcriptional activation of the C₄ *Ppc* promoter, possibly through competitive binding interactions with the Dof1 protein.

G2-like (*Glk*) genes encode transcriptional factors that can affect the development of chloroplasts in maize BS cells (Cribb et al., 2001; Rossini et al., 2001). The maize G2-like proteins most likely affect plastid development indirectly, by functioning as transcription factors that regulate or interact with other genes involved in photosynthetic function. The first member of this family to be identified, *Golden2* (*G2*), was originally named *bundle sheath defective 1* (*bsd1*), due to its disruptive effects on chloroplast development and the expression of some BS cell-specific genes, including *rbcL*, *RbcS*, and C₄ NADP-ME (Roth et al., 1996). In maize leaves, *G2* is expressed and effects plastid development primarily in BS cells whereas another member of the G2-like family (*Glk*), *ZmGlk1*, is active in MP cell plastid development. Transcripts for both proteins are increased by light, and are active in C₃-like as well as C₄ tissues of maize. These proteins are also present in C₃ plants, where they appear to function redundantly in leaves and other photosynthetic tissues to promote chloroplast development.

The search for additional trans-acting regulatory factors that determine MP and BS cell-type specific transcription of C₄ photosynthetic genes is still in its early stages. Yeast one-hybrid analysis as well as DNA-binding/gel shift analyses with BS or MP nuclear extracts have identified potential candidate proteins involved in the expression of C₄ *Ppc* genes in maize and *Flaveria* (Taniguchi et al., 2000b; Westhoff and Gowik, 2004). These proteins interact specifically with DNA sequences that confer specificity to leaf MP cells, such as the PR and MEM-1 elements of the *F. trinervia* *PpcA1* promoter. Some of these potential regulatory proteins are localized specifically to MP cells, and may be involved with activation of C₄ *Ppc* expression, while others are found in BS cells, and could act in the repression of transcription. Functional determination of the role of such proteins will require additional biochemical characterization and analysis in transgenic C₄ plants.

B. Post-transcriptional Control C₄ Gene Expression

Post-transcriptional regulation of gene expression is relatively common in plants, and has been reported for both plastid- and nuclear-encoded genes (for reviews, see Gallie, 1993, 2002; Zerges, 2000;

Cheng and Chen, 2004; Manuell et al., 2004). In plants, plastid- as well as nuclear-encoded mRNAs possess specific cis-acting sequences that are involved in their post-transcriptional regulation. These control regions are usually located within the 5' or 3' untranslated regions (UTRs) of the mRNA, or more rarely within the coding region. The regulatory sequences are often recognized by RNA binding proteins that may affect a transcript's translation, processing, localization, or stability.

Many examples of C_4 gene expression in BS and MP cells are described in this chapter and elsewhere (Furbank and Taylor, 1995; Berry et al., 1997; Sheen, 1999; Markelz et al., 2003). As described in previous sections, under certain conditions or developmental stages the accumulation of some C_4 mRNAs does not correlate with accumulation of their corresponding proteins. Such a lack of correlation is often indicative of post-transcriptional control at the level of translation. The regulation of Rubisco gene expression by light provides a good example of this control mechanism. Genes encoding this enzyme are post-transcriptionally regulated by light in the C_4 plants amaranth, *F. bidentis*, and maize, and translational control by light has been well documented (Nelson et al., 1984; Berry et al., 1985, 1986, 1988, 1990; McCormac et al., 2001; Markelz et al., 2003; M. Patel and J.O. Berry, unpublished, 2008). In the C_4 dicots, *rbcL* and *RbcS* mRNAs accumulate in the presence or absence of light, but Rubisco synthesis occurs only when these plants are illuminated. There is no synthesis of either Rubisco subunit in the absence of light. When dark-grown seedlings are transferred to light, synthesis of the LSU and SSU polypeptides is induced very rapidly, and this occurs without corresponding increases in levels of their transcripts. Further analysis has shown that *rbcL* and *RbcS* mRNAs associate with polysomes in light-grown seedlings, but not in dark-grown seedlings. When dark-grown seedlings are transferred to light, the Rubisco mRNAs rapidly associate with polysomes, and synthesis of the Rubisco subunits is initiated. Such findings clearly demonstrate light-mediated regulation of translational initiation. Another set of experiments has shown that synthesis of both Rubisco subunits can also be rapidly repressed in the absence of light at the level of translational elongation.

In some C_4 plants, control of transcript stability appears to play a major role in determining BS-specific accumulation of both Rubisco transcripts. Initial evidence for this control mechanism came from earlier studies which demonstrated that, as in intact leaves, the *rbcL* transcripts accumulated only in BS chloroplasts, and not in MP chloroplasts, that had been isolated and separated from mature amaranth leaves. However, run-on transcription analysis using these same isolated plastid populations showed that *rbcL* mRNAs were transcribed at similar levels in both chloroplast types (Boinski et al., 1993). Similar findings were reported for *rbcL* mRNA accumulation in the C_4 monocots maize and sorghum (Kubicki et al., 1994). Run-on transcription analysis using nuclei from separated BS and MP cells have demonstrated that specific accumulation of *RbcS* transcripts in C_4 maize leaves also involves differences in mRNA stability in the two cell types (Schäffner and Sheen, 1992; Sheen, 1999).

Recent findings have demonstrated that 5' and 3' UTRs of heterologous C_4 amaranth *AhRbcS1* mRNA, in themselves, conferred partial BS-specific expression to a *gusA* reporter gene at the level of mRNA accumulation when constitutively expressed from a CaMV promoter in stable transgenic lines of *F. bidentis* (Patel et al., 2004). Interestingly, the heterologous amaranth UTRs were able to mediate normal tissue-specific mRNA accumulation, suggesting that post-transcriptional regulatory mechanisms that determine organ and tissue specific mRNA accumulation may be more highly conserved than those determining BS cell-specific expression. Most significantly, our most recent study has shown that the 5' and 3' UTRs of an endogenous *F. bidentis* *RbcS* mRNA (*FbRbcS1*), in themselves, conferred strong BS-specific expression to *gfpA* reporter gene at the level of mRNA accumulation when transcribed from a constitutive CaMV promoter (Patel et al., 2006). This study, which is summarized in Fig. 2, clearly demonstrates that characteristic C_4 BS-specific *RbcS* gene expression patterns can be established in the absence of any cell-type specific transcription elements, and are mediated by the UTRs of the *FbRbcS1* mRNAs. Taken together, findings reviewed here indicate that both *rbcL* and *RbcS* transcripts have different stabilities in BS versus MP cells of mature C_4 leaves. These observations have provided strong evidence that post-transcriptional

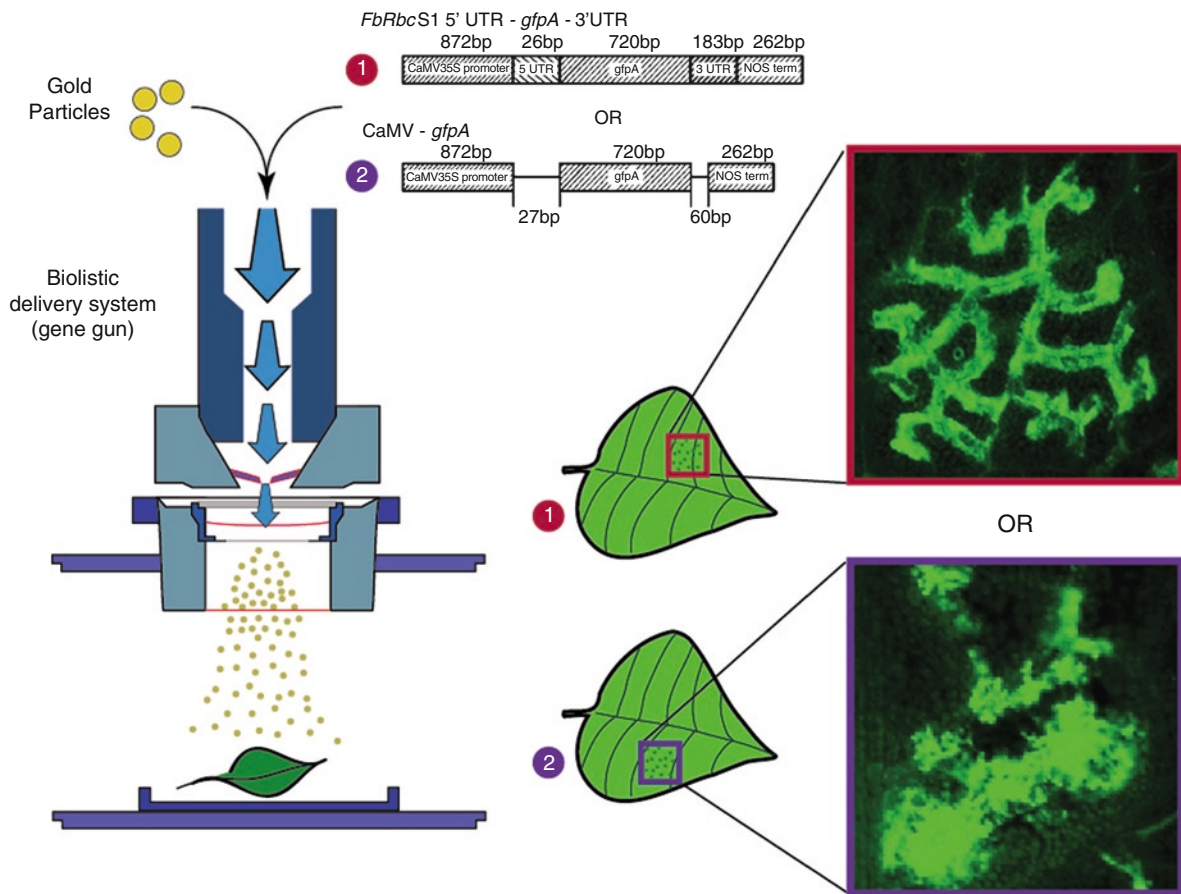


Fig. 2. Transient expression assay demonstrating post-transcriptional control of BS cell-specific *RbcS* gene expression. An experimental DNA expression construct (*FbRbcS1* 5'UTR-*gfpA*-3'UTR) containing 5' and 3' untranslated regions from the *F. bidentis* *FbRbcS1* mRNA linked to *gfpA* and constitutively transcribed from a CaMV promoter (1), or a control construct (CaMV-*gfpA*) lacking the *FbRbcS1* mRNA UTRs, also transcribed from a CaMV promoter (2), were precipitated onto 1 μ M gold microprojectiles. These were bombarded into intact, detached, *F. bidentis* leaves by using biolistic delivery system ("gene gun"). Following bombardment, transformation foci produced by the *FbRbcS1* 5'UTR-*gfpA*-3'UTR construct (within red squares) or the control CaMV-*gfpA* construct (within purple squares) were identified and visualized by fluorescent microscopy. The two panels located to the right of the figure show fluorescent images taken from the surface of C₄ *F. bidentis* leaf transformation foci that were transiently expressing either experimental or control constructs. The top right panel shows green fluorescent protein (GFP) expression from the *FbRbcS1* 5'UTR-*gfpA*-3'UTR that is highly specific to the layer of bundle sheath cells surrounding the leaf veins, demonstrating post-transcriptional control of BS cell-specificity. The bottom right panel shows no cell-type specific GFP fluorescence in leaves bombarded with the CaMV-*gfpA* control construct, which lacked the *FbRbcS1* UTRs. These transient expression studies demonstrate that the 5' and 3' UTRs from an *F. bidentis* *RbcS* mRNA are sufficient, in themselves, to mediate BS cell-specific gene expression in the leaves of this C₄ dicot (Experimental design and modified images from Patel et al., 2006).

control of cell type-specific Rubisco mRNA accumulation in C₄ plants is mediated in large part by cell-type specific degradation of these transcripts in MP cells, or by specific stabilization of these transcripts in BS cells.

In an effort to isolate mRNA binding proteins involved in the post-transcriptional regulation of C₄ Rubisco gene expression,

biochemical analysis was used to identify proteins that interact with 5' regions of *rbcL* mRNA in vivo and in purified plastid extracts of amaranth and *F. bidentis* (McCormac et al., 2001; J.O. Berry and M. Patel, unpublished results). Mapping of protein binding sites in the 5' region of *rbcL* mRNA identified only three principle sites of mRNA-protein interaction that corre-

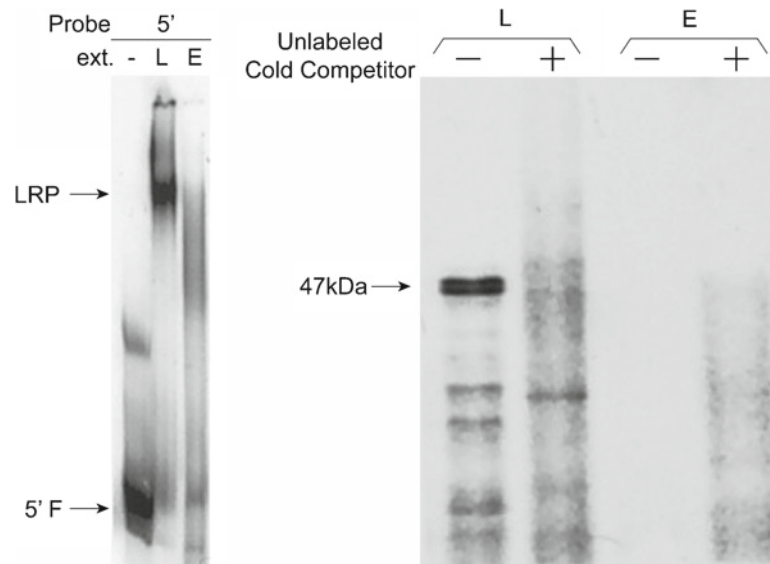


Fig. 3. Two methods used to identify binding proteins (possible post-transcriptional regulatory factors) specific to *rbcL* mRNA in light-grown *C₄* plants. *Left panel*, gel mobility shift analysis. In vitro labeled RNAs corresponding to the 5' portion of amaranth *rbcL* mRNA were incubated with plastid extracts prepared from light-grown (L) or etiolated (E) amaranth plants. The control reaction (–) was incubated in the absence of any plastid extract. Note that extracts from L plastids produced a clear shift in the mobility of the *rbcL* 5' RNA, relative to the control lane, and distinguishable from the diffuse smear produced from the E extracts. The 5' RNA–protein complex observed by the mobility shift in L (5'LRP) is indicative of an interaction between *rbcL* mRNA and one or more proteins present in the extract. Note that uppermost band in the free 5' RNA lane (first lane) represents an additional secondary structure that this RNA adopts under nondenaturing conditions. *Right panel*, UV crosslinking of p47 to *rbcL* 5'RNA. Plastid extracts from light-grown (L) or etiolated (E) plants were incubated and crosslinked with ³²P-5'RNA in the absence (–) or presence (+) of unlabeled self-competitor, and then digested with RNase cocktail. Plastid extracts from L plants contain binding activity for the 5' portion of *rbcL* mRNA. This binding is not observed in plastid extracts from D plants. The most prominent activity is observed as a protein of approximately 47 kDa in size. Background signal in (+) competitor lanes was due to incomplete RNase digestion in the presence of excess of competitor RNA (*Right panel* from McCormac et al., 2001).

lated with ribosome-association and activation of *rbcL* translation. If protein binding at these sites is involved with light-mediated activation of *rbcL* translation, then the function of these binding proteins could possibly overlap with post-transcriptional activation of *rbcL* expression in BS chloroplasts. Gel-shift and UV crosslinking studies (Fig. 3) have identified an *rbcL*-specific mRNA binding protein with specificity for the 5' portion of the transcript, with an approximate molecular mass of 47 kDa (designated p47). These findings have indicated three possible functions for this protein (McCormac et al., 2001). First, p47 could be responsible for translation-associated processing of *rbcL* mRNA to the –66 position (relative to the translation start codon). Second, p47 could be responsible for capping and protecting the polysome-bound *rbcL* mRNAs from degradation following cleavage at the –66 site. Third, p47 could be one of a

small group of regulatory proteins that specifically mobilize these transcripts to polysomes. These potential functions are not exclusive; p47 could have multiple roles in the regulation of *rbcL* mRNA translation/stabilization. A focus for future research will be to determine if cytoplasmic- and plastid-localized RNA binding proteins such as p47, which appear to be involved in the light-mediated expression, might also be involved in determining the specificity of *rbcL* mRNA translation/stability in BS cells.

VII. Conclusions, Future Directions, and Molecular Engineering of *C₄* Capability

A review of previous and ongoing studies of photosynthetic gene expression in BS and MP cells of *C₄* plants, as presented in this chapter, reveals a considerable amount of information

about the types of regulation responsible for this unique CO₂ assimilation pathway. Despite the information gained so far, studies of regulatory mechanisms controlling the different genes in C₄ plants cells have lagged behind studies of gene expression in C₃ plants and other more basic photosynthetic organisms. This is likely due in part to the challenges of investigating gene regulation in complex C₄ systems, as well as the tendency to focus much of plant gene regulation studies on more amenable C₃ and algal model systems. It is now important to apply knowledge from such model systems to the more specialized processes in C₄ plants. Findings to date present a complex and interactive system of C₄ gene expression, involving multiple levels of regulation occurring in different cellular compartments within the specialized leaf cell types. While it is apparent that both transcriptional and post-transcriptional control mechanisms contribute to C₄ gene expression, it is likely that the relative contributions of each level of regulation will vary between different C₄ genes, and between different species of C₄ plant. Superimposed on this complexity is the apparent involvement of numerous regulatory factors, which have yet to be identified, and which ultimately underlie the C₄ gene expression characteristics of BS and MP cells. It is likely that similar cis acting regulatory sequences on DNA and RNA, as well as the regulatory factors that interact with them, will occur in both C₃ and C₄ plants. However, at some level regulation will have diverged between these groups, either through the functional modification of pre-existing regulatory factors, or through the acquisition of completely novel processes not present in C₃ species.

If mechanisms responsible for high-level, cell-specific gene expression patterns can be elucidated in C₄ plants, then ultimately it may be possible to engineer C₄ photosynthetic capability into agriculturally-important C₃ crop plants such as rice. The goal would be to improved CO₂ assimilation and/or environmental adaptability by introducing either full C₄ capacity, or more likely artificial C₃-C₄ intermediates with some C₄ traits, such as elevated, cell-specific accumulation of some photosynthetic proteins (Matsuoka et al., 2001; Taniguchi et al., 2008). However, when considering the prospect of introducing C₄ capacity into C₃ crop species, it

is important to consider the challenges likely to be encountered in the engineering of complex C₄ systems, including the involvement of many genes, and coordination of these processes within and between the involved cell types. Reorganizing the functional existing genetic systems within a C₃ plant into a C₄ system will require a better understanding of BS and MP cell-specific molecular recognition systems and associated regulatory components. Based on the information currently available, it is likely that multiple stages of plant engineering would be required to introduce full, or even intermediate, C₄ capacity into a C₃ plant species. However, the observation that C₄-like capability occurs within the vascular cells of non-leaf tissues in C₃ plants (Hibberd and Quick, 2002) has provided new information about how C₄ capability in leaves might have originated from processes already existing and functional processes in C₃ species. In addition, it is apparent that groups of genes encoding C₄-like enzymes have been recruited together in some plant species to function in certain other specialized processes, such as heat production in thermogenic *Arum* species (Chivasa et al., 1999). Thus, it is possible that coordinate modification of existing regulatory processes, to achieve new gene expression patterns directed towards a specialized function, may involve less extensive modification of gene expression than anticipated. In spite of the apparent complexity, perhaps only a few changes to as yet uncharacterized regulatory processes would be required to reorganize existing C₃ processes and lead to the development of full C₄ capability. Ongoing research efforts to identify regulatory sequences for BS and MP cell-specific genes, and the binding proteins they interact with, are essential for understanding conserved and divergent regulatory processes responsible for photosynthetic function at the molecular level in C₄ plants.

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Chapter 13

C₄-Phosphoenolpyruvate Carboxylase

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Summary	257
I. Phosphoenolpyruvate Carboxylase: An Overview	258
A. Origin of Plant PEPCs	258
B. Genes and Gene Families	258
C. The Enzyme: Biochemistry and Regulation	260
D. Differences Between C ₄ and Non-photosynthetic ppc Genes	263
II. Evolutionary Origin of C ₄ PEPCs	263
III. Molecular Evolution of C ₄ PEPCs	266
A. Protein Properties	266
B. Changes in Gene Expression	269
IV. Outlook	272
References	272

Summary

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is one of the enzymes indispensable for all variants of the C₄ photosynthetic pathway. C₄ photosynthesis evolved polyphyletically implying that the genes encoding the C₄ PEPC originated several times independently from non-photosynthetic ancestral genes. During the evolution of C₄ photosynthesis the photosynthetic PEPCs acquired distinct properties that distinguish them considerably from other PEPCs of higher plants. These changes include the modification of kinetic and regulatory properties of the enzyme as well as the high and cell-specific expression of C₄ PEPC genes. In this review, beside a brief introduction to general aspects of plant PEPCs, we discuss the evolutionary origin of C₄ PEPCs and how their specific properties might have been realised on the molecular level.

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I. Phosphoenolpyruvate Carboxylase: An Overview

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a cytosolic enzyme and catalyzes the irreversible carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate and phosphate. The enzyme is involved in a variety of important physiological tasks in the metabolic and photosynthetic contexts of bacteria, algae and higher plants, including photosynthetic and anaplerotic CO₂ fixation, production of carbon skeletons in symbiotic nitrogen fixation, modulation of turgor in stomatal guard cells, maintenance of ion balance, pH regulation, and others (Ting and Osmond, 1973a; Latzko and Kelly, 1983; Winter, 1985; Melzer and O'Leary, 1987; Schuller et al., 1990; Cushman and Bohnert, 1999).

PEPC was isolated for the first time in 1953 from spinach leaves (Bandurski and Greiner, 1953) and since then PEPC was found in all so far investigated plants, algae and bacteria, but not in any animals or fungi. Recently PEPC genes were even identified in the genomes of several *Archaea* species. However, these archaeal-type enzymes differ strongly from plant and other bacterial PEPCs as they are much smaller and lack typical regulatory properties and the allosteric sites responsible for this regulation (Ettema et al., 2004; Patel et al., 2004; Matsumura et al., 2006).

A. Origin of Plant PEPCs

The origin of the eukaryotic PEPCs is not clear so far. Based on their occurrence only in higher and lower plants one would assume that PEPC genes (*ppc* genes) were introduced into the plant genome via endosymbiotic gene transfer from the eubacterial ancestor of chloroplasts (Lepiniec et al., 1994). If this would be true, cyanobacterial PEPCs should show the highest similarity to the eukaryotic PEPCs. However this is not the case, the plant enzymes are more similar to PEPCs from γ -proteobacteria than from cyanobacteria

(Fig. 1 (Cushman and Bohnert, 1999; Gehrig et al. 2001; Sánchez and Cejudo, 2003)). Such a relationship was observed for other plant genes before (Schnarrenberger and Martin, 2002) and indicates that some lateral gene transfer must have been taken place very early in the history of the plant lineage. Alternatively, the α -proteobacterial progenitor of the mitochondria or, more likely, the cyanobacterial progenitor of the chloroplast must have had acquired a γ -proteobacterial *ppc* gene via lateral gene transfer before endosymbiosis (Schnarrenberger and Martin, 2002).

B. Genes and Gene Families

PEPCs of higher plants consist of up to 1,000 amino acids and have a molecular mass of about 110 kDa (Lepiniec et al., 1994; Rajagopalan et al., 1994). The largest PEPC catalytic subunit reported to date is the CrPpc2 protein from *Chlamydomonas reinhardtii*, consisting out of 1,221 amino acids, with a molecular mass of 131 kDa (Mamedov et al., 2005). The active form of plant PEPCs, as of all other so far known PEPCs, is a homotetramer with four active sites. Most plant *ppc* genes investigated so far exhibit a very conserved structure consisting of 10–11 exons and nine to ten introns whose positions are also very conserved. The divergences in exon and intron numbers is due to the presence or absence of an intron in the 5' untranslated regions of some *ppc* genes (Hermans and Westhoff, 1992).

In most higher plants small *ppc* gene families with three to four gene classes exist while each gene class may consist of one to several individual genes. For instance, four *ppc* genes were found in *Arabidopsis thaliana*, six in rice and *Sorghum bicolor*, three to four in *Flaveria trinervia* and sugarcane, and three genes were found in maize, *Brassica napus* or *Alternanthera pungens* (Ernst and Westhoff, 1996; Dong et al., 1998; Besnard et al., 2003; Sánchez and Cejudo, 2003; Gowik et al., 2006; Paterson et al., 2009; Wang et al., 2009).

In plant species whose genomes were fully sequenced like *Arabidopsis thaliana* and *Oryza sativa*, a stand-alone class of plant PEPCs was discovered. The genomes of both plants harbour a *ppc* gene with a drastically different gene structure compared to other plant *ppc* genes. While the *Arabidopsis* gene (*Atppc4*) consists of 20 exons,

Abbreviations: DCDP – 3,3-Dichloro-2-dihydroxyphosphinomethy-2-propenoate, a PEP analogue; MEM1 – Mesophyll expression module 1; PEP – Phosphoenolpyruvate; PEPC – Phosphoenolpyruvate carboxylase; PEPCCK – PEPC-kinase; *ppc* – PEPC gene

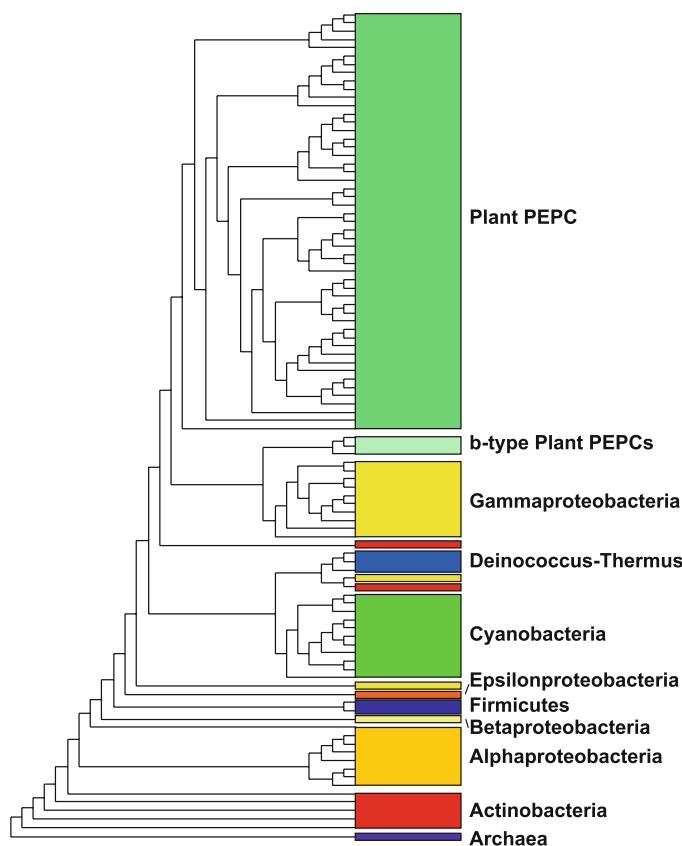


Fig. 1. Phylogenetic analysis of representative phosphoenolpyruvate carboxylases from eukaryotes and diverse groups of bacteria. Protein sequences were retrieved from GenBank and aligned with Clustal X 1.83 (Thompson et al., 1997). The phylogenetic tree was calculated with the Neighbour-Joining method as implemented in PAUP 4.10b (Swofford, 2002).

16 exons were found in the rice gene *Osppc-b* (Sánchez and Cejudo, 2003). The enzymes encoded by these genes show a high similarity to each other (70%) and are more similar to bacterial PEPCs (42% similarity between *Atppc4* and the *Escherichia coli* PEPC) than to other plant PEPCs (39–40% similarity between *Atppc4* and the other *Arabidopsis* PEPCs) (Sánchez and Cejudo, 2003). A N-terminal phosphorylation motif conserved in all other plant PEPCs is missing in these two enzymes as in all known bacterial PEPCs. Therefore these two plant PEPCs were designated as bacterial-type PEPCs (Sánchez and Cejudo, 2003). Meanwhile bacterial-type PEPCs were also detected in *Vitis vinifera*, *Ricinus communis*, *Glycine max* and *Sorghum bicolor* (GenBank accession: AM424873, EF634318) (Sullivan et al., 2004; Wang et al., 2009). It is not clear so far whether these bacterial-type *ppc* genes exist in all

other plant species. On the one hand this seems to be unlikely since the *ppc* gene families of many plant species have been examined in detail without finding a bacterial-type *ppc* gene, on the other hand the sequences of the so far known bacterial-type *ppc* genes are quite different from plant *ppc* genes and the bacterial-type *ppc* genes are expressed only very weakly (Sánchez and Cejudo, 2003). It might be possible, therefore that these genes have escaped detection when *ppc* gene families were analysed by PCR or hybridisation methods. It was demonstrated that a bacterial PEPC is involved in forming a hetero-octameric class-2 PEPC complex in developing castor oil seeds (Gennidakis et al., 2007). The function and biological relevance of such class-2 PEPC complexes is largely unknown so far.

A phylogenetic analysis revealed that both types of plant PEPCs diverged early during the

evolution of plants from a common ancestor gene, related to PEPC genes from γ -proteobacteria (Sánchez and Cejudo, 2003). The existence of bacterial-type *ppc* genes both in mono- and dicotyledonous plants lead to the assumption that these genes exist or at least have existed in all groups of higher plants.

C. The Enzyme: Biochemistry and Regulation

PEPC uses Mg^{2+} as cofactor and the active form of the enzyme is normally a homotetramer with four active sites (Izui et al., 2004). PEP and bicarbonate serve as substrates to produce oxaloacetate and phosphate (Fig. 2). PEPC activity is controlled by various factors. Glucose-6-phosphate is an activator, aspartate and malate are feedback inhibitors (Bauwe and Chollet, 1986; Andreo et al., 1987). PEPCs of monocots are also activated by glycine, while PEPCs of dicotyledonous plants are not (O'Leary, 1982). Additionally, PEPC is regulated by reversible phosphorylation at a conserved serine residue near the amino terminus of the enzyme (Jiao and Chollet, 1991; Duff and Chollet, 1995). Since high resolution three-dimensional structures of the photosynthetic maize PEPC as well as of the *E. coli* PEPC are available and careful studies including site directed mutagenesis and kinetic measurements have been performed, the mechanisms of catalysis and some of the regulatory properties could be elucidated at the molecular level.

The PEPC monomer consists of an eight-strand β -barrel and about 40 α -helices (in case of the maize C_4 PEPC) (Fig. 3). The catalytic

site is located at the C-terminal region of the β -barrel. The three dimensional structure of this catalytic site, and especially the structure of the Mg^{2+} binding site, is very similar to the catalytic sites of pyruvate kinase and pyruvate orthophosphate dikinase although there are no significant sequence similarities between these three enzymes (Izui et al., 2004). Pyruvate kinase and pyruvate orthophosphate dikinase utilize PEP and require a bivalent cation like Mg^{2+} and their catalytic sites are located at the C-terminal region of a β -barrel.

Based on the three-dimensional structures of the *E. coli* protein that was crystallized with Mn^{2+} and/or the PEP analogon DCDP (3,3-dichloro-2-dihydroxyphosphinomethy-2-propenoate) a ordered reaction mechanism was proposed with the bivalent cation, in vivo normally Mg^{2+} , binding first followed by PEP and finally bicarbonate (Kai et al., 2003; Izui et al., 2004). Mg^{2+} is bound to the carboxyl groups of glutamate 566 (numbering of amino acids corresponds to the maize enzyme) and aspartate 603. PEP is bound to the arginine residues 456, 647, 759 and 773. Arginine 647 is near the phosphate group and partially neutralizes its negative charge. Bicarbonate is bound by the lysine residues 606 and 762 and the arginine residues 763 and 764. The methylene group of PEP is incorporated in a hydrophobic pocket consisting of histidine 177, tryptophane 288, leucine 564 and methionine 598. Histidine 177 is indispensable for catalysis and supposed to stabilize a carboxyphosphate intermediate, emerging during the reaction, and abstracting a proton from its carboxyl group. Based on kinetic measurements it was proposed earlier that Mg^{2+} and PEP do not

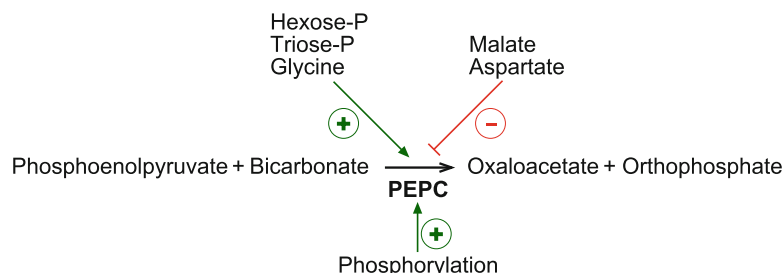


Fig. 2. Enzymatic reaction and regulation of phosphoenolpyruvate carboxylase. Metabolite products of photosynthesis such as sugar phosphates stimulate the enzyme, resulting in a lowered K_m for the substrate PEP. Glycine is a specific activator of monocot but not of dicot PEPCs. Malate and other four-carbon organic acids (oxaloacetate, aspartate) are feedback inhibitors affecting non-photosynthetic PEPCs more severely than C_4 isozymes. Phosphorylation of the N-terminal serine residue affects the impact of both metabolite activators and inhibitors.



Fig. 3. Amino acid sequence of the *ppc*-C4 PEPC of maize. Amino acid residues of experimentally proven function (According to Kai et al., 2003) are labelled by grey boxes. Secondary structure elements (According to Kai et al., 1999) are indicated by orange cylinders (α-helices) and yellow arrows (β-strands).

bind separately to the enzyme but that a Mg–PEP complex is the actual substrate of PEPC (Tovar-Mendez et al., 1998, 2000). So far it is not clear which of the suggested mechanisms of substrate binding is correct.

Phosphorylation at a N-terminal serine residue leads to an activation of the enzyme by reducing its sensitivity towards the allosteric inhibitors malate and aspartate (Vidal and Chollet, 1997; Nimmo, 2000). The phosphorylation site is conserved in

eukaryotic PEPCs but not found in the PEPCs of bacteria. The enzyme is phosphorylated by a Ca²⁺-independent serine/threonine kinase that shows a high substrate specificity and therefore was named PEPC-kinase (PEPCK) (Hartwell et al., 1999). PEPCKs are encoded by small gene families. They have a molecular weight of approximately 30 kDa and are the smallest protein kinases reported yet. At first, PEPCK activity was thought to be regulated by de novo synthesis and constant rapid

degradation (Chollet et al., 1996). Meanwhile other regulatory mechanisms like a PEPCK inhibitor protein and a possible redox regulation were discovered (Nimmo et al., 2001; Saze et al., 2001). However, the *in vivo* significance of these mechanisms is not clear so far.

Previously it was proposed that the phosphoryl group at the N-terminus could reach and block the binding site of malate and aspartate and by this prevent the binding of these allosteric inhibitors to the enzyme. This model is in line with the three-dimensional structure of the enzyme as well as with the observed effects after PEPC phosphorylation (Kai et al., 1999). Nevertheless this model is questioned by experiments modifying the PEPC N-terminus and measuring the effects on the enzyme's regulation. Among others a modified version of the C_4 maize PEPC with a N-terminal truncation of 33 amino acids was investigated. This enzyme showed a marked desensitization towards malate and aspartate comparable with a phosphorylated enzyme. The fact that truncation of the N-terminus mimics the effect of the phosphorylation is of course in contrast with the above described model (Izui, et al. 2004). There is some evidence that phosphorylation of the enzyme interferes in some way with allosteric regulatory mechanisms of the enzyme. Modified PEPCs which were insensitive to the allosteric activator glucose-6-phosphate, caused by the mutation of arginine residues involved in the glucose-6-phosphate binding (see below), were also insensitive to phosphorylation, indicating the involvement of these arginine residues also in activation by phosphorylation (Takahashi-Terada et al., 2005). In the moment, therefore a simple and consistent mechanistic model for the activation of plant PEPCs by phosphorylation is not available.

A analysis of *Flaveria bidentis* (C_4) plants with massively reduced PEPCK activity, caused by RNAi or antisense techniques, raises doubts about the importance of this regulatory mechanism in planta. Although PEPC phosphorylation was drastically reduced and the PEPC isolated from these plants exhibited an increased sensitivity towards malate the transgenic plants showed no aberrant phenotypes under green house conditions (Furumoto et al., 2007). No differences in growth, in the CO_2 and light response of CO_2 assimilation rates between these plants and wild

type plants were observed. This suggests that regulatory phosphorylation is not that important for the function of at least the photosynthetic PEPCs of C_4 plants (Furumoto et al., 2007).

In addition to phosphorylation plant PEPCs are regulated by metabolites. Aspartate and malate are allosteric inhibitors of the enzyme and act competitively to the substrate PEP. The three-dimensional structure of the *Escherichia coli* PEPC crystallized together with bound aspartate indicates that the binding site for aspartate is about 2 nm away from the catalytic site of the enzyme (Kai et al., 1999). The completely conserved four residues, R647, K835, R894 and N968 (Fig. 3) participate directly in the binding of aspartate. The residue R647 is part of a mobile loop and also involved in PEP binding and the catalytic activity (Izui et al., 2004). On inhibitor binding R647 is trapped away from the catalytic site which explains that aspartate acts competitively to PEP. Since site directed mutagenesis of either K835 or R894 of the maize C_4 PEPC caused marked desensitization to aspartate as well as to malate it is highly probable that malate and aspartate bind to the same site of the enzyme and that both metabolites share a common mechanism of inhibition (Izui et al., 2004).

Sugar-phosphates like glucose-6-phosphate and triose-phosphates as well as some amino acids like glycine or alanine act as allosteric activators (Tovar-Méndez et al., 2000). Activation via glucose-6-phosphate is achieved by elevating the enzyme's affinity for its substrate PEP as well as by altering the affinity to allosteric inhibitors. While the effect on PEP affinity is equal for all PEPCs investigated so far, it may differ regarding the action of allosteric inhibitors. The photosynthetic PEPC of maize as well as the *ppcA* PEPCs of *F. pringlei* (C_3) and *F. pubescens* (C_3/C_4 intermediate) are desensitized towards malate in the presence of glucose-6-phosphate (Engelmann et al., 2003; Takahashi-Terada et al., 2005). In contrast, the *ppcA* PEPCs of *F. trinervia* (C_4) and *F. brownii* (C_4 -like) are more sensible towards malate after activation by glucose-6-phosphate (Engelmann et al., 2003).

The residues constituting the glucose-6-phosphate binding site can be inferred from the three-dimensional structure of the C_4 maize PEPC, that has been crystallized together with sulphate, which acts as an analogue of glucose-6-phosphate

(Matsumura et al., 2002; Takahashi-Terada et al., 2005). The tetrameric PEPC holoenzyme is organized in a “dimer-of-dimers” structure. The glucose-6-phosphate binding site is located at the dimer interface. The arginine residues 183, 184 and 231 are involved in the binding of glucose-6-phosphate within each monomer. Additionally arginine 372 from the adjacent subunit of the dimer contributes to the binding site (Kai et al., 2003; Takahashi-Terada et al., 2005). Substitution of one of these arginine residues by glutamine led to a marked (R231) or complete (R183, R184 and R372) desensitization towards glucose-6-phosphate (Takahashi-Terada et al., 2005). Interestingly, these mutations did not alter the sensitivity of the enzyme towards glycine (Takahashi-Terada et al., 2005). This indicates different binding sites as well as different mechanisms of activation for glycine and glucose-6-phosphate, explaining that glucose-6-phosphate activates dicot as well as monocot PEPCs whereas the effect of glycine is restricted to monocot enzymes (O’Leary, 1982).

The different mechanisms of PEPC regulation cannot be understood independently but are closely interrelated. As mentioned above the sensitivity of the enzyme towards allosteric inhibitors is influenced by activation via glucose-6-phosphate or phosphorylation. Mutations affecting the binding of glucose-6-phosphate also alter the effects of phosphorylation or the glycine triggered activation of the C₄ maize enzyme. The regulation of PEPC becomes even more complicated since kinetic studies hint strongly that binding of the substrate PEP, perhaps at a site different from the catalytic site, can also interfere with glucose-6-phosphate binding. At least in case of the C₄ maize enzyme, this leads to a stronger activation of this PEPC (Tovar-Méndez et al., 2000; Yuan et al., 2006).

D. Differences Between C₄ and Non-photosynthetic *ppc* Genes

During evolution of C₄ photosynthesis the photosynthetic PEPCs acquired distinct properties that distinguish them clearly from other PEPCs of higher plants. These changes include the modification of the kinetic properties of the enzyme as well as the expression of photosynthetic *ppc* genes.

The PEPCs involved in C₄ photosynthesis bind PEP with a lower affinity than the non-photosynthetic PEPCs (Svensson et al., 1997; Dong et al.,

1998; Gowik et al., 2006; Lara et al., 2006). On the other hand the affinity to their other substrate bicarbonate is higher (Bauwe, 1986). C₄ PEPCs are more tolerant towards the allosteric inhibitors aspartate and malate and that they are more strongly affected by the allosteric activators glucose-6-phosphate or glycine (Dong et al., 1998; Svensson et al., 2003; Gowik et al., 2006). While substrate saturation curves for the substrate PEP of most PEPCs (including the C₄ PEPC of maize) follow a Michaelis–Menten kinetic, the C₄, but not the non-photosynthetic PEPCs of the genera *Flaveria* and *Alternanthera*, show a sigmoid saturation kinetic for PEP, indicating cooperative PEP binding. When activated with glucose-6-phosphate the cooperativity disappears, and the PEP saturation curves of these enzymes become hyperbolic, too (Svensson et al., 1997; Gowik et al., 2006). From what is known so far it appears that C₄ PEPCs react on phosphorylation in the same way as non-photosynthetic PEPCs. However, the PEPCKs of C₄ plants are regulated differentially compared to PEPCKs of C₃ plants. As a consequence C₄ PEPCs are phosphorylated and activated in the light, whereas non-photosynthetic PEPCs are phosphorylated in the dark (Vidal and Chollet, 1997; Fukayama et al., 2003; Bailey et al., 2007).

C₄ photosynthesis is characterized by the division of labour between mesophyll and bundle sheath cells that relies on the differential expression of the carboxylases PEPC and RUBISCO in these two cell types. It must be ensured therefore, that C₄ PEPCs are only expressed in the mesophyll cells of the leaves. In addition, high PEPC levels are required in the mesophyll cells of leaves to accomplish the high fluxes of metabolites needed for proper function of C₄ photosynthesis. In contrast, in the leaves of C₃ plants PEPC is expressed at a much lower level and not in a cell-specific manner (Sheen, 1999). There is convincing evidence that the C₄-type mode of *ppc* gene expression evolved by changes in the transcriptional control of these genes (Stockhaus et al., 1997; Sheen, 1999).

II. Evolutionary Origin of C₄ PEPCs

C₄ photosynthesis arose several times independently during the evolution of higher plants. According to Sage (2004) it evolved at least

32 times in eudicots and 16 times in monocots. Consequently, photosynthetic PEPCs must have evolved from non-photosynthetic isoforms at least 48 times (Sage, 2004; Muhaidat et al., 2007). Phylogenetic analyses support this inference (Gehrig et al., 1998, 2001; Fig. 4). The dicot C_4 PEPCs do not group together but with non-photosynthetic PEPCs from the same or closely related species. This indicates that they evolved independently from non-photosynthetic ancestor enzymes (Fig. 4). It is assumed that the evolution of C_4 PEPCs as the evolution of the other C_4 cycle enzymes required gene duplication with subsequent diversification through neo-functionalization (Monson, 2003). This acquisition of new functions by a pre-existing gene through gene duplication events and changes in expression patterns and/or functional modifications of the encoded protein of one of the gene copies is known as gene co-option (Olson, 2006).

This mode of gene evolution can easily be followed for the evolution of the photosynthetic PEPCs in the genus *Flaveria*. In C_4 *Flaveria* species the C_4 PEPCs are encoded by the *ppcA* genes. These genes are also present in C_3 *Flaveria* species where they encode non-photosynthetic isoforms in these species (Bläsing et al., 2002). The *ppcA* gene arose by duplication of a *ppcB*-like gene leading to the present *ppcA* and *ppcB* genes. It follows that both genes must have been existed already in the last common ancestor of the present C_3 and C_4 *Flaverias* (Bläsing et al., 2002).

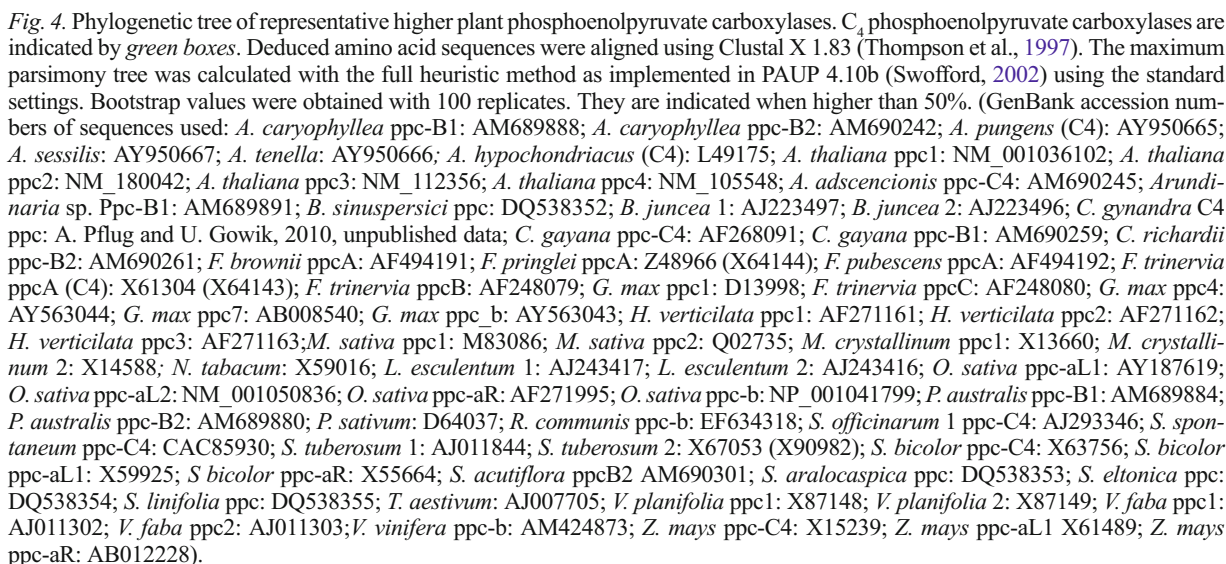
The functions of the *ppcB* and *ppcC* genes are not precisely known but according to their accumulation patterns and kinetic properties one can assume that *ppcB* encodes the housekeeping- and *ppcC* the root-isoform PEPC of *Flaveria* (Ernst and Westhoff, 1996; Bläsing et al., 2002).

The function of the non-photosynthetic *ppcA* PEPC in C_3 plants is completely unclear. The gene is expressed only very weakly in leaves stems and roots with no apparent organ specificity (Ernst and Westhoff, 1996). According to the phylogeny of the genus *Flaveria*, which was, based on both morphological and molecular characters all C_4 and C_4 -like species form a distinct clade (clade A), while most of the C_3/C_4 intermediate species of the genus, with exception of *F. ramosissima* and *F. sonorensis*, are contained within clade B (McKown et al., 2005). The C_3

species *F. pringlei* and *F. cronquistii* as well as *F. robusta* are basal (McKown et al., 2005). The *ppcA* genes of the C_3/C_4 intermediate species *F. pubescens* and C_4 -like C_3/C_4 intermediate species *F. brownii*, both belonging to clade B, still show C_4 properties, e.g. a higher expression level and typical C_4 specific kinetic properties of the encoded enzymes (Engelmann et al., 2003). One has to infer that co-option of the *ppcA* gene and first development of C_4 properties happened before the partition of the A and the B clade.

The photosynthetic PEPC of *Cleome gynandra*, a C_4 plant form the genus *Cleome*, the most closely related genus to the C_3 model plant *Arabidopsis thaliana* (Brown et al., 2005; Marshall et al., 2007), shows the highest similarity to the *ppc2* gene of *A. thaliana* (Fig. 4; A. Pflug and U. Gowik, 2010, unpublished data). Of all four known *Arabidopsis ppc* genes *ppc2* shows the highest expression in leaves, whereas *ppc1* is expressed ubiquitously and *ppc3* is preferentially expressed in the roots. *Ppc4* represents the bacterial-type *ppc* gene of *Arabidopsis* and is expressed mainly in flowers and siliques (Sánchez and Cejudo, 2003). Assuming that the orthologous genes of C_3 *Cleome* species show similar expression patterns, it appears that the *ppc* gene recruited for development of C_4 traits was already highly expressed in leaves but not in other organs. Interestingly, in phylogenetic analyses the *ppc2* genes of *Arabidopsis* and *Cleome* do not cluster with other *ppc* genes from the Brassicales, but constitute their own branch (Fig. 4; (Sánchez and Cejudo, 2003)). This indicates the existence of a special *ppc* gene class within the Brassicales which differed clearly in its sequence from the other *ppc* genes and was at least once recruited for the evolution of C_4 traits.

Within the grasses six different *ppc* gene classes can be distinguished, where genes from the same class, but from distantly related species, are more closely related to each other than genes from closely related species belonging to different classes. This indicates that these different PEPC classes must have formed very early in grass evolution and that they have already existed in the last common ancestor of all recent grass species. One of these *ppc* classes, which exists solely in C_4 grasses, consists exclusively of photosynthetic PEPCs (Christin et al., 2007; Fig. 4). This seems to contradict the fact that C_4 photosynthesis



evolved eleven times independently in the grasses (Sage, 2004). However, the species tree deduced from the C_4 *ppc* genes is incongruent with phylogenetic trees inferred by the use of other genes sequences. It could be shown that the high degree of similarity of the grass C_4 PEPCs is the result of positive selection (Christin et al., 2007). All C_4 PEPCs developed from *ppc-B2* genes which show a high similarity to the *ppc-C4* genes and which are absent in most C_4 species (Christin et al., 2007; Fig. 4). A strong positive selection was found for 21 amino acid positions. When this fact was taken into account for the calculation of phylogenetic trees it was found that the C_4 PEPCs evolved at least eight times independently from non C_4 PEPCs. Thus the high degree of similarity between the C_4 PEPCs of the grasses is caused by convergent evolution (Christin et al., 2007).

Only two of the 21 amino acid positions under positive selection in grass PEPCs (H/N/R/L 665 and A/S 780, numbering corresponds to the C_4 maize enzyme) seem to be important also for the evolution of dicot C_4 PEPCs. This could indicate special requirements for grass C_4 PEPCs when compared to dicot C_4 PEPCs. On the other site this might also reflect the fact that most of the dicot C_4 lineages are very young compared to the first origins of C_4 photosynthesis within the grasses (Ehleringer et al., 1997; Sage, 2004). One may infer therefore, that the C_4 PEPCs of grasses are much more optimized for their role C_4 in photosynthesis than their dicot counterparts, which explains the higher degree of convergence within the photosynthetic PEPCs of the grasses.

III. Molecular Evolution of C_4 PEPCs

A. Protein Properties

C_4 and non-photosynthetic PEPCs differ in their kinetic and regulatory properties. C_4 PEPCs exhibit substrate saturation constants (K_m) for PEP that are usually about ten times larger than those of their C_3 counterparts. The saturation constant for bicarbonate, the second substrate, is lower in C_4 than C_3 PEPCs (Ting and Osmond, 1973b; Bauwe, 1986). The C_4 PEPCs are more tolerant to the inhibitor malate and more sensitive to the activator glucose-6-phosphate (Dong et al., 1998; Bläsing et al., 2002). This is also true

for the orthologous *ppcA* enzymes of *Flaveria trinervia* (C_4) and *F. pringlei* (C_3) or of *Alternanthera pungens* (C_4) and *A. sessilis* (C_3) (Svensson et al., 1997; Gowik et al., 2006). The differences in substrate affinity and accordingly the reaction towards allosteric effectors suggest that C_4 PEPCs harbour specific C_4 determinants that were acquired during the evolution of C_4 photosynthesis. To identify such C_4 determinants at the molecular level the *ppcA* PEPCs of the genus *Flaveria* were used as a model.

In the C_4 plant *F. trinervia* *ppcA* encodes the photosynthetic PEPC while the function of the orthologous gene in the C_3 plant *F. pringlei* is not clear so far. To get an insight how PEPC enzyme characteristics changed during evolution towards C_4 photosynthesis, *ppcA* PEPCs from the C_3/C_4 intermediate plant *F. pubescens* and the C_4 -like C_3/C_4 intermediate *F. brownii* were investigated additionally. Both the K_m (PEP) values and the malate inhibition constants (K_i) of these *ppcA* PEPCs were found to be intermediate between the C_3 and C_4 *ppcA* PEPCs with the *F. brownii* enzyme being more C_4 -like than that of *F. pubescens*. This indicates that the C_3 PEPC evolved step by step into a C_4 enzyme (Engelmann et al., 2003).

Since the C_3 and C_4 *ppcA* isoforms shared 96% identical amino acid positions it was feasible to pinpoint changes in the amino acid sequence responsible for the C_4 characteristics (Svensson et al., 1997). To locate regions and amino acid residues in the enzyme that influence the K_m of PEP, reciprocal domain swapping experiments combined with site-specific mutagenesis were conducted with the two *ppcA* PEPCs of *F. trinervia* and *F. pringlei* (Bläsing et al., 2000). Using this approach two regions, from amino acids 296 to 437 (region 2) and from amino acids 645 to 966 (region 5), were identified that contain the major C_4 determinants for the saturation kinetics of the substrate PEP. The C_4 -specific properties in region 5 could be confined to a single amino acid, serine 774 (Fig. 5) (Bläsing et al., 2000). Insertion of region 2 of the C_4 enzyme and the C_4 -specific serine into an otherwise C_3 background resulted in a chimerical enzyme possessing about two thirds of C_4 PEPC characteristics with respect to K_m (PEP) (Engelmann et al., 2002).

Slightly different results were obtained when the effect of the allosteric inhibitor malate towards the chimeric enzymes was measured. Regions 2

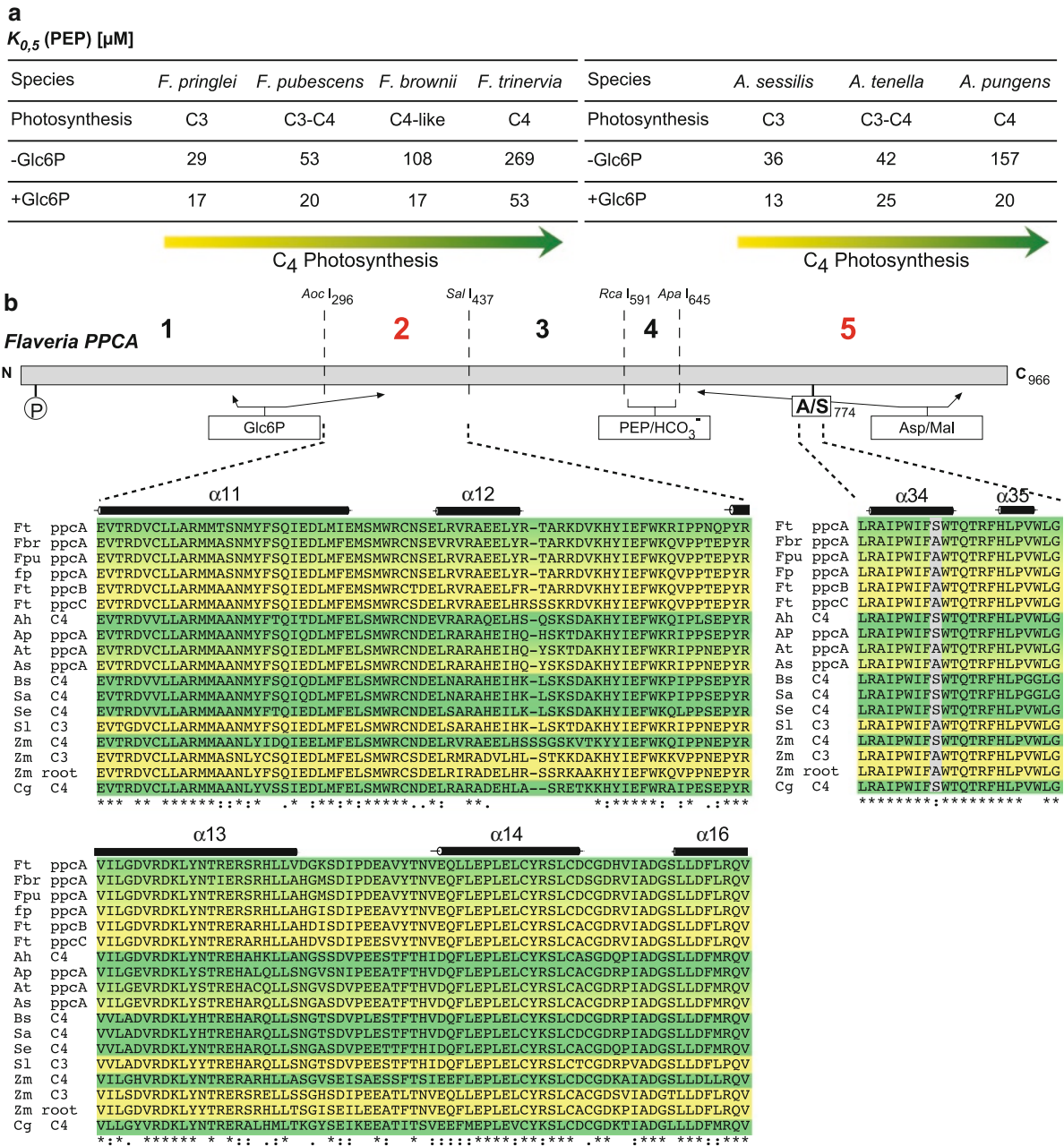


Fig. 5. Kinetic properties of C₃ and C₄ PEPC of *Flaveria* and *Alternanthera* and molecular properties of closely related C₃ and C₄ PEPCs. From five investigated enzyme domains (in *Flaveria*), region 2 (positions 296–437) and region 5 (amino acids 645–966) contain the major C₄ determinants for the saturation kinetics of PEP. P indicates the target phosphorylation site at position 11. The secondary structures indicated on top of the sequence alignments (black bars) were obtained from the 3D structure of the C₄ PEPC of *Zea mays* (Matsumura et al., 2002). Sequence positions, which are identical in all shown PEPCs, are marked by stars below the strings of sequences. At position 774 (grey column) serine occurs only in C₄ PEPCs, while PEPCs from C₃ and C₃/C₄ intermediate plants contain an alanine at this position. The amino acid numbering follows that of the *F. trinervia* protein. Ft: *Flaveria trinervia*; Fbr: *F. brownii*; Fpu: *F. pubescens*; fp: *F. pringlei*; Ah: *Amaranthus hypochondriacus*; Ap: *Alternanthera pungens*; At: *A. tenella*; As: *A. sessilis*; Bs: *Bienertia sinuspersici*; Sa: *Suaeda aralocaspica*; Se: *S. eltonica*; Sl: *S. linifolia*; Zm: *Zea mays*; Cg: *Cleome gynandra*.

and 5 also contain the main determinants for the C_4 -specific high tolerance towards malate (Jacobs et al., 2008), but in case of malate tolerance exchange of serine 774 to alanine or vice versa had no effect. This indicates that this amino acid exchange was not important for the acquisition high tolerance towards malate (Jacobs et al., 2008).

The effect of region 2 on malate tolerance becomes particular obvious when the chimeric enzymes are activated by glucose-6-phosphate. Since the identity of region 2 also strongly affects the modulation of PEP affinity by glucose-6-phosphate (Bläsing et al., 2000; Engelmann et al., 2002) one has to assume that determinants responsible for the effect of glucose-6-phosphate are located in this region 2. The identity of region 2 influences the enzyme's affinity towards the substrate PEP and its reaction towards the allosteric effectors glucose-6-phosphate and malate and one has to conclude that region 2 plays a significant role in the regulation of the enzyme. This can be understood from the three-dimensional structure of the PEPC. Several amino acids located in region 2 are part of the PEPC dimer interface, within the dimer-of-dimer structure of the active PEPC tetramer (Kai et al., 2003), and might therefore be involved in transmitting allosteric interactions beyond this interface.

Sixteen amino acid differences were detected between the C_3 and C_4 *ppcA* PEPCs of *Flaveria* in region 2 (Fig. 5). There is only one amino acid residue, a lysine at position 347, which both *F. trinervia* and *F. brownii* enzymes (C_4 and C_4 -like kinetic properties respectively) have in common and which differs from the arginine in this position in the *F. pubescens* and *F. pringlei* enzymes (more C_3 -like and C_3 kinetic properties, respectively) (Engelmann et al., 2003). This lysine is also conserved in the C_4 PEPC of maize (Matsumura et al., 2002). In contrast in the corresponding region of the *Alternanthera* enzymes (aa 297–438) no corresponding amino acid exchanges could be detected. Nevertheless in *Alternanthera* as in *Flaveria* the C_4 enzyme has a lower affinity to the substrate PEP than the respective C_3 enzyme, indicating that some alterations of the enzyme kinetic properties were realized by different modifications at the molecular level in both genera (Gowik et al., 2006).

The distinct serine residue in the carboxyterminus (serine 774 in the *F. trinervia* and serine 775

in the *A. pungsens* enzyme) is the main determinant for the high K_m with respect to PEP and is very well conserved in C_4 PEPCs. All C_4 enzymes studied to date contain a serine at this position while in all non-photosynthetic and CAM PEPCs this site is occupied by an alanine (Fig. 5, Svensson et al., 2003; Christin et al., 2007). It has to be concluded that serine 774 is of central importance for the evolution of C_4 characteristics, at least with regard to the K_m (PEP). Since all investigated C_3 / C_4 intermediate PEPCs, even from the C_4 -like species *F. brownii*, still show an alanine at this position (Engelmann et al., 2003), one has to infer that the change from alanine to serine occurred only recently during evolution from C_3 to C_4 photosynthesis.

One wonders why this change occurred so late during evolution and, more importantly, why it apparently had to occur? Changing the alanine to a serine residue increases the K_m (PEP) (Bläsing et al., 2000). When, in addition, residues 296–437 are swapped from C_3 to C_4 the K_m (PEP) value raises further, almost reaching that of the C_4 enzyme (Engelmann et al., 2002). Is this increase in K_m (PEP) important to become a kinetically efficient C_4 PEPC or is it rather required because of regulatory characteristics of the C_4 isoform? It is possible that the rise in K_m (PEP) is an even unavoidable side effect, for instance of creating a lower K_m for bicarbonate. An increase in K_m (PEP) may have been necessary to adequately regulate C_4 PEPC, since the PEP concentration in vivo is significantly higher around C_4 PEPCs than around non-photosynthetic PEPCs (Leegood and Walker, 1999). This is supported by experiments with transgenic C_3 plants that constitutively overexpress PEPCs with high affinity to PEP and/or high malate tolerance. These plants were severely disturbed in carbon and nitrogen metabolism suggesting that high levels of PEPCs with low K_m (PEP) or the combination of low K_m (PEP) with high K_i malate interferes with the basic cell metabolism (Rademacher et al., 2002); (Chen et al., 2004). On the other hand the importance of a lower K_m for bicarbonate may be more vital than the apparent disadvantage of a higher K_m (PEP). Consequently, if serine 774 is important for that characteristic, a higher K_m (PEP) might be “the price to pay.”

All plant-type PEPCs identified so far possess a serine residue at the N-terminus which can be

phosphorylated thereby change the regulatory properties of the enzyme. It has not been investigated yet with the isolated *ppcA* PEPCs from C₃, C₃/C₄ and C₄ *Flaveria* species, how phosphorylation of this amino terminal serine influences enzyme characteristics. It was shown for the photosynthetic enzymes of maize and sorghum that phosphorylated PEPCs are less sensitive to the inhibitor malate and more sensitive to the activator glucose-6-phosphate (Vidal and Chollet, 1997). However, as described above C₄ PEPC phosphorylation appears to be of minor importance, at least under controlled environmental conditions in *Flaveria* (Furumoto et al., 2007).

To fully understand the function and regulation of C₄ PEPCs the available recombinant enzymes from C₃, C₃/C₄, and C₄ *Flaverias* and *Alternantheras* or other closely related C₃ and C₄ species will be crucial. Thorough investigation of the kinetic and regulatory properties of these enzymes, with and without phosphorylation combined with activators and inhibitors (Tovar-Méndez et al., 2000), should provide detailed information about the evolutionary steps during C₃ to C₄ PEPC evolution.

Since all in vitro studies suffer from inherent limitations, at the end in vivo analyses will be necessary to critically test the predictions inferred from in vitro enzyme studies. Such studies could involve a knockout of the C₄ PEPC gene in a C₄ plant combined with its replacement by a PEPC gene whose properties is to be assessed. A transformation system for the C₄ plant *F. bidentis* is currently available (Chitty et al., 1994), and therefore *Flaveria* would be the study system of choice in which to pursue this in vivo approach.

B. Changes in Gene Expression

For an efficient function of C₄ photosynthesis it is imperative that the two carboxylases involved in this metabolic pathway are separated from each other. Consequently RUBISCO is exclusively expressed in bundle sheath cells whereas PEPC expression is restricted to the mesophyll compartment. Since non-C₄ PEPCs are usually expressed at low levels in all tissues of the leaves of C₃ plants, the expression of PEPC genes must have been altered during C₄ evolution. PEPC expression appears to be largely regulated transcriptionally (Sheen, 1999) which is

supported by a detailed analysis of the promoter of the photosynthetic PEPC gene (*ppcA*) of the C₄ plant *Flaveria trinervia*.

In the last years more and more evidence was collected that changes in the spatio-temporal expression of genes were the starting point for the development of novel biochemical or morphological traits and that such changes in spatio-temporal gene expression are often realized by the modification of *cis*-regulatory elements (Doebley and Lukens, 1998).

The promoter of the *ppc-C₄* gene of maize was studied in great detail using transgenic maize as well as transgenic rice plants. In both species the promoter shows a high and mesophyll specific activity and is regulated by light (Matsuoka et al., 1994). This indicates that all *trans*-regulatory factors needed for a C₄ specific expression of this gene are present in the C₃ plant rice. So far the *cis*-regulatory elements responsible for this activity pattern could not be identified at the molecular level. Recently it was shown that the activity of the maize *ppc-C₄* promoter is regulated by chromatin modifications like histone acetylation and methylation (Offermann et al., 2006; Danker et al., 2008).

In contrast, the full-size C₄-*ppcA* promoter of *F. trinervia* does not show any cell-specificity in the leaves of the heterologous C₃ dicot *A. thaliana*, i.e. the promoter is active in all leaf parenchyma cells (Akyildiz et al., 2007). When analyzed in *Nicotiana tabacum*, a member of the Solanaceae, the promoter was found to be active in the palisade but not the spongy parenchyma cells. No expression was observed in the vascular bundles (Stockhaus et al., 1994). Thus in both heterologous C₃ backgrounds the mesophyll specificity of expression is not maintained. This implies that the *trans*-regulatory systems operating in the leaf cells of the two species *A. thaliana* and *N. tabacum* differ from that of *F. bidentis* and that this difference causes the non-specific expression of the C₄-*ppcA* promoter. It is likely that the multiple origin of C₄ photosynthesis in the angiosperms involved multiple independent selections of *cis*-regulatory modules for cell-specific gene expression, and therefore the mesophyll-specificity module of the C₄-*ppcA* promoter could be specific for *Flaveria*. Also within the Poaceae the expression of C₄ genes from the Panicoid C₄ grasses maize and *Panicum miliaceum* (Matsuoka et al., 1994;

Nomura et al., 2005a) and the Chloridoid C_4 grass *Zoysia japonica* (Nomura et al., 2005b) in the C_3 grass *Oryza sativa* did not generally result in the maintenance of cell specificity. While some C_4 gene promoters maintain their cell-specificity of expression like the maize *ppc*- C_4 promoter, others do not.

To identify putative *cis*-regulatory elements, relevant for mesophyll specific and high expression in *Flaveria*, the promoter sequences of *ppcA* genes from *Flaveria* species with a C_3 , C_4 or C_3 - C_4 intermediate type of photosynthesis were compared and experimentally analyzed by stable transformation of the C_4 species *F. bidentis* (Stockhaus et al., 1997; Gowik et al., 2004; Akyildiz et al., 2007).

The mesophyll-specific expression of the *ppcA* gene of *F. trinervia* is indeed controlled at the transcriptional level. About 2,200 base pairs of 5'flanking sequences (with reference to the AUG translational start codon) are sufficient to cause high β -glucuronidase (GUS) expression exclusively in the mesophyll cells (Stockhaus et al., 1997) (Fig. 6). In contrast, the 2,538 base pairs (with reference to the AUG start codon) of the 5'flanking sequences of the *ppcA* gene of *F. pringlei* were found to be a weak promoter and did not direct any organ- or cell-specific expression (Stockhaus et al., 1997) (Fig. 6). Both promoters thus exhibited the attributes expected from the accumulation patterns of their corresponding RNAs and proteins. The increase in PEPC gene expression, but exclusively in the leaves, and the confinement of expression to the mesophyll cells must be caused by differences between these two promoter sequences.

As revealed by promoter deletion and recombination studies, a 41-base-pair segment, named MEM1 (mesophyll expression module 1) (Fig. 6), located in the distal segment of the *F. trinervia* promoter in combination with the proximal promoter segment, was sufficient to confer mesophyll specificity of expression to the GUS reporter gene. The proximal promoter part alone leads only to a weak expression in mesophyll and bundle sheath cells and appears to function as a basal, i.e. core promoter (Gowik et al., 2004).

MEM1 homologous sequences were also detected in the *ppcA* promoters of *F. pringlei* as well as of other C_3 , C_4 and C_4 -like *Flaveria* species like *F. cronquistii* (C_3), *F. bidentis* (C_4),

F. palmeri (C_4 -like), *F. vaginata* (C_4 -like), *F. brownii* (C_4 -like C_3/C_4) or *F. pubescens* (C_3/C_4) (Fig. 6) (Gowik et al., 2004; Akyildiz et al., 2007). Their comparison revealed that MEM1 sequences consist of two parts, A and B, which are contiguous in *F. trinervia*, but are separated by 97–108 base pairs in the other *Flaveria* promoters (Fig. 6) (Gowik et al., 2004). The A-parts of all C_4 and C_4 -like species show a guanine at their first nucleotide position, while an adenine is present in the A-homologues of the two C_3 species. A similar C_4 to C_3 associated difference is also found for the tetranucleotide CACT. This assemblage is present in the B parts of all C_4 and C_4 -like species but absent in both C_3 promoters. Interestingly, an intermediate situation was found in the MEM1 sequences of the C_3/C_4 intermediate species *F. pubescens* and the C_4 -like C_3/C_4 intermediate species *F. brownii*. The B submodules of these species show the C_4 specific CACT insertion, while the C_3 specific adenine is still found in the A submodule.

These C_4 to C_3 correlated differences in MEM1 composition are parts of *cis*-regulatory elements within MEM1 critical for mesophyll-specific gene expression. It was found that the *F. trinervia* MEM1 lost its ability to direct mesophyll specific expression when one submodule was deleted or one of the two submodules was converted to the C_3 state. On the other hand, the *F. pringlei* MEM1 acted as a mesophyll specificity element, if the C_4 motives were included (Akyildiz et al., 2007).

MEM1 acts as transcriptional repressor. The addition of this *cis*-regulatory element to the proximal *ppcA* promoter of *F. trinervia*, which shows a low activity in the mesophyll, the bundle sheath and the vascular tissue, leads to a suppression of the activity of this promoter in bundle-sheath cells and the vascular bundle (Akyildiz et al., 2007). To convert a weak promoter with no apparent cell specificity to a strong and cell-specific promoter it may be appropriate in a first step to increase the overall activity. In a second step *cis*-regulatory repressors could be added to ensure that the promoter is exclusively active in the desired cell type.

The C_4 -specific properties of the *ppcA* promoters appear to be implemented in the genus *Flaveria* following the scheme described above. An step-wise increase in *ppcA* transcript levels can even be observed in *Flaveria* species with only weakly

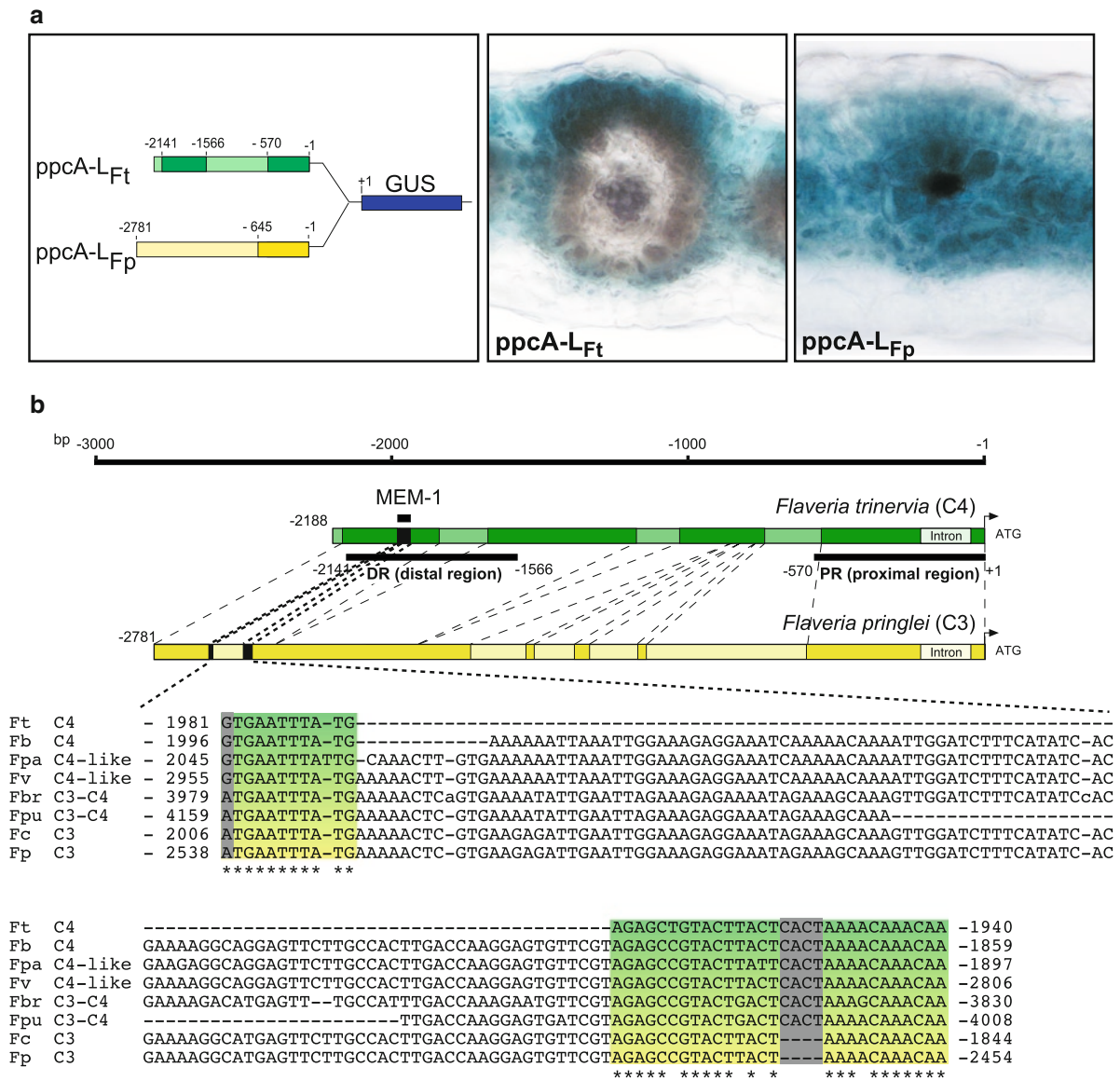


Fig. 6. Molecular evolution of the *ppcA* promoter within the genus *Flaveria*. **(a)** Histochemical analysis of the activities of the *ppcA* promoters of *F. trinervia* (C₄) and *F. pringlei* (C₃) in transgenic *F. bidentis* (C₄) (cf. Stockhaus et al., 1997). **(b)** The structures of the *ppcA* promoters from *F. trinervia* (C₄) and *F. pringlei* (C₃) and the nucleotide composition of the mesophyll expression module MEM1 in C₄, C₄-like C₃/C₄ intermediate and C₃ *Flaverias*. The numbers of nucleotides refer to the translation initiation codon. Dark colours mark regions with high similarity (>60% identical nucleotides). The positions of MEM1 and its homologues in *F. pringlei* are marked by black boxes. Asterisks label identical nucleotides in the **(a)** or **(b)** segments of MEM1. The C/T difference in the **(b)** segment is not correlated with C₃/C₄ photosynthesis, because all C₄ *Flaverias* except *F. trinervia* contain a C at that position (Gowik et al., 2004).

evolved C₄ properties (Engelmann et al., 2003). A fully functional MEM1, with the ability to repress gene expression in the vascular bundle and bundle sheath cells, was only found in C₄ and C₄-like species of the A-clade of *Flaveria* which are widely evolved towards C₄ (Akyildiz et al., 2007).

Basic leucine zipper proteins that interact with MEM1 of *F. trinervia* but not with the MEM1 homologue of *F. pringlei* were isolated by a DNA protein interaction screen with the yeast one-hybrid system (Li and Herskowitz, 1993) using MEM1 as a bait (Akyildiz, 2007). The CACT

tetranucleotide is embedded in a sequence context (TTACTCACTAA) that can form an imperfect palindrome and resembles a binding site for a GCN4-like basic leucine zipper transcription factor (Arndt and Fink, 1986; Oñate et al., 1999; Matys et al., 2003). The precise function of this DNA/protein interaction must be further investigated by gene knockout and/or overexpression experiments and biochemical approaches as well as other trans-regulatory factors involved in C_4 -specific gene expression are to be identified. It might be very interesting to study the function of the orthologous of these transcription factors in C_3 plants. That could provide information about the primary function of this transcription factors prior they were recruited to control the expression of C_4 genes. A detailed knowledge of these interrelationships would help to better understand the development of the regulatory network responsible for cell-specific gene expression during the evolution of C_4 photosynthesis.

IV. Outlook

The evolution of C_4 *ppc* genes from non-photosynthetic isogenes in the genus *Flaveria* required only small changes at the molecular level. Since such small changes likely occur quite easily in plant genomes, it is conceivable that C_4 photosynthesis arose many times independently during the evolution of angiosperms. It would be therefore of interest to investigate those changes in the promoters and structural part of *ppc* genes of as many genera as possible.

It is known from sequence comparisons of C_4 PEPCs that most of the amino acid exchanges are not conserved between different lineages of C_4 plants. The work of Christin et al. (2007) presents a good starting point to identify and characterize C_4 specific amino acid exchanges in the grass PEPCs. Since different amino acid replacements might lead to similar alterations in the tertiary and quaternary structure of a protein, progress in modelling the three-dimensional protein structures might allow to detect general differences of the overall protein structure between C_4 and non-photosynthetic PEPCs and help to fully understand the changes of C_4 PEPC kinetic properties.

Similarly, it would be interesting to know how cell-specific gene expression can be established in C_4 plants. Is MEM1 a universal *cis*-regulatory module for mesophyll-specific gene expression in *Flaveria*? The analysis of other genes expressed specifically in mesophyll cells, for instance the C_4 carbonic anhydrase of *Flaveria*, is a good choice to answer this question (Tetu et al., 2007). It may be even more interesting to investigate how mesophyll-specific gene expression was achieved in other families of the angiosperms that evolved C_4 species. Since the genomes of the Brassicaceae are presently intensively studied, the genus *Cleome* with its C_4 and C_3 species might be a good model system for a comparative analysis at the genome level.

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C₄ Decarboxylases: Different Solutions for the Same Biochemical Problem, the Provision of CO₂ to Rubisco in the Bundle Sheath Cells

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Summary	277
I. Introduction	278
II. NADP-Malic Enzyme, the Most Studied C ₄ Decarboxylase	280
A. The Photosynthetic Chloroplastic C ₄ -NADP-ME	281
B. Non-photosynthetic Plastidic and Cytosolic Isoforms in C ₄ -NADP-ME Plants	283
C. Non-photosynthetic Plastidic and Cytosolic NADP-ME Isoforms in C ₃ Plants	285
D. Phylogenetic Relationships Among Plant NADP-ME Sequences	286
III. Plant Mitochondrial NAD-E, a Hetero-Oligomeric Malic Enzyme	286
A. Photosynthetic Mitochondrial C ₄ Plant NAD-ME	288
B. NAD-ME from Non-photosynthetic Tissues of C ₄ Plants	288
C. Non-photosynthetic NAD-ME from C ₃ Plants	289
D. Phylogenetic Relationship Among Plant NAD-ME Sequences	289
IV. Plant PEPCK: the Cytosolic Gluconeogenic Enzyme Involved in C ₄ Photosynthesis	290
A. The Photosynthetic PEPCK Isoform	291
B. Non-photosynthetic PEPCK Isoforms from C ₄ Plants	292
C. Non-photosynthetic PEPCK Isoforms from C ₃ Species	292
D. Phylogenetic Relationship Among Plant PEPCK Sequences	293
V. Future Perspectives	295
Acknowledgments	295
References	295

Summary

The decarboxylation of C₄ acids in the bundle sheath cells (BSCs) is a key step in the C₄ photosynthetic carbon assimilation pathway. Depending on the particular subtype of C₄-species, this process can be mediated by different enzymes: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and/or phosphoenolpyruvate carboxykinase (PEPCK), and each enzyme has a different subcellular compartmentalization within the BSCs. Thus, the C₄ subtype cycle mediated by each decarboxylase displays

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distinguishing features in leaf anatomy, biochemistry and physiology. In some cases, the operation of more than one type of decarboxylating enzyme in the C_4 photosynthetic process has been described. During the last few years, remarkable advances have been made in the characterization of different isoforms of each C_4 decarboxylase. In most cases, non-photosynthetic isoforms of the C_4 -decarboxylating enzymes involved in primary and/or secondary metabolisms were characterized. These non- C_4 isoforms were for sure the starting point for the evolution of the C_4 -specific decarboxylases through the gaining of characteristics that make them more suitable to fulfill the requirements of the photosynthetic process. For each decarboxylating enzyme, the analysis of phylogenetic relationships reveals several features of the molecular evolution of the C_4 process which accomplished the same biochemical aim: the generation of CO_2 in the vicinity of Rubisco in BSCs, reducing photorespiration and enhancing photosynthesis.

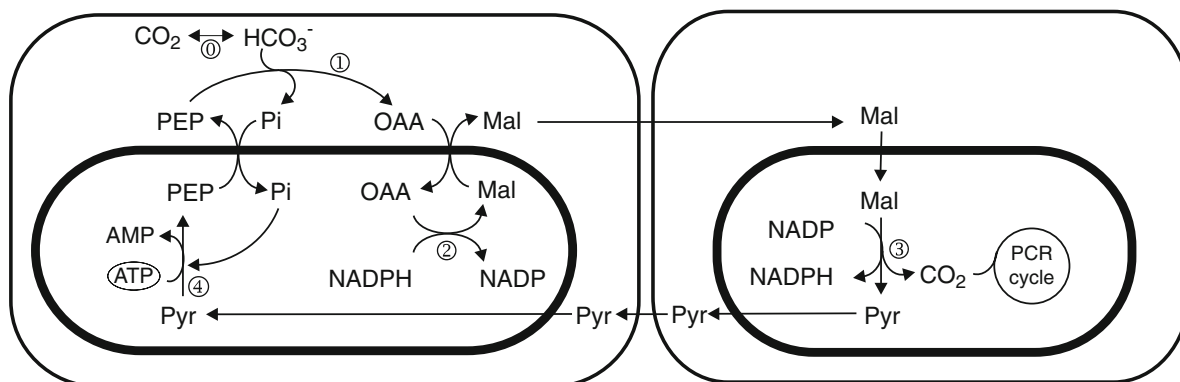
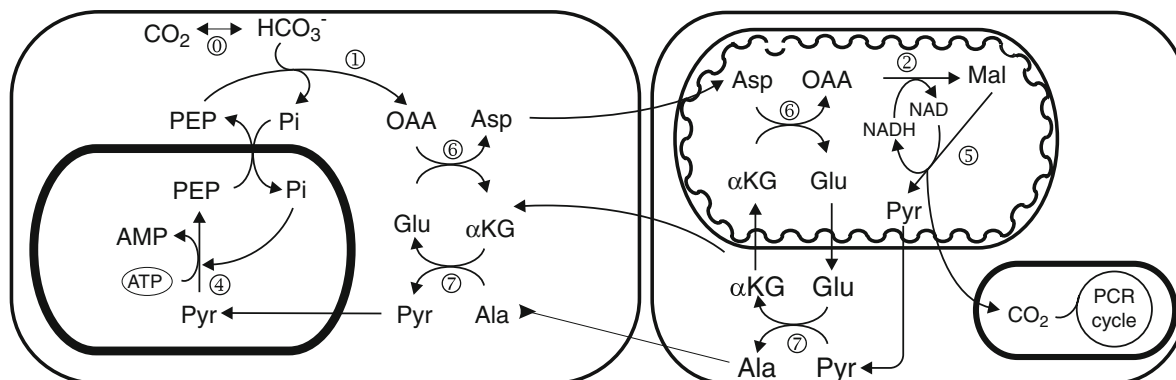
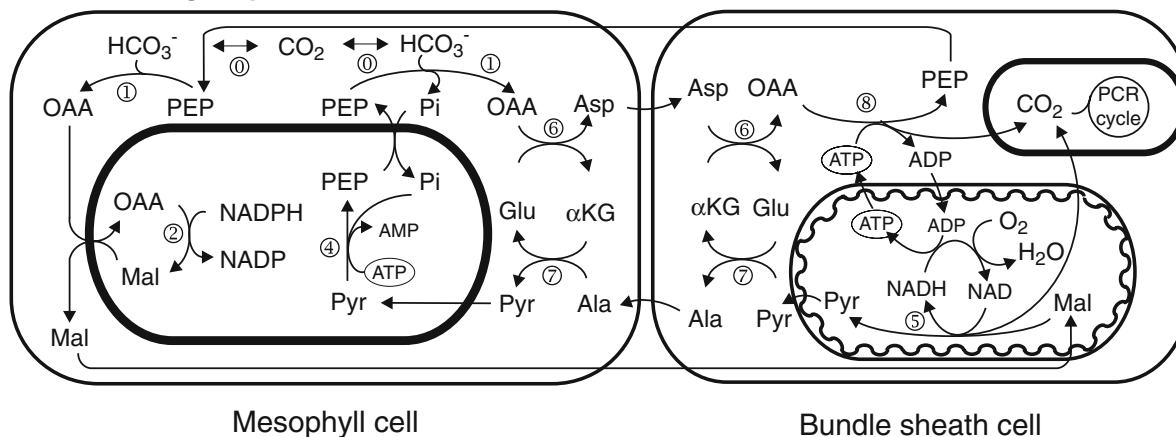
I. Introduction

C_4 plants spatially separate the initial fixation of CO_2 in mesophyll cells (MCs) and its subsequent conversion to carbohydrates in bundle sheath cells (BSCs). Differentiation of these two cell types is essential for the operation of C_4 photosynthesis, although special cases for the operation of the C_4 cycle within only one type of photosynthetic cell have been found (see relevant chapters, and Lara et al., 2002; Edwards et al., 2004). C_4 photosynthesis has a number of distinct properties that enable the capture of CO_2 and its concentration in the vicinity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the BSCs. This CO_2 pump reduces the oxygenase activity of Rubisco and hence the rate of photorespiration. In this way, the efficient operation of C_4 photosynthesis requires the strict, selective compartmentation of a set of enzymes in the MCs and the BSCs.

The first requirement for the CO_2 pump operating in C_4 plants is the fixation of CO_2 , in the form of HCO_3^- , in MCs by an enzyme that is not sensitive to O_2 (Phosphoenolpyruvate carboxylase, PEPc). The immediate product of PEPc, oxaloacetate (OAA), is highly reactive

and it is converted to C_4 acids such as malate and/or aspartate, which are transient stores of fixed CO_2 . The relative proportion of malate and aspartate formed from OAA is especially dependent on the decarboxylating enzyme that operates in BSC. The release of inorganic carbon in the form of CO_2 , substrate of Rubisco, from the intermediate pool of C_4 acids in BSCs is mediated by three enzymes: NADP-malic enzyme (NADP-ME); NAD-malic enzyme (NAD-ME) or phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 1). C_4 plants have been traditionally grouped into three biochemical subtypes depending on the major decarboxylase used (C_4 -NADP-ME subtype; C_4 -NAD-ME subtype or C_4 -PEPCK subtype). Each C_4 subgroup possesses particular structural features, biochemistry and physiology, and also differences in the mechanism used to regenerate phosphoenolpyruvate (PEP), the substrate of PEPc in MCs (Fig. 1). Nevertheless, although C_4 plants have been traditionally classified into these three subtypes, it is now becoming apparent that, in several cases, more than one decarboxylase operates at the same time. Apart from the recognized case of C_4 -PEPCK-subtype, where PEPCK works in tandem with NAD-ME (Fig. 1, Burnell and Hatch, 1988), other cases of co-existence of more than one decarboxylating enzyme have been identified. For example, in maize, a typical NADP-ME subtype, decarboxylation of aspartate by PEPCK also occurs in BSCs (Furumoto et al., 1999; Wingler et al., 1999). Other examples suggestive of a second decarboxylating enzyme, in addition to the main C_4 -decarboxylase have already been found. For example, PEPCK has been suggested to function in the case of sugar

Abbreviations: 3-PGA – 3-Phosphoglycerate; BSC – Bundle sheath cell; CAM – Crassulacean acid metabolism; MC – Mesophyll cell; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; OAA – Oxaloacetate; PEP – Phosphoenolpyruvate; PEPc – Phosphoenolpyruvate carboxylase; PEPCK – Phosphoenolpyruvate carboxykinase; PPDK – Pyruvate orthophosphate dikinase; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; TCA – Tricarboxylic acid cycle

NADP-ME subgroup**NAD-ME subgroup****PEP-CK subgroup**

Mesophyll cell

Bundle sheath cell

Fig. 1. Operation of the C₄ pathway and its intracellular compartmentalization in each of the three subgroups of C₄ plants. The numbers indicate the different enzymes involved: 0: carbonic anhydrase; 1: phosphoenolpyruvate carboxylase; 2: NADP-malate dehydrogenase; 3: NADP-malic enzyme; 4: pyruvate orthophosphate dikinase; 5: NAD-malic enzyme; 6: aspartate aminotransferase; 7: alanine aminotransferase; 8: phosphoenolpyruvate carboxykinase. PCR: photosynthetic carbon reduction cycle including Rubisco.

cane based on transcript analysis (Calsa and Filgueira, 2007), and in several eudicots based on enzymatic assays (Muhaidat et al., 2007). Further studies are needed to directly assess whether a second decarboxylase coexists and functions in each C_4 -subtype and under which condition(s) it could be relevant.

The different C_4 -subtypes have considerable differences in energy requirements (ATP and NADPH per CO_2 fixed, and requirements in MCs and BSCs) and in metabolite transport (Hatch, 1987; Furbank et al., 1990). The advantages or disadvantages of a particular decarboxylating pathway are yet unclear. In the same way, up to now it is not known whether each C_4 -subtype confers some photosynthetic advantage under certain environmental conditions. Moreover, differences in photosynthetic nitrogen use efficiency among C_4 -subtypes seem to occur in the background of substantial interspecific variation (Taub and Lerda, 2000). Nonetheless, it is true that for each C_4 -subtype, particular arrangements in terms of cellular structure and biochemistry must have occurred to allow the operation of the C_4 -cycle. For example, in C_4 -NADP-ME-subtype species, the BSC chloroplasts possess rudimentary or deficient grana with no, or reduced, Photosystem II expression; in C_4 -NAD-ME-subtype species, BSC mitochondria have a very high C_4 acid decarboxylating capacity, possibly showing unusual respiratory characteristics; and, finally, the cytosol of C_4 -PEPCK subtype species, must be capable of generating enough ATP to allow the decarboxylation of OAA in this compartment. In the latter case, it seems that phosphorylation linked to malate oxidation in mitochondria by NAD-ME is the source of ATP for PEPCK (Hatch, 1987).

The occurrence of different decarboxylases in C_4 plants offers alternative solutions to the same dilemma: how to generate CO_2 around Rubisco to prevent high levels of photorespiration. In this relation, the question arises as to why different enzymes are used for the same biochemical purpose. The existence of distinct subtypes among C_4 plants is in accordance to the multiple evolutionary origins of C_4 photosynthesis. Moreover, this diversity probably reflects the flexibility of plant metabolism. This suggests that the adaptation of each decarboxylase to the C_4 photosynthetic process occurred independently and on many

occasions during evolution, and that the starting point towards the C_4 decarboxylases isoforms must have been the housekeeping enzymes not involved in photosynthesis, which were already present in C_3 plants.

The purpose of the present chapter is to provide an update on the occurrence of different isoforms for each C_4 decarboxylase towards the goal of unravelling the evolution of C_4 isoforms from non-photosynthetic ancestors. For this, phylogenetic trees showing relationships for each decarboxylase have been constructed to gain insight into the origin of each C_4 -isoform. Since previous reviews on each decarboxylase (NADP-ME: Drincovich et al., 2001; NAD-ME: Wedding, 1989; PEPCK: Leegood and Walker, 2003) several advances have been made in characterizing the distinct properties and prevalence of each enzyme. Thus, we will mostly focus on recent achievements in the characterization of isoforms of each decarboxylase in different types of plants, and in the comparison of C_4 and C_3 decarboxylases in order to determine the mechanisms that might have been involved in the evolution of the photosynthetic isoforms.

II. NADP-Malic Enzyme, the Most Studied C_4 Decarboxylase

NADP-malic enzyme (NADP-ME; L-malate: NADP oxidoreductase [OAA decarboxylating], EC 1.1.1.40) is a widely distributed enzyme involved in different metabolic pathways in various animal and plant tissues, as well as in prokaryotic and eukaryotic microorganisms. It catalyses the oxidative decarboxylation of L-malate to yield pyruvate, CO_2 and NADPH. Depending on the source of the enzyme, either Mg^{2+} or Mn^{2+} serves as metal cofactor. Although the decarboxylation reaction is favoured, it is now becoming apparent that some isoforms are able to catalize the reverse reaction: the reductive carboxylation of pyruvate (Gerrard Wheeler et al., 2008).

Crystal structures of human mitochondrial NAD(P)-dependent malic enzyme (EC 1.1.1.39; Xu et al., 1999) and pigeon cytosolic NADP-malic enzyme (EC 1.1.1.40; Yang et al., 2002) have been resolved. The structures revealed that this type of malic enzyme belongs to a new class

of oxidative decarboxylases. Although the amino acid residues sequences of human versus maize chloroplastic C₄ NADP-ME are highly conserved (44% identity), there are several kinetic differences between them, e.g. the specificity and mode of interaction of the substrates and metabolic regulation.

Many C₄ species (e.g. maize, sugarcane and sorghum) possess NADP-ME in the chloroplasts of BSCs as the unique or major decarboxylase. In NADP-ME C₄ plants, malate is the predominant C₄ acid formed during photosynthesis (Fig. 1). Nevertheless, in some C₄-NADP-ME species aspartate is also a significant transient store of fixed CO₂. This is the case of maize, where PEPCK is also localized in BSC and contributes to the generation of CO₂ for Rubisco (Wingler et al., 1999). In other NADP-ME species, such as the dicot *Flaveria bidentis*, approximately equal amounts of malate and aspartate are formed and utilized during photosynthesis, although in this case it was suggested that aspartate is metabolized to malate and then decarboxylated by chloroplastic NADP-ME (Meister et al., 1996).

Typical C₄-NADP-ME species have BSC chloroplasts with a gradation of structure from chloroplasts with rudimentary grana, as in maize, to completely agranal, as in sugarcane and sorghum. Nevertheless, some dicot C₄-NADP-ME species have well-developed granal stacks in BSC chloroplasts. It was suggested that there may be a correlation between the use of aspartate instead of malate with the development of grana in BSC chloroplast, taking into account that when malate is used, an increase in reducing equivalents are transferred, along with CO₂, to BSC (Meister et al., 1996). Moreover, the degree of grana development in C₄ NADP-ME subtype species and thus, the differences in O₂ production from photosystem II, seems to be correlated with the potential BSC photorespiratory capacity (Ueno et al., 2005). The high activity of the C₄ NADP-ME in BSC chloroplasts was suggested also to affect chloroplast development, as transgenic rice expressing the maize C₄ NADP-ME also showed an aberrant structure (Takeuchi et al., 2000).

Since the last two reviews on plant NADP-ME (Edwards and Andreo, 1992; Drincovich et al., 2001) remarkable advances have been made in

the characterization of different isoforms from a wide variety of plant sources and a considerable number of nucleotide sequences of plant cDNAs and genomic structures are now known. The most studied isoform of plant NADP-ME is the one involved in C₄ photosynthesis, which is exclusively localized in BSC chloroplasts to provide CO₂ for Rubisco. Another photosynthetic isoform of NADP-ME is found in certain Crassulacean acid metabolism (CAM) plants; however, in this case the enzyme is cytosolic.

Apart from these specialized roles in photosynthesis, several cytosolic and/or plastidic NADP-ME isoforms have been found in different tissues of C₃, C₄ and CAM plants, playing non-photosynthetic roles and having particular sub-cellular localizations depending on the species. In some cases more than one isoform are present in the same cellular compartment.

A. The Photosynthetic Chloroplastic C₄-NADP-ME

This specialized isoform of NADP-ME displays unique kinetic and regulatory properties, and particular localization -exclusive in BSC chloroplasts, where it is up-regulated by light (Maurino et al., 1996, 1997; Drincovich et al., 1998; Tausta et al., 2002).

The maize C₄-NADP-ME was first structurally and kinetically characterized after purification from mature green leaves and further used for chemical modification studies (Drincovich et al., 1992; Drincovich and Andreo, 1994). Subsequently, this enzyme was obtained in large amounts as a recombinant protein, which allowed further characterization and mutagenesis and the identification of the amino acid residues and/or domains responsible for the C₄-specific properties (Detarsio et al., 2003, 2004, 2007). The kinetic parameters of the recombinant NADP-ME are essentially the same as those of the purified enzyme, although the recombinant form has more than six times higher k_{cat} , which may be due to partial inactivation of the enzyme during the long purification procedure from maize green leaves (Detarsio et al., 2003).

Recombinant maize NADP-ME displays very high intrinsic activity and affinity for the substrates, presenting also activity with NAD, although the k_{cat}/K_m value is much lower with NAD

(Detarsio et al., 2003). The maize C₄-NADP-ME has all the well-conserved sites (from I to V) shared by other plant isoforms (Drincovich et al., 2001). Two of these sites (site II and V) possess the typical signature motif GXGXXG, which is diagnostic of a dinucleotide-binding fold. The three-dimensional model of the maize C₄-NADP-ME, based on the structure of the crystallized human and pigeon NADP-ME as templates (Detarsio et al., 2003, 2004), shows that only site V adopts a typical Rossmann fold. Nevertheless, site II is also near the nicotinamide ring of the associated NAD(P) molecule. In an attempt to analyze the involvement of these two sites in the binding of NADP, they were disrupted by mutagenesis, resulting in abortive mutants. Thus, both are part of the active site of NADP-ME and that is the reason for the high degree of conservation among all NAD(P)-ME (Drincovich et al., 2001; Detarsio et al., 2003). Further site directed mutagenesis of candidate residues involved in catalysis and/or substrate binding, showed the participation of Ala392 in coenzyme specificity and of Ala387 and Arg237 in the catalytic mechanism (Detarsio et al., 2003). Similar studies indicated also that the basic residues Lys255 and Arg237 play key roles in catalysis and that Lys435 and/or Lys436 are implicated in the coenzyme specificity (Detarsio et al., 2004).

C₄-NADP-ME assembles in different oligomeric states depending on the media used. The purified isoforms from both sugarcane and maize are present as homotetramers at pH 8.0 and as homodimers at pH 7.0. Both forms are active but the tetramer is the most active form of the enzyme (Iglesias and Andreo, 1989). These results were confirmed by native gel electrophoresis of maize leaf crude extracts and the recombinant enzyme (Saigo et al., 2004). In both cases, gels analysed for NADP-ME activity at pH 7.5 showed only a band with activity corresponding to the tetramer whilst Western blot analyses detected both the tetrameric and dimeric states.

Recently, in order to identify the domains responsible for the structural and kinetic differences between maize C₄- and non-C₄-NADP-ME, several chimeras between these isoforms were constructed and analysed (Detarsio et al., 2007). In maize, the plastidic non-photosynthetic NADP-ME represents the more recent and direct ancestor of the C₄-NADP-ME, with both

proteins having a high degree of identity (85%, Tausta et al., 2002). Among the structural differences identified between these two isoforms expressed as recombinant proteins (Detarsio et al., 2004; Saigo et al., 2004), the most relevant is the oligomeric state: the C₄-NADP-ME assembles as a tetramer and the non-C₄-NADP-ME as a dimer. By characterizing various chimeras between these two isoforms, the region flanked by amino acid residues 102 and 247 was found to be responsible for the tetrameric state of C₄-NADP-ME. In this way, the oligomerization strategy of maize NADP-ME isoforms differs markedly from the one present in non-plant NADP-ME with known crystal structures, where a segment of approximately 20 residues at the C-terminus of the enzyme's monomer was found to be critical for the tetramer-interaction (Chang and Tong, 2003). This segment is not found in plant NADP-MEs (Detarsio et al., 2007).

The chimera strategy between the maize C₄ and the non-C₄-NADP-ME isoforms was also used to identify segments involved in kinetic differences between these two isoforms. In this regard, one of the most outstanding difference is the inhibition of the C₄-NADP-ME by the substrate malate in a pH-dependent way; the inhibition occurs at pH 7.0 but not at pH 8.0 (Drincovich et al., 1991; Detarsio et al., 2007). On the other hand, the non-C₄-NADP-ME is not inhibited by high concentrations of this substrate (Saigo et al., 2004). By the chimera approach, the region from residue 248 to the C-terminal end of the C₄ isoform was found to be involved in the allosteric inhibition by high malate concentrations at pH 7.0. It is worth mentioning that the purified enzymes from *Flaveria bidentis* (Ashton, 1997) and sugarcane (Iglesias and Andreo, 1990) were also inhibited by high malate concentrations. In this way, although more C₄-NADP-ME family members should be analyzed, it is probable that this inhibition may be important for the C₄-pathway regulation in vivo. Accordingly, NADP-ME activity would be high when photosynthesis is in progress, a regulatory property that seems to be not necessary for the non-C₄ isoforms. Thus, the high level of malate concentration found in C₄ plant tissues, along with the decrease in pH of the stroma of chloroplasts when photosynthesis is not occurring,

would produce a decrease in NADP-ME activity when carbon fixation is not active.

The cDNA of maize C₄-NADP-ME was the first plant NADP-ME cDNA to be sequenced (Rothermel and Nelson, 1989) and the processing site of the precursor (between the residues Ser65 and Asn66) was obtained by N-terminal sequencing of the purified enzyme (Maurino et al., 1996). Interestingly, the overexpression of maize C₄-NADP-ME under the control of the CaMV promoter in chloroplasts of *Arabidopsis thaliana* causes a photoperiod dependant phenotype (Fahnenstich et al., 2008; Zell et al., unpublished results). While no visible phenotypic changes are evident in the transgenic plants growing in long days, an increase in the plastidic NADP-ME activity led to a green pale phenotype in plants growing in short days. Plants growing in both conditions have a disturbed metabolic profile and thus, this transformant offered a way to manipulate the levels of malate and to analyse the physiological consequences. Metabolic analysis and complementation assays demonstrated that these transgenic plants enter dark induced senescence more rapidly than the wild-type due to an accelerated starvation caused by extremely low levels of malate and fumarate (Fahnenstich et al., 2007). In contrast to these results, the overexpression of NADP-ME in chloroplasts of rice under the control of the rice cab promoter showed serious deteriorative effects on plant growth, such as bleaching of leaves and growth hindrance under autotrophic conditions (Takeuchi et al., 2000; Tsuchida et al., 2001). In this case the authors suggested that the high NADP-ME activity in the chloroplasts could affect the development of this organelle by generating excessive reducing power. As *Arabidopsis* is a dicot and rice is a monocot, the introduction of the highly active C₄-NADP-ME might have induced different physiological disturbances in each species most probably due to the metabolic differences occurring in these plants.

The nucleotide sequence of the mature C₄-NADP-ME from *Flaveria trinervia* shows an extensive similarity with the maize C₄ enzyme, whilst the primary structure of the transit peptides differs considerably. This suggests an independent origin for the NADP-ME gene during the evolution of monocots and dicots. The expression of the gene encoding the C₄ NADP-ME in *Flaveria*,

increases in leaves with high C₄ pathway activity (Marshall et al., 1996; Lai et al., 2002a). Moreover, Western blot screening and immunolocalization studies on 13 *Flaveria* species (belonging to C₃, C₃-C₄ and C₄ species), indicated that the degree of C₄ photosynthesis positively correlates with the occurrence of the C₄-NADP-ME isoform and its localization in BSC (Drincovich et al., 1998). Interestingly, in the C₃ species *F. pringlei*, the C₄-NADP-ME expression is transient and limited to early leaf development and the enzyme was suggested to act in CO₂ refixation schemes in both C₃ and C₄ species (Lai et al., 2002a). Using transgenic *F. bidentis* it was shown that the 5' region of the C₄-NADP-ME gene determines the BSC specific expression and the 3' region contains the enhancer-like elements that confer high expression level in leaves (Marshall et al., 1997). However such a 3' requirement was not observed for the *F. trinervia* gene (Lai et al., 2002a).

The kinetic and structural properties of *Haloxylon persicum* (C₄ species) and *F. floridana* (a C₃-C₄ intermediate species) C₄-NADP-ME were also reported (Casati et al., 1999a, b). A 67 kDa isoform purified from *H. persicum* shoots was identified as a C₄ NADP-ME due to its high specific activity, low Km values for its substrates malate and NADP, and characteristic pH optimum. However, it presents different molecular mass and isoelectric point than those of other studied C₄ isoforms. *H. persicum* has C₃ isopalisade non-Kranz cotyledons and C₄ Salsoloid-type green stems in which C₄-NADP-ME is localized in BSC chloroplasts (Voznesenskaya et al., 1999). In the case of *A. floridana*, although three immunoreactive bands were detected in leaf extracts only one isoform could be purified to homogeneity. The purified enzyme shows intermediate kinetic characteristics between those of the C₃ and C₄ isoforms, with a molecular mass similar to the C₄ isoform and with BSC chloroplastic localization (Casati et al., 1999b).

B. Non-photosynthetic Plastidic and Cytosolic Isoforms in C₄-NADP-ME Plants

Plastidic non-C₄ isoforms of NADP-ME have been identified in maize (Maurino et al., 1996, 1997, 2001; Tausta et al., 2002) and in C₄ *Flaveria* species (Marshall et al., 1996; Lai et al., 2002a).

The characterization of these isoforms was of great relevance towards understanding how and why new enzyme products were created to fulfil the C_4 pathway requirements during the course of evolution, as C_4 plants already possessed plastidic non-photosynthetic NADP-ME. Apart from gaining high level of expression and localization in BSC, the C_4 isoforms acquired particular kinetic and structural features that make them much more suitable to accomplish the photosynthetic requirements, although photosynthetic and non-photosynthetic isoforms share a high degree of homology.

In maize, a cDNA that encodes a plastidic 66 kDa NADP-ME was isolated by screening a root cDNA library and by amplification by reverse-transcription PCR using mRNA from green leaves as template (Maurino et al., 2001). The maize plastidic non- C_4 NADP-ME, which accumulates constitutively at low levels, is not influenced by light or by the developmental stages of the plastids and is transcriptionally and/or post-transcriptionally regulated by effectors related to plant defence responses (Maurino et al., 2001).

The plastidic non-photosynthetic NADP-ME was obtained in high amounts as a recombinant protein (Saigo et al., 2004). The recombinant product displays an unexpected high intrinsic NADP-ME activity and assembles as a dimer, although a higher molecular mass oligomeric state is also found in maize etiolated leaves and roots (Saigo et al., 2004). This last finding may be related to the occurrence of yet unidentified cytosolic and/or plastidic isoform(s). Native gel electrophoresis of *Arabidopsis* leaves overexpressing the maize non- C_4 plastidic NADP-ME also shows a band with activity corresponding to a dimer (M. Saigo et al., unpublished results). This observation indicates that the plastidic non-photosynthetic NADP-ME has the same quaternary structure, whether it is expressed in a prokaryotic or eukaryotic system (Saigo et al., 2004; unpublished results). In contrast to the C_4 isoform, the non-photosynthetic plastidic NADP-ME is not inhibited by malate at pH 7.0 and displays lower affinity for the substrates (Saigo et al., 2004; Detarsio et al., 2007).

Previous work identified a 72-kDa protein as the non-photosynthetic NADP-ME in C_4 plants such as maize (Maurino et al., 1996, 1997; Tausta et al., 2002) and in several C_3 species, such as different *Flaveria* species (Drincovich et al., 1998), wheat (Casati et al., 1997; Maurino et al.,

1997) and *Aloe arborescens* (Honda et al., 2000). At least three different batches of antibodies against NADP-ME purified from maize leaves (Maurino et al., 1996; Tausta et al., 2002) and *Mesembryanthemum crystallinum* (Honda et al., 2000) reacted to this 72 kDa protein. Moreover, a protein purified from maize etiolated leaves and roots with an apparent molecular mass of 72 kDa, as assessed by Coomassie stained SDS-PAGE, showed NADP-ME activity (Maurino et al., 1996, 2001). Proteins with the same molecular mass were also purified from wheat stems (Casati et al., 1997), *Egeria densa* leaves (Casati et al., 2000), *Aptenia cordifolia* leaves (Falcone et al., 2003) and *Ricinus communis* cotyledons (Colombo et al., 1997). Nevertheless, the fact that a sequence encoding a putative 72-kDa NADP-ME was not found, neither among the known cDNAs encoding for NADP-MEs nor in the *Arabidopsis thaliana* and *Oryza sativa* genomes, opened the question about the identity of the 72 kDa protein. Further studies indicated that the 72 kDa protein is not actually a NADP-ME but in fact a heat shock protein (Hsp70), which associates with NADP-MEs (Lara et al., 2005). Probably, NADP-ME/Hsp70 association takes place when preparing crude extracts, leading to a co-purification of the proteins, and thus explaining the cross-reaction of the antibodies. This Hsp70 was found also to associate in vitro to the recombinant NADP-ME (Lara et al., 2005). Thus, the identity of the so-called "72 kDa NADP-ME" from maize roots was recently revisited and shown to be an Hsp70 protein. Thus, the functions assigned to the "72 kDa NADP-ME" from maize roots (Drincovich et al., 2001; Maurino et al., 2001; Tausta et al., 2002) should be attributed to the 66 kDa NADP-ME characterized by Saigo et al. (2004).

A genomic sequence encoding a non-photosynthetic plastidic NADP-ME was also characterized in the C_4 *Flaveria bidentis* (Marshall et al., 1996). This gene is expressed concurrent with stages in chloroplast biogenesis (Lai et al., 2002a) and at low, but similar, level in all organs of different C_3 , C_4 and C_3 - C_4 intermediate *Flaveria* species, a fact that suggests that the product of this gene is a housekeeping enzyme (Marshall et al., 1996; Lai et al., 2002a).

Although still speculative, the role of the plastidic non photosynthetic NADP-ME might be to provide a burst of NADPH and pyruvate for

plastid biogenesis and/or for plastid-localized protein and lipid biosynthesis, possible using the malate generated by the tricarboxylic acid (TCA) cycle as the substrate (Lai et al., 2002a). A strong expression of this enzyme in the vascular tissue of stems and roots was also correlated with a role in the uptake and transport of ions (Martinoia and Rentsch, 1994).

On the other hand, cytosolic NADP-ME in C₄ plants have also been identified in several species, e.g. in some *Flaveria* species, where it is encoded by a single gene (Lai et al., 2002b). In *F. trinervia*, the cytosolic NADP-ME expression was uniformly found in all vegetative organs and accumulated by mechanical injury. Nevertheless, the corresponding cytosolic NADP-ME was neither isolated nor characterized at the enzymatic level.

In maize, a third cDNA encoding for a NADP-ME highly expressed in epidermis of embryogenic roots has been isolated (Lopez Becerra et al., 1998). The recombinant protein of this non-C₄-NADP-ME displays very distinct properties from that of the other maize NADP-ME isoforms already characterized (Detarsio et al., 2008). Moreover, at least two more isoforms can be detected in maize, one of which is constitutively expressed in all the tissues tested (E. Detarsio et al., unpublished results). In *Sorghum bicolor*, a NADP-ME, which is closely related to the non-C₄-NADP-ME from maize embryogenic roots, increases in response to osmotic stress and abscisic acid treatments (Buchanan et al., 2005).

Several roles can be suggested for the cytosolic NADP-ME isoform, including the supply of NADPH for the cytosolic metabolism, the support of wound responses and the balance of intracellular pH (Detarsio et al., 2008).

C. Non-photosynthetic Plastidic and Cytosolic NADP-ME Isoforms in C₃ Plants

Both cytosolic and plastidic NADP-ME isoforms have been characterized from C₃ plants. cDNAs for putative cytosolic NADP-ME have been obtained, e.g. from bean, poplar and grape berries (see Drincovich et al., 2001). On the other hand, plastidic isoforms of NADP-ME have been identified in C₃ plants by either cloning the corresponding cDNAs having putative

plastidic transit peptides (e.g. in *Flaveria pringlei*) or by detecting the enzyme in isolated chloroplasts (e.g. in *Cucurbita pepo* and *Glycine max*, *Hydrilla verticillata* and *Egeria densa*; see Drincovich et al., 2001).

Recently, the complete set of the C₃ *A. thaliana* NADP-ME isoforms has been analyzed (Gerrard Wheeler et al., 2005). The *Arabidopsis* genome contains four *NADP-ME* genes, three of which encode cytosolic products (NADP-ME1-3), while only one encodes a plastidic located enzyme (NADP-ME4). The characterization of insertion mutants defective in particular NADP-ME isoforms, as well as the tissue-specific expression of the individual genes, indicated differential expression patterns for each isoform. In this way, while NADP-ME2 and -4 are constitutively expressed in mature organs, NADP-ME1 is restricted to secondary roots and NADP-ME3 to trichomes and pollen. Moreover, during embryogenesis and germination, the four genes show considerable differences in expression (Gerrard Wheeler et al., 2005). The four recombinant isoforms show NADP-ME activity and the K_m for each substrate has been analyzed in both the direct and reverse reactions (Gerrard Wheeler et al., 2005, 2008).

The occurrence of four NADP-ME isoforms in the monocot rice has also been reported (Chi et al., 2004), three of which are putative cytosolic and only one plastidic, as in *Arabidopsis*. The expression of one of these isoforms (NADP-ME2) was induced by several environmental stresses and its over-expression in *Arabidopsis* was found to confer high salt and osmotic stress tolerance (Liu et al., 2007), probably by balancing the level of reactive oxygen species under stresses that alter their expression.

In view of the coexistence of both cytosolic and plastidic isoforms in the C₃ species whose genomes are known, it is possible to speculate that all C₃ species possess both types of isoforms. It has been postulated that cytosolic C₃-NADP-MEs are involved in plant defence responses (Casati et al., 1999c) and that the plastidic isoform is involved in lipid biosynthesis by providing pyruvate and NADPH. In fruit tissues of tomato and grape berries, NADP-ME was implicated in respiration during ripening, providing pyruvate and/or NADPH as substrate for respiration (Famiani et al., 2000).

D. Phylogenetic Relationships Among Plant NADP-ME Sequences

A phylogenetic tree was constructed with a multiple sequence alignment using all known NADP-ME sequences (Fig. 2). In addition to the sequences previously analysed (Drincovich et al., 2001; Gerrard Wheeler et al., 2005), six new NADP-ME sequences from *Sorghum bicolor*, *Nicotiana tabacum* and *Hydrilla verticillata* have been included (Fig. 2).

The phylogenetic tree can be divided into four groups: 1 – cytosolic dicot NADP-ME group; 2 – plastidic dicot NADP-ME group; 3 – monocot NADP-MEs; and 4 – a group composed of both monocot and dicot NADP-MEs (Fig. 2). The existence of two separate groups within the dicot, one for cytosolic and one for plastidic isoforms, suggests that both groups may have originated from two different ancestral genes. It is now becoming apparent that all dicots possess both types of NADP-ME, although in some species only one isoform has been sequenced (Fig. 2). It is also clear that both cytosolic and plastidic ancestral genes had the potential to originate photosynthetic NADP-MEs independently; the cytosolic CAM-NADP-ME and the chloroplastic C_4 -NADP-ME are found in these two separate groups.

On the other hand, both plastidic and cytosolic monocot NADP-MEs group together (Fig. 2). Four of the sequences now added to the previously constructed tree (Gerrard Wheeler et al., 2005) are within this group: the photosynthetic C_4 -NADP-ME from sorghum and the three isoforms from *H. verticillata*, from which two are putative cytosolic and one plastidic (Fig. 2). *H. verticillata* is a submersed aquatic macrophyte that is able to develop a Kranz-less C_4 metabolism under certain environmental conditions, especially when the CO_2 concentration decreases (Magnin et al., 1997). Nevertheless, it is not currently known which of the three NADP-ME isoforms is involved in the proposed C_4 cycle. It is also obvious that the monocot NADP-ME ancestral genes also had the potential to originate photosynthetic NADP-MEs, as the maize and sorghum C_4 isoforms are comprised within this group.

Finally, a fourth group of very distinct NADP-MEs can be found in the phylogenetic tree.

It includes the cytosolic maize NADP-ME that is expressed in embryo roots (Lopez Becerra et al., 1998; Detarsio et al., 2008), an *Arabidopsis* cytosolic NADP-ME, which is expressed only in embryo roots (Gerrard Wheeler et al., 2005) a cytosolic NADP-ME isoforms from rice, and two uncharacterized cytosolic NADP-ME from poplar and grape (Fig. 2). Although more isoforms from this group need to be characterized, it seems that the phylogenetic association may correspond with a particular physiological function that this isoforms may fulfil in vivo.

III. Plant Mitochondrial NAD-E, a Hetero-Oligomeric Malic Enzyme

NAD-malic enzyme (NAD-ME; EC 1.1.1.38) catalyses the oxidative decarboxylation of malate to yield pyruvate and CO_2 in the presence of NAD and a divalent cation. The enzyme is exclusively localized to mitochondria of different eukaryotic organisms.

NAD-ME can use NADP to varying extents but it prefers NAD, and has an absolute requirement for a divalent cation (Mn^{2+} or Mg^{2+}). CoA is a potent activator of NAD-ME. Several intermediates of the TCA cycle and glycolysis were reported to be activators of the enzyme, e.g. fumarate, fructose-1, 6-bisphosphate and acetyl-CoA (Hatch et al., 1974; Wedding, 1989). Plant NAD-MEs have been classified as EC 1.1.1.39 due to their inability to decarboxylate OAA, suggesting a different kinetic mechanism from other NAD-MEs which decarboxylate OAA (E.C. 1.1.1.38.) as part of the chemical mechanism of catalysis (Wedding, 1989).

Crystal structures of human NAD(P)-ME (EC 1.1.1.39; Xu et al., 1999) and *Ascaris suum* NAD-ME (E.C. 1.1.1.38; Coleman et al., 2002) were resolved. Both of these non-plant NAD-MEs are homotetramers with a dimer of dimers quaternary structure. Nevertheless, plant NAD-MEs seem to be very different from these non-plant isoforms, as they have been reported to exist as hetero-oligomers, composed of two different subunits with distinct molecular masses.

In some C_4 plants, NAD-ME functions in C_4 metabolism by providing CO_2 for the Calvin cycle, showing an activity of around 50 times higher than that found in C_3 plants. In these

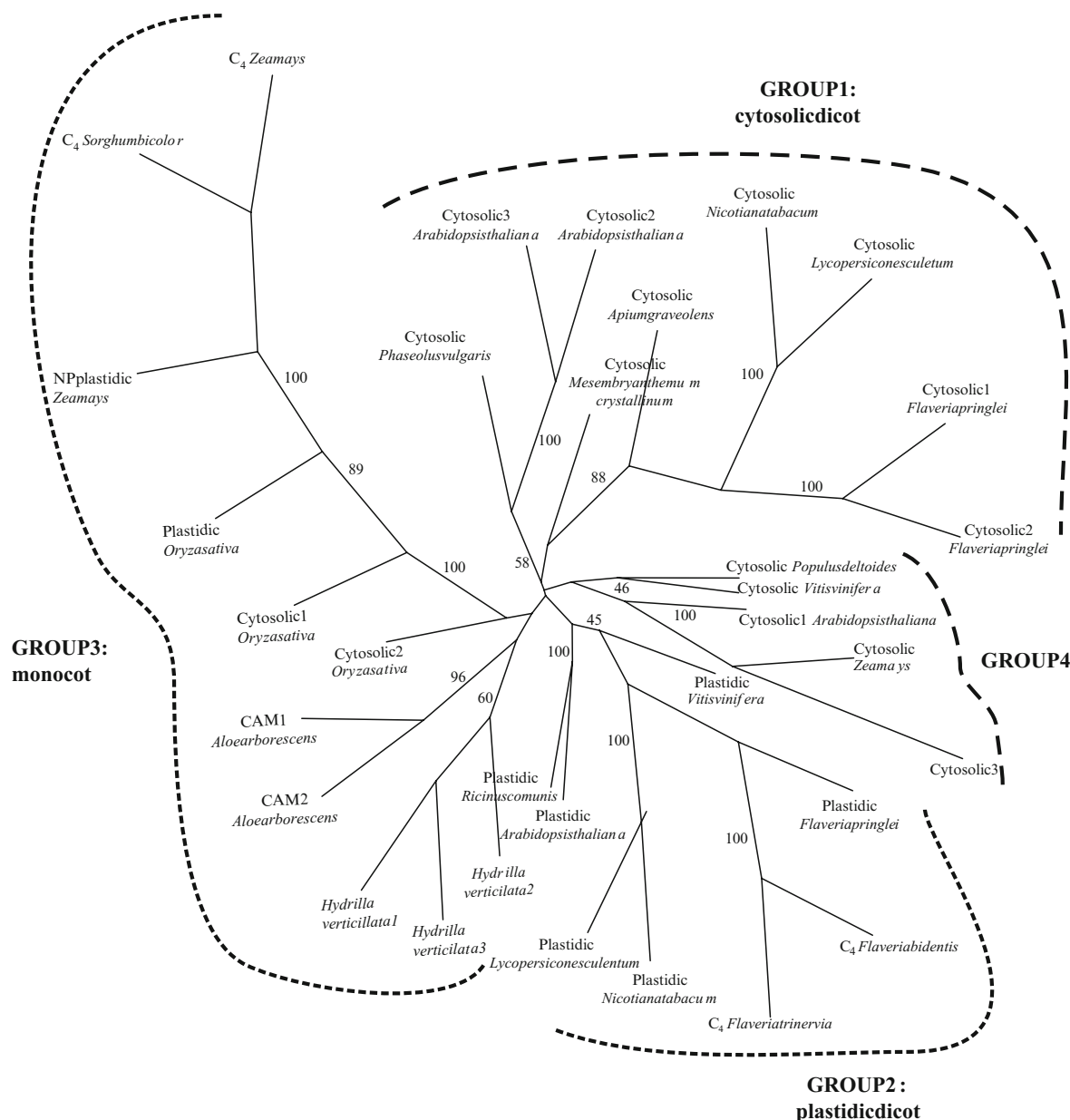


Fig. 2. Phylogenetic tree of plant NADP-MEs. The existence of transit peptides in each NADP-ME sequence was predicted by ChloroP1.1 software (<http://www.cbs.dtu.dk/services/ChloroP/>). Mature proteins were aligned using ClustalW (1.81) multiple alignment program (Thompson et al., 1994) and the alignment obtained was modified by visual inspection to exclude the sites containing gaps. The phylogenetic tree was constructed by the Neighbour-Joining (NJ) method using the Phylip software package (Felsenstein, 1989). Statistical significance of each branch of the tree was evaluated by bootstrap analysis by 100 iterations of bootstrap samplings and reconstruction of trees by the NJ method. The topology obtained by this method is shown, along with statistical significance of each branch. The sequences included, apart from the sequences previously included (Drincovich et al., 2001; Gerrard Wheeler et al., 2005) are the following: Photosynthetic NADP-ME from *Sorghum bicolor* (AY274836); non-photosynthetic cytosolic and plastidic NADP-ME from *Nicotiana tabacum* (DQ923118 and DQ923119); and three NADP-ME sequences from *Hydrilla verticillata* (AY594687, AY594688 and AY594689). NP: non-photosynthetic.

species, the photosynthetic isoform is found in BSC mitochondria. In some CAM plants, the diurnal decarboxylation of malate is achieved by the dual action of NAD-ME in the mitochondria and NADP-ME in the cytosol.

NAD-ME type C_4 plants possess also high activities of aspartate and alanine aminotransferases, both in MC and BSC. Aspartate rather than malate is the major C_4 acid which is decarboxylated in BSC mitochondria (Fig. 1). This C_4 subtype has very large number of mitochondria in BSC, which are closely associated with the chloroplasts, in order to achieve a high photosynthetic efficiency.

The carbon flux through BSC mitochondria in NAD-ME C_4 subtype plants is equivalent to the rate of photosynthesis. Thus, the BSC mitochondria of NAD-ME C_4 plants must have special features to allow high rates of malate decarboxylation, specially during the day, when photosynthesis is active (Agostino et al., 1996).

In the present chapter, we will describe the advances in characterizing NAD-ME from different sources, especially since the last two reviews on plant NAD-ME (Artus and Edwards, 1985; Wedding, 1989).

A. Photosynthetic Mitochondrial C_4 Plant NAD-ME

Photosynthetic NAD-MEs from *Crassula argentea* (CAM), *Urochloa panicoides* (C_4 -PEPCK) and *Amaranthus hypochondriacus* (C_4 -NAD-ME) are composed of two dissimilar subunits with distinct molecular masses, called α and β (Grover and Wedding, 1982; Willeford and Wedding, 1987; Long et al., 1994). In the case of *U. panicoides* and *C. argentea*, the purified enzymes exist mainly as octamers composed of the two subunits in a 1:1 ratio, with both subunits being required for activity (Wedding and Black, 1983; Burnell, 1987). On the contrary, the C_4 NAD-ME purified from *Eleusine coracana*, *Panicum dichotomiflorum* and *A. tricolor* is an octamer of identical subunits (Oshui and Murata, 1980; Murata et al., 1989).

The α and β subunits of the C_4 NAD-ME from *A. hypochondriacus* were purified, and immunolocalization of the NAD-ME α protein showed reaction only in BSC mitochondria. The cDNA encoding for the α subunit of NAD-ME was isolated and

sequenced, presenting all the motifs required for a complete and functional NAD-ME (Long et al., 1994). Synthesis of the α subunit of NAD-ME is light-dependent, although it is also influenced by seedling development (Long and Berry, 1996).

It was proposed that diurnal regulation of the enzyme in vivo is mediated by pH and the malate level without change in the oligomeric form of the enzyme (Artus and Edwards, 1985). Contrary to this, Grover and Wedding (1984) suggested that the drop in NAD-ME activity observed as the pH increases above 7.0 may be due to a shift to the low activate dimer form. The control by pH of the equilibrium between malate dehydrogenase and NAD-ME activities in the mitochondria was confirmed by the relative activity of both enzymes under changing pH conditions (Wedding and Whatley, 1984).

A homozygous mutant of the C_4 plant *Amaranthus edulis* containing only 5% of the wild-type NAD-ME activity can grow in elevated CO_2 but is unable to carry out photosynthetic CO_2 assimilation in normal air (Dever et al., 1998). No changes in the content of α and β NAD-ME and in the activity of other C_4 enzymes were detected in these plants. However, in contrast to the wild type that possesses only the octameric form of the enzyme (more active), a higher proportion of the tetrameric form (less active) was found in the mutant plants. It is possible that the presence of less-aggregated forms in the mutants is responsible for the reduced rate of CO_2 assimilation (5% of the wild-type) and their consequent poor growth under atmospheric CO_2 (98% reduction). Little difference in photosynthetic rate of heterozygous mutants suggests that NAD-ME exerts little control over the rate of C_4 photosynthesis and that the activity loss can be compensated by regulatory mechanisms that increase the activity of the enzyme (Dever et al., 1998).

B. NAD-ME from Non-photosynthetic Tissues of C_4 Plants

Although non-photosynthetic isoforms of NAD-ME in C_4 plants have not been characterized at all, small amounts of the α subunit of NAD-ME were detected in Amaranth tissues including stem, petiole, flower and root. It is not clear if the signal detected corresponds to the C_4 -NAD-ME isoform or to other isoforms that are able to

hybridize to the C₄ probe (Long and Berry, 1996). Further studies are needed to determine whether the C₄ isoform of NAD-ME is expressed in all tissues in NAD-ME C₄ plants or whether other isoform exists. If only the C₄ isoform is present, then enhanced expression in leaves, specific localization in BSC and light-dependent expression was gained by the same NAD-ME encoding gene in order to fulfil the requirements of C₄ photosynthesis.

C. Non-photosynthetic NAD-ME from C₃ Plants

The universal presence of NAD-ME in plants is due to the key role that it plays in malate respiration. Malate enters the plant mitochondria either via glycolysis or from vacuolar malate reserves. There is a branch point of malate metabolism in mitochondria, it can be metabolized by malate dehydrogenase or by the NAD-ME. In the latter case, malate is decarboxylated to pyruvate allowing repeated cycling of carbon skeletons through the TCA cycle. By providing a means of generating acetyl-CoA and thus, ATP and carbon skeletons, NAD-ME is assumed to play a central role in the management of carbon flux through the TCA cycle (Grover et al., 1981).

It is likely that the duplication of a nuclear gene encoding for NAD-ME, such as that found in C₃ plants, might have created the BSC specific and highly expressed enzyme involved in C₄ photosynthesis. As in the case of maize Pyruvate orthophosphate dikinase (PPDK) gene, it is possible that genetic rearrangements may have led to separated upstream regions that differentially regulate the transcription of the same coding sequence (Sheen, 1991).

NAD-ME from *Solanum tuberosum* (C₃) is composed of two dissimilar subunits (α and β) in a 1:1 molar ratio (Grover and Wedding, 1982; Willeford and Wedding, 1987). No activity has been associated with the separated subunits, but activity could be found in a reconstituted system (Willeford and Wedding, 1987). The cDNAs encoding both subunits were isolated from potato (Winning et al., 1994). These subunits display 65% identity at the amino acid level and are immunological different. Northern blot analysis from several potato tissues has revealed equivalent steady state levels of the two subunits,

suggesting that they are coordinately expressed (Winning et al., 1994).

In a biotechnological approach, potato plants were transformed with the NAD-ME β -subunit in antisense orientation under the control of either the CaMV 35S promoter or the patatin promoter (Jenner et al., 2001). Tubers with NAD-ME activity as low as 40% of the wild-type were obtained. The mutant plants showed no phenotypical differences and biochemical analyses of tubers indicated no detectable effects on flux through the TCA cycle. However, increased concentrations of 3-Phosphoglycerate (3-PGA), PEP and starch were found. The authors suggested that the increased 3-PGA content in the tubers of these plants may stimulate ADP-glucose pyrophosphorylase, resulting in an increased rate of starch synthesis. Although there is a universal presence and function of NAD-ME in mitochondrial metabolism, plants can apparently cope with substantial reductions in the activity of this enzyme without detrimental effects on plant development.

By analysing the C₃ *Arabidopsis* NAD-ME, only two genes present homology to potato NAD-MEs, one being more similar to the α subunit and the second to the β (Tronconi et al., 2008). Studies with the recombinant proteins indicated that each subunit in the absence of the other, can catalyse the NAD-ME reaction. More interesting, only the NAD-ME more similar to the potato β subunit was activated by CoA. Nevertheless, although both *A. thaliana* NAD-ME subunits are catalytically active, an association between these two proteins is occurring, with the major native form being a heterodimer (Tronconi et al., 2008).

D. Phylogenetic Relationship Among Plant NAD-ME Sequences

All complete α and β sequences from plant NAD-ME (from *Arabidopsis*, *Oryza sativa*, *Solanum tuberosum* and the α subunit from the C₄ plant *Amaranthus hypochondriacus*) were aligned (not shown). The higher degree of protein identity was found among the alpha subunits, between 79% and 85%. The beta subunits showed identities between 76% and 78%. On the other hand, the degree of identity between the α and β subunits ranges from 64% to 70%.

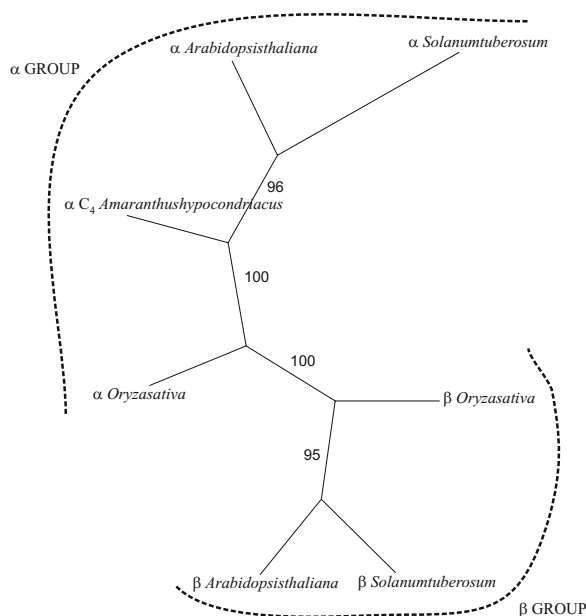


Fig. 3. Phylogenetic tree of plant NAD-MEs. The existence of transit peptides in each NADP-ME sequence was predicted by MITOPROT software (<http://ihg.gsf.de/ihg/mitoprot.html>). The tree was constructed as described in Fig. 2. The sequences included are the following: α subunits from *Amaranthus hypochondriacus* (U01162; photosynthetic NAD-ME), *Arabidopsis thaliana* (At2g13560) and *Solanum tuberosum* (Z23023), *Oryza sativa* (NM_001066235) and β subunits from *A. thaliana* (At4g00570), *O. sativa* (NM_001071533) and *Solanum tuberosum* (Z23002).

Although very few sequences from plant NAD-ME are known, a phylogenetic tree was constructed with the multiple sequence alignment obtained. The phylogenetic tree shows that the α and β sequences group separately (α and β group, Fig. 3). The only C_4 NAD-ME sequence known (from *A. hypochondriacus*) groups within the α subgroup. It remains to be clarified if new NAD-ME isoforms were created to fulfil the C_4 requirements, or whether a higher level and compartmentalized expression of the same isoform found in non-photosynthetic tissues was sufficient to function in the C_4 cycle.

IV. Plant PEPCK: the Cytosolic Gluconeogenic Enzyme Involved in C_4 Photosynthesis

PEPCK (ATP[GTP]:OAA carboxylase[transphosphorylase]) catalyzes the reversible carboxylation of PEP to OAA in the presence of a divalent cation

and a nucleoside triphosphate (Utter and Kolenbrander, 1972). There are two classes of PEPCKs depending on their nucleotide specificities: those which have a specific, or preferential, use of adenine dinucleotides (ATP-PEPCK: EC 4.1.1.49) and those which use guanine or sometimes inosine nucleotides (GTP-PEPCK: EC 4.1.1.32, Leegood and Walker, 2003).

The apparent universal presence of PEPCK suggests that it has a function broader than its specialized purpose in C_4 and CAM photosynthesis. The GTP-dependent enzyme is found in animals, birds, fish, insects, molluscs, nematodes, chytrids and bacteria and shows maximal activity in the presence of Mg^{2+} and Mn^{2+} (Walker and Chen, 2002). The ATP-dependent isoform is found in flowering plants, ascomycetes, basidiomycetes, brown, red and green algae, diatoms, dinoflagellates, trypanosomes and in many bacteria and has a strict requirement for Mn^{2+} (Leegood and Walker, 2003).

It is well known that the GTP-dependent isoform catalyzes the first committed step in gluconeogenesis and functions as an anaplerotic reaction (Utter and Kolenbrander, 1972). However, the function of the enzyme in many animal tissues is uncertain and more complex than originally thought (Croninger et al., 2002). In mammals, PEPCK is an important enzyme that helps to regulate blood glucose levels (Hundal et al., 2000).

In plants, ATP-PEPCK is a cytosolic enzyme occurring in a diverse range of plant tissues, including both developing and germinating seeds, various flower parts, fruits, trichomes, stomatal guard cells, phloem, roots, latex producing ducts and leaves of some CAM and C_4 plants (Kim and Smith, 1994; Borland et al., 1998; Leegood and Walker, 1999, 2003; Walker and Chen, 2002; Walker et al., 1999, 2001; Chen et al., 2004; Delgado Alvarado et al., 2007; Malone et al., 2007). Interestingly, the enzyme from diatoms is localized to chloroplasts (Cabello-Pasini et al., 2001).

Although the enzyme has been purified from a wide variety of animals, plants and bacteria, the crystal structures from only some sources excluding plants are available: ATP-PEPCK from *E. coli* (Matte et al., 1996), *Trypanosoma cruzi* (Trapani et al., 2001), *Anaerobiospirillum succiniciproducens* (Cotelesage et al., 2005), *Thermus thermophilus* (PDB 1j3b) and

Actinobacillus succinogenes (Leduc et al., 2005) and GTP-PEPCK from human (Dunten et al., 2002) and from *Corynebacterium glutamicum* (Aich et al., 2003).

A. The Photosynthetic PEPCK Isoform

The key role of PEPCK in the photosynthetic CO₂-concentrating mechanisms in some C₄ and CAM leaves and in some algae and diatoms providing CO₂ to the Calvin Cycle is well understood (Edwards et al., 1971; Ditttrich et al., 1973; Burnell and Hatch, 1988; Reiskind and Bowes, 1991; Leegood et al., 1996; Girodano et al., 2005; Roberts et al., 2007). In PEPCK-type C₄ plants, the enzyme is located in the cytosol of BSCs and works in tandem with the NAD-ME, which seems to supply the ATP required by PEPCK (Fig. 1, Hatch, 1987). It has also been reported that maize leaves contain appreciable amounts of PEPCK, though maize is a NADP-ME type C₄ plant (Walker et al., 1997). In a number of other C₄ grasses with NADP-ME type C₄-photosynthesis, PEPCK has also been found (Walker et al., 1997; Voznesenskaya et al., 2006). In maize, PEPCK is specifically expressed in BSCs (Furumoto et al., 1999) where it is involved in the decarboxylation of aspartate (Wingler et al., 1999). The predicted protein has an N-terminal extension, characteristic of plant PEPCKs, which may be involved in the regulation of PEPCK activity in vivo through some modification such as reversible phosphorylation (Furumoto et al., 1999). In this way, PEPCK may also play a role in C₄ photosynthesis in maize. In addition, evidence that PEPCK plays a role in decarboxylating C₄ acids via a partial C₄ cycle in the vascular system of C₃ plants has also been suggested (Hibberd and Quick, 2002).

Plant PEPCK is a multimeric enzyme of identical subunits (Burnell, 1986; Walker et al., 1995). In PEPCK-type C₄ plants, the leaf enzyme involved in photosynthesis is hexameric, as in *Urochloa panicoides* (Burnell, 1986). The N terminus of the leaf subunit is extremely labile and is rapidly cleaved off during preparation of cell extracts (Finnegan and Burnell, 1995; Walker and Leegood, 1996; Walker et al., 1997). In *U. panicoides*, the native peptide of 68 kDa is proteolysed to polypeptides of 62 and 64 kDa (Finnegan and Burnell, 1995). This proteolytic process may have

taken place in previous purifications in which catalytic properties of the enzyme have been studied (Leegood and Walker, 2003).

Compared with mammalian and yeast PEPCKs, plant isoforms have special features. PEPCKs of *U. panicoides* and some other C₄ plants are inhibited by photosynthetic intermediates like 3-phosphoglycerate, fructose-6-phosphate, fructose-1,6-bisphosphate and dihydroxyacetone phosphate (Hatch and Mau, 1977; Burnell, 1986), whereas mammalian and yeast PEPCKs are not affected (Utter and Kolenbrander, 1972). Additionally, plant (ATP-dependent) and non-plant (GTP-dependent) PEPCKs are inactivated by thiol-modifying reagents (Chang and Lane, 1966; Cardemil et al., 1990; Lewis et al., 1993).

As mentioned before, PEPCK has an absolute requirement for Mn²⁺. Assays conducted at non-physiological concentrations of Mn²⁺ showed that the enzyme is inhibited by millimolar concentrations of Mg²⁺ (Burnell, 1986; Walker et al., 1997). This finding contrasts with the results with the enzyme from yeast, trypanosome and rat liver (Cannata and Stoppani, 1963; Foster et al., 1967). However, Chen et al. (2002) later demonstrated that the enzyme of Guinea grass *Panicum maximum*, is activated by physiological concentrations of Mg²⁺.

The equilibrium of PEPCK reaction favours decarboxylation. Due to PEPCK low affinity for CO₂ it has always been proposed that the enzyme acts as a decarboxylase in vivo (Ray and Black, 1976; Urbina and Avilan, 1989), as in gluconeogenesis and in C₄ and CAM photosynthesis. However, in some aquatic plants and algae it has also been proposed that PEPCK acts as a carboxylase (Reiskind and Bowes, 1991). In fact, in previous studies PEPCK was assayed at non-physiological concentrations of metal ions, which together with the occurrence of the N terminal proteolysis, could be the reason for the low CO₂ affinity reported (Walker et al., 1997, 2002). Chen et al. (2002) demonstrated that at concentrations of Mn²⁺/Mg²⁺ similar to those found in the cytosol, and at physiological ATP to ADP ratios, PEPCK from *P. maximum* showed a K_m (CO₂) as low as 20 μM CO₂, similar to that of Rubisco from different sources. These findings support the notion that PEPCK may function as a carboxylase in vivo (Leegood and Walker, 2003). In addition, X-Ray diffraction of ATP-PEPCK from *E. coli* has

been accomplished under different conditions and the CO₂ binding site essential for the carboxylation reaction has been characterized (Matte et al., 1996; Cotelesage et al., 2007).

The enzyme from leaves of CAM plants and C₄ species like *P. maximum* is susceptible to phosphorylation during the dark (Walker and Leegood, 1995, 1996). In PEPCK-type CAM plants, this process is reversed by illumination (Walker and Leegood, 1995; Walker et al., 1997). This phosphorylation is unique to plants and takes place in an additional N-terminal sequence, which is not found in the protein isolated from other organisms. On the contrary, the enzyme from *U. panicoides* and several other C₄ grasses is not susceptible to phosphorylation (Walker and Leegood, 1996). In the case of maize, PEPCK is weakly phosphorylated by a cAMP-dependent protein kinase in vitro (Furumoto et al., 1999); but in vivo it is not regulated by phosphorylation showing no light/dark regulation of the activity (Walker et al., 2002).

Okadaic acid (inhibitor of protein phosphatase) promotes the phosphorylation of PEPCK from Guinea grass, indicating that PP2A may be involved in the dephosphorylation process of the enzyme. Apparently, the phosphorylation is controlled by PEPCK kinase turnover (Bailey et al., 2007). Both characteristics are also typical for PEPC (Bakrim et al., 1992; Carter et al., 1990). In addition, the phosphorylation state of PEPC and PEPCK from *P. maximum* showed a similar, but inverse pattern in relation to changes in light and CO₂ (Bailey et al., 2007). In this way, the phosphorylation state of both enzymes is coordinated in vivo, with both enzymes being activated in the light in C₄ plants.

The ratio of adenylates modulates PEPCK activity. It has been initially proposed that adenylates interact with PEPCK at an allosteric site (Burnell, 1986; Urbina and Avilan, 1989). Walker et al. (2002) showed that the response to PEP and OAA strongly depends on the adenylate ratio and changes in the phosphorylation state. Studies on the unphosphorylated form of PEPC from *P. maximum* showed that when assayed in the carboxylation direction in the absence of ATP, the response of the enzyme to increasing PEP was hyperbolic, but increasing the amount of ATP strongly inhibited and induced a sigmoidal behaviour. Comparison of proteolytically cleaved PEPCK in crude extracts of Guinea grass leaves, which

causes loss of the phosphorylation site (Walker and Leegood, 1996), showed a large difference between illuminated and darkened leaves when measured at low concentration of PEP and a high ratio of ATP to ADP, (Walker et al., 2002). A similar response to PEP was shown by PEPCK from yeast, PEPCK from *U. panicoides* that lacks the phosphorylation site, and by PEPCK from *P. maximum* without the N-terminal extension.

B. Non-photosynthetic PEPCK Isoforms from C₄ Plants

Up to date, only one gene encoding PEPCK has been found in many species carrying out C₄ photosynthesis such as *Spartina anglica* (E12730), *Zoysia japonica* (AB199899, Nomura et al., 2005) and *P. maximum* (AF532733). In the case of *Flaveria trinervia*, two cDNA sequences are available in databases but they probably derived from the same gene (AB050471 and AB050472). However, PEPCK in *U. panicoides* is encoded by at least four genes, *pepck1* (AF136161) and *pepck2* (UP09241) are most abundant in leaf tissue, whereas *pepck3* and *pepck4* are most abundant in root tissue (AF136162 and AF136163, Finnegan et al., 1999, Suzuki and Burnell, 2003). This clearly shows that in C₄ species, PEPCK may be found in photosynthetic and non-photosynthetic tissues where it probably carries out functions similar to those in C₃ plants (see next section). Nevertheless, currently there is scarce information in this respect, and future studies may help to elucidate the functions of non-photosynthetic isoforms in C₄ plants.

C. Non-photosynthetic PEPCK Isoforms from C₃ Species

Besides its photosynthetic role, the reaction catalyzed by PEPCK has a main role in metabolism, by linking the pathways of amino acids, organic acids, sugars and lipids. The gluconeogenic form of PEPCK is widely distributed among C₃ plants where it plays an important role in the conversion of lipid into sugar during the germination of fat-storing seeds, mobilizing, in this way, reduced carbon from lipids for use in other tissues of the seedling (Leegood and ap Rees, 1978; Trevanion et al., 1995). PEPCK may play a key role in amino acid metabolism as the enzyme is associated with

tissues in which the metabolism of nitrogenous compounds is enhanced (Walker et al., 1999; Lea et al., 2001; Walker and Chen, 2002). In some tissues, the enzyme has a peak of activity which matches with the deposition of storage proteins (Walker et al., 1999; Delgado Alvarado et al., 2007). PEPCK is involved in the conversion of the carbon skeleton of asparagine/aspartate to glutamate/glutamine, is particularly important in transport systems, seed development and fruit ripening of higher plants (Bahrami et al., 2001; Leegood and Walker, 2003; Chen et al., 2004; Delgado Alvarado et al., 2007; Malone et al., 2007). In *Arabidopsis*, the enzyme is involved in the transfer of assimilates to developing ovules and seeds (Malone et al., 2007), and the function of PEPCK1 in gluconeogenesis in germinating seeds has been clearly demonstrated by down regulation of the gene (Ryllot et al., 2003; Penfield et al., 2004). PEPCK may be involved in the transport of nitrogenous assimilates out of senescing cucumber (*Cucumis sativus*) cotyledons (Chen et al., 2000) and in the transport and metabolism of assimilates in pea seeds (Delgado Alvarado et al., 2007). Walker et al. (2001) provided evidence that PEPCK may be also involved in the regulation of intracellular pH in tissues active in nitrogen metabolism by decarboxylating OAA derived from malate. Chen et al. (2004) showed that PEPCK is increased by treatments that lower intracellular pH and may function in the conversion of amino acids to PEP as respiratory or gluconeogenic substrates, according to energetic requirements, with the concomitant consumption of protons, and thus regulating pH.

The enzyme found in gluconeogenic cucumber cotyledons is tetrameric in contrast to the hexameric form involved in photosynthesis (Walker and Leegood, 1995; Walker et al., 1995). Depending on the species, the molecular mass of the polypeptides varies between 61 and 78 kDa, for example in cucumber cotyledons and other seedlings the protein subunits are of 74 kDa (Walker et al., 1995; Walker and Leegood, 1996), in *Arabidopsis*, the protein products of the two genes encoding PEPCK are of 73.4 and 72.9 kDa (Malone et al., 2007), and in rice of 73.2 and 70.9 kDa.

As in the case of photosynthetic isoforms, the enzyme purified from cucumber cotyledons and endosperm of germinating seedlings is phosphorylated in the N terminus and this process is

reversed by illumination (Walker and Leegood, 1995; Walker et al., 1997).

D. Phylogenetic Relationship Among Plant PEPCK Sequences

The first PEPCK to be cloned was that from *Cucumis sativus* (AF481231, Kim and Smith, 1994). Currently, only one gene has also been found in many other species (Fig. 4), while the PEPCK in *U. panicoides* is encoded by at least four genes (Finnegan et al., 1999; Suzuki and Burnell, 2003). There are two genes in the *Arabidopsis* genome (At5g65690.1, At4g37870.1); although both transcripts are found in a range of tissues, pepck1 mRNA is more abundant and is present in a wider range of tissues (Malone et al., 2007). The rice genome also has two PEPCKs (Os03g0255500 and Os10g0204400). PEPCKs from *Brassica napus*, *B. oleracea* and *B. campestris* are also encoded by several genes as shown by Southern blot hybridization (Saez-Vasquez et al., 1995).

There are significant amino acid sequence identities within ATP and GTP dependents PEPCK, but no significant similarity in the amino acid sequence between the two classes of PEPCKs, although the active-site residues are conserved in the enzymes from all sources (Dunten et al., 2002). In addition, there is a great similarity in kinetic properties and tertiary structures. Many residues and motives are conserved along the whole sequence, suggesting that they evolved from a common ancestor (Matte et al., 1997; Trapani et al., 2001; Leegood and Walker, 2003).

A phylogenetic tree was constructed with the multiple sequence alignment of the full length PEPCK cDNAs available. The tree (Fig. 4) shows that the sequences from monocots and dicots group separately, with the enzyme from C₄ species being present in both groups. Interestingly, among the monocot group, both forms of the enzyme from the C₃ monocot rice are the ones closer to the dicotyledonous group. In addition, within the dicots there is no subgrouping for the C₄-PEPCK type (Fig. 4). It is suggested that the C₄ photosynthetic genes have evolved from a set of pre-existing genes that had not been utilized for photosynthesis in ancestral C₃ plants (Ku et al., 1996). Modification of the ancestral genes and development of Kranz anatomy with

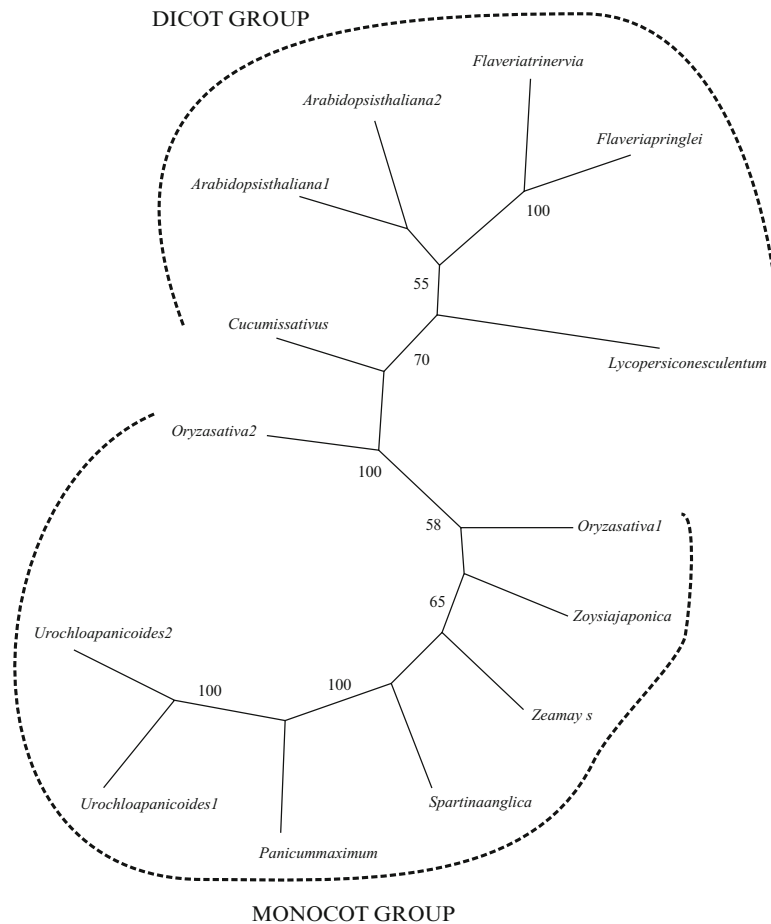


Fig. 4. Phylogenetic tree of plant PEPCK. The tree was constructed as described in Fig. 2. The sequences included are the following: *Arabidopsis thaliana* PEPCK1 and PEPCK2 (At4g37870 and At5g65690, respectively), *Cucumis sativus* (L31899), *Flaveria pringlei* (AB050473), *F. trinervia* (AB050471), *Lycopersicon esculentum* (AY007226), *O. sativa* PEPCK1 and PEPCK2 (Os03g0255500 and Os10g0204400, respectively), *Panicum maximum* (recently placed in the *Urochloa* genus as *Urochloa maxima*; AF532733), *Spartina anglica* (E12730), *U. panicoides* PEPCK1 and PEPCK2 (U09241 and AF136161, respectively), *Zea mays* (AB018744) and *Zoysia japonica* (AB199899).

differentiated photosynthetic BSC must have allowed high-level, light-dependent, and cell-specific expression of those genes to facilitate the evolution of C_4 plants from their C_3 ancestors and this process may have taken place probably after the monocot–dicot diversification.

The N-terminal extension of plant PEPCKs contains two potential phosphorylation sites, a cAMP-dependent protein kinase site and a consensus sequence for the SNF-1 related protein kinases which may be implicated in the regulation of Sucrose-P synthase, nitrate reductase, and 3-hydroxy-3-methyl glutaryl coenzyme A reductase (Leegood and Walker, 2003). Both sites are present in PEPCK from cucumber

(Walker and Leegood, 1995), while they are absent in PEPCK from *U. panicoides* (Finnegan and Burnell, 1995). In contrast, *P. maximum* has only the cAMP-dependent protein kinase site (Walker and Leegood, 1996). The Ser residue that undergoes phosphorylation in vivo is within the sequence of amino acids: Gln-Lys-Lys-Arg-Ser-Thr (residues 63–68 in *C. sativus*), which is similar to the phosphorylation motif of PEPC-kinase (Walker and Leegood, 1995). This motif is present among all the dicot sequences included in the phylogenetic tree. Within the group of monocotyledonous species, this motif is absent in *Z. mays* and in one of the rice sequences (Os10g0204400) and it is rather different and lacking the Ser

residue in both sequences of *U. panicoides*. It seems that during the evolution, some monocot species, especially those performing C₄ photosynthesis, have lost this regulatory phosphorylation site. This is a preliminary hypothesis and more sequences should be obtained and analyzed in order to confirm it. It has been proposed that during evolution, the regulatory phosphorylation of PEPCK has been maintained in CAM plants to prevent a futile cycle of carboxylation/decarboxylation, as both PEPC and PEPCK are present in the cytosol. In C₄ plants, both enzymes are present in different cells and levels of OAA, adenylates and other metabolites might be important in the regulation of PEPCK activity, which may explain why some C₄ PEPCK are not phosphorylated (Leegood and Walker, 2003). In addition, its function in concert with NAD-ME may also control the activity of PEPCK by providing ATP through mitochondrial respiration (Burnell and Hatch, 1988; Agostino et al., 1996). Nevertheless, it has been shown that phosphorylation of PEPCK and PEPC is coordinated (Bailey et al., 2007). In addition, the role of the phosphorylation of the enzyme in relation to its function in gluconeogenesis is even more unclear (Leegood and Walker, 2003).

From the data presented in this chapter it is clear that more studies are needed to understand the regulatory properties of PEPCK, especially under conditions that prevent its proteolysis during isolation. Clarification of whether during the course of evolution of C₄ species, a new isoform of the enzyme was acquired to participate in the CO₂ concentration mechanism and why only in some cases the regulation by phosphorylation has been lost are also important topics that need to be addressed.

V. Future Perspectives

During the last years, much knowledge was acquired about C₄ acid decarboxylases operating in plants. The existence of different subgroups among C₄ plants is in agreement with the multiple evolutionary origins of C₄ photosynthesis. The adaptation of each decarboxylase to the CO₂ concentration mechanism occurred independently and in many occasions during evolution and after the diversification of monocot and dicot species. It seems that the preexisting forms of these decarboxylases in C₃

plants may have served as starting points for the evolution of the photosynthetic isoforms.

Among the three decarboxylases, NADP-ME is the most widely studied. Much has been learnt about the kinetic and structure properties of the photosynthetic isoforms and a great amount of information is being obtained on the non-photosynthetic counterparts occurring in C₃ as well as in C₄ plants. Both cytosolic and plastidic ancestral genes may have had the potential to originate photosynthetic NADP-MEs independently.

With respect to NAD-ME and PEPCK, there is still deficiency in knowledge regarding functioning of non-photosynthetic isoforms in C₄ plants. In addition, more studies are needed to understand the regulatory properties of both enzymes in relation to photosynthesis. In contrast to PEPCK and NADP-ME, only few photosynthetic NAD-MEs have been studied. It remains unclear if during the course of evolution, new NAD-ME and PEPCK isoforms were created to fulfil the photosynthetic requirements or whether a high level and compartmentalized expression of the housekeeping isoforms in non-photosynthetic tissues was sufficient to function in the C₄ cycle. For both types of decarboxylases, the study of phylogenetic relationships and the characterization of more isoforms in different species will help to elucidate these questions.

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Structure, Function, and Post-translational Regulation of C₄ Pyruvate Orthophosphate Dikinase

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Summary	301
I. Introduction.....	302
A. Role of PPDK in C ₄ Plants.....	302
B. PPDK Enzyme Properties	302
1. Catalysis as Related to Structure.....	302
2. Oligomeric Structure and Tetramer Dissociation at Cool Temperatures.....	304
3. Substrate K _m for C ₄ PPDK.....	304
C. PPDK as a Rate-Limiting Enzyme of the C ₄ Pathway	304
II. Post-translational Regulation of C ₄ PPDK	305
A. Light/Dark Regulation of C ₄ PPDK Activity by Reversible Phosphorylation.....	305
1. Discovery of the PPDK Regulatory Protein, RP.....	305
2. PPDK RP: Enzyme Properties.....	305
3. The PPDK Phosphoryl-Inactivation Mechanism	306
4. Regulation of RP's Opposing Activities.....	307
B. Other Post-translational Components Governing PPDK Activity In Vivo	310
III. Functional and Bioinformatic Analysis of Cloned Maize C ₄ and <i>Arabidopsis</i> C ₄ -Like PPDK-Regulatory Protein	310
A. Cloning of RP from Maize and <i>Arabidopsis</i>	310
B. Functional Properties of Recombinant Maize C ₄ - and <i>Arabidopsis</i> C ₄ -Like RP.....	311
C. Bioinformatic Analysis of RP Primary Amino Acid Sequence	312
1. RP Is Highly Conserved in C ₃ and C ₄ Plants.....	312
2. RP Represents a Fundamentally New Structural Class of Regulatory Protein Kinase.....	312
IV. Future Directions.....	313
Acknowledgments	313
References	313

Summary

Pyruvate orthophosphate dikinase is a cardinal enzyme of the C₄ pathway. Its role in C₄ photosynthesis is to catalyze the regeneration of PEP, the primary carboxylation substrate from pyruvate, Pi, and ATP in the chloroplast stroma of leaf-mesophyll cells. It is the most abundant of C₄ enzymes, comprising up to 10% of the soluble protein of C₄ leaves, and thus may exert a limitation on the rate of CO₂ assimilation into the C₄-cycle. Studies dating back to the 1970s documented its biochemical properties as related to its role in C₄ photosynthetic process. Later studies originating in the early 1980s discovered how the enzyme is regulated in a light/dark manner by reversible phosphorylation of an active-site threonine.

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A bifunctional protein kinase/protein phosphatase with unprecedented properties, the PPDK Regulatory Protein (RP), was identified as the enzyme catalyzing this reversible phosphorylation event. However, the gene encoding this unusual enzyme had eluded cloning for some two decades until modern cloning methods allowed its recent isolation from maize. Although the enzyme properties of C_4 -PPDK are well understood, the molecular basis of its post-translational light/dark regulation by RP is poorly understood. Because of the significance of PPDK regulation to the C_4 -photosynthetic process, this chapter addresses the current state-of-knowledge on how C_4 -PPDK is post-translationally regulated by its companion regulatory enzyme, RP. This includes proposed models that describe how phosphorylation of PPDK by RP leads to complete inactivation of enzyme activity and the mechanism regulating the direction of RP's opposing PPDK-dephosphorylation and PPDK-phosphorylation activities. Also reviewed are the recent bioinformatic analyses of the RP polypeptide primary structure. These revealed that vascular plant RP represents a fundamentally new and novel kind of protein kinase with evolutionary origins in PPDK-containing anaerobic bacteria.

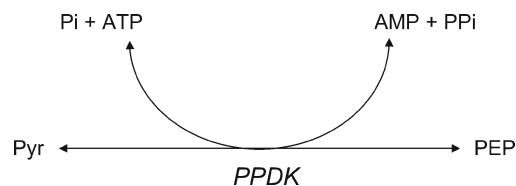
I. Introduction

Pyruvate orthophosphate dikinase (PPDK, E.C. 2.7.9.1) is an ancient enzyme found in a diverse group of microorganisms that includes the archaea (Tjaden et al., 2006), eubacteria (Pocalyko et al., 1990; Herzberg et al., 1996), amitochondriate protozoa (Bringaud et al., 1998) and green algae (Chastain and Chollet, 2003). It is absent in cyanobacteria and metazoans, but is evidently present in lower fungi (Marshall et al., 2001). Its evolution in (C_3) plants and recruitment into the C_4 pathway has been proposed to be the result of modifications of the gene promoter to confer cell specific expression (Sheen, 1991). In this regard, its transcriptional regulation, as with other key C_4 enzymes, is an important overall component of C_4 photosynthesis regulation. This aspect of PPDK regulation is covered in Chapter 12. This chapter will focus on the functional aspects of PPDK in the C_4 pathway and the more recent findings concerning its post-translational regulation.

A. Role of PPDK in C_4 Plants

In the C_4 pathway, PPDK catalyzes the conversion of 3-carbon pyruvate into phosphoenolpyruvate

(PEP) in the stroma of leaf-mesophyll cell chloroplasts:



Although its catalysis is freely reversible, the reaction is maintained in the PEP forming direction by the abundant pyrophosphatase and adenylate kinase activities in this organelle as well as the physiochemical factors prevailing during illumination such as stromal alkaline pH (Jenkins and Hatch, 1985; Ashton et al., 1990). It is the sole PEP regenerating mechanism for photosynthetic PEP carboxylase (PEPc) fixation in $NADP^+$ - and NAD^+ ME- type C_4 plants and contributes to C_4 photosynthetic PEP supply in PEPcK-type C_4 plants (Ashton et al., 1990).

B. PPDK Enzyme Properties

1. Catalysis as Related to Structure

Most of what is known concerning the structural aspects of the PPDK catalytic mechanism originate from studies of crystallized PPDK homodimer from the bacterium *Clostridium symbiosum* (Pocalyko et al., 1990; Herzberg et al., 1996; Lin et al., 2006; Lim et al., 2007). Although comparable studies of crystallized plant PPDK are not as yet available, the *C. symbiosum* structural model

Abbreviations: aa – amino acid; GFP – green fluorescent protein; NADP MDH – NADP malate dehydrogenase; NADP ME – NADP malic enzyme; ORF – open reading frame; Pi – inorganic phosphate; PPi – pyrophosphate; PEP – phosphoenolpyruvate; PEPc – PEP carboxylase; PPDK – pyruvate orthophosphate dikinase; Pyr – pyruvate; RP – regulatory protein;

is considered to be homologous to that of the plant enzyme as indicated by a high degree of conserved primary structure between plant and bacterial PPDKs (Pocalyko et al., 1990), and an identical reaction mechanism (Carroll et al., 1990). Furthermore, the first reported plant PPDK crystal structure (of a maize C₄ PPDK dimer complexed with PEP) is very similar to the three dimensional structure of the *C. symbiosum* enzyme (Nakanishi et al., 2005).

PPDK is a member of the PEP-utilizing enzyme family that catalyze Pyr/PEP interconversions using a highly conserved His residue for catalytic phosphoryl group transfer. Typically, enzymes of the PEP-utilizing family are structured into three major catalytic domains that facilitate the overall reversible catalysis (Herzberg et al., 1996; Lin et al., 2006; Tjaden et al., 2006; Lim et al., 2007). In the case of PPDK, the structural basis for the reversible reaction mechanism, as deduced from the *C. symbiosum* enzyme (Fig. 1), involves the dynamic interaction of a central “swiveling” phospho-transfer domain with flanking N- and C-terminal substrate binding domains (Herzberg

et al., 1996; Lin et al., 2006; Lim et al., 2007). A key element in this mechanism is the ability of the central domain to freely pivot or swivel between the remote N- and C-terminal domains upon flanking “hinge-like” peptide linkers. Thus, as viewed in this structural context, catalysis proceeds within these domains through a 3-step partial reaction sequence as illustrated in Fig. 1. In the C₄ PEP-forming direction, the first partial reaction is initiated by ATP binding to the N-terminal nucleotide-binding domain. This is followed by pyrophosphorylation of the central domain catalytic His residue (E-His) with the β- and γ- phosphates of ATP during interdomain docking to form an E-HisP^βP^γ intermediate. In the second partial reaction, the γ phosphate from the E-HisP^βP^γ catalytic intermediate is transferred to a free phosphate yielding pyrophosphate (PPi), AMP, and the E-HisP^β catalytic intermediate. In the third partial reaction, the central phospho-transfer domain pivots to the active-site of the C-terminal pyruvate-binding domain where subsequent transfer of the His bound P^β to pyruvate takes place to form PEP.

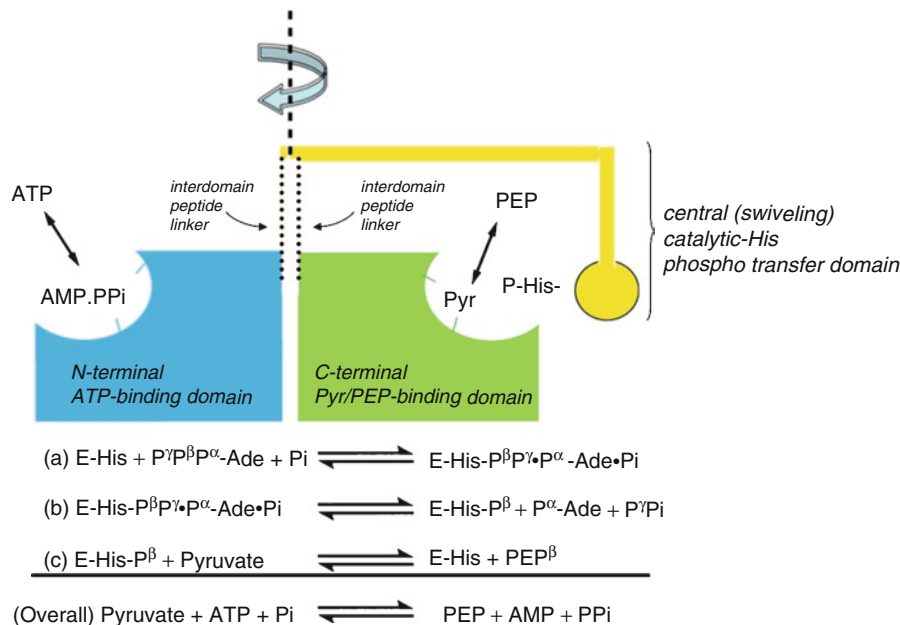


Fig. 1. The reversible three-domain enzyme reaction mechanism of PPDK. PPDK catalysis proceeds via a three-step partial reaction sequence that involves the interaction of a swiveling central catalytic phospho-transfer domain with remote N- and C-terminal ATP and Pyr/PEP substrate binding domains, respectively (Herzberg et al., 1996; Lin et al., 2006; Lim et al., 2007). The central catalytic phospho-transfer domain can freely pivot back-and-forth on flexible interdomain peptide linkers of ~15–30 residues in length, enabling either reaction direction energetically feasible.

Table 1. Representative PPDK Substrate K_m (μ M): PEP forming direction.

C ₄ leaf source	Pyruvate	ATP	Pi
Maize	82 ^a ; 158 ^b /65 ^b	32 ^a ; 95 ^b /47 ^b	380 ^a ; 408 ^b /134 ^b
<i>Flaveria bidentis</i>	73 ^b /59 ^b	25 ^b /49 ^b	118 ^b /138 ^b

Source of data are superscripted: ^aEdwards et al. (1985); ^bOhta et al. (1997).

Paired K_m values are: K_m native leaf enzyme (numerator)/ K_m recombinantly produced enzyme (denominator).

2. Oligomeric Structure and Tetramer Dissociation at Cool Temperatures

C₄ PPDK is active as a homotetramer of ~95 kDa subunits. Tetramerization to form active enzyme requires free Mg²⁺. In planta and in vitro, it has been long known that C₄ PPDK dissociates into inactive dimers and monomers when subjected to cold temperatures (e.g., ≤12°C) (Shirahashi et al., 1978). The single known exception to this phenomenon occurs in the C₄-like NADP-ME dicot species *Flaveria brownii* where its cold-stable PPDK retains tetrameric structure at temperatures down to 0°C in vitro (Burnell, 1990). A later study utilizing amino acid substitutions of recombinantly expressed *F. brownii* PPDK identified three hydrophobic residues within the extreme C-terminal portion of the polypeptide that were responsible for conferring cold-stability of the tetramer in vitro (Ohta et al., 1997). A proposed mechanism by which these three closely spaced residues allow cold-stabilization of active *F. brownii* PPDK tetramer centers around how the respective hydrophobic side-chains may increase interaction between PPDK monomers (and hence tetramer stabilization). Evidence confirming this proposed mechanism will ultimately require a three dimensional structure of *F. brownii* wild-type and mutant enzyme. Nevertheless, these investigations provide convincing evidence that *F. brownii* C₄ PPDK has acquired resistance to cool, suboptimal temperatures solely by minor structural changes in the enzymes' C-terminal PEP/Pyr binding domain.

3. Substrate K_m for C₄ PPDK

Earlier investigations into the biochemical and kinetic properties of maize C₄ PPDK largely established the enzymes' biochemical and kinetic properties (reviewed in Edwards et al., 1985; Carroll et al., 1990). Substrate binding constants

determined by these studies for the PEP-forming reaction are summarized in Table 1. Comparable extensive studies with recombinantly produced C₄ PPDK have yet to be performed, although Ohta et al. (1997) found that recombinantly produced maize and *F. bidentis* (C₄) PPDK had substrate K_m that were similar to the respective species enzyme isolated from leaves (Table 1). For example, in maize, the reported K_m for pyruvate ranged from 82–158 μ M for enzyme extracted from leaves, while a K_m of 65 μ M was reported for cloned, recombinantly expressed maize PPDK. Likewise, the K_m for ATP from these same sources ranged from 32–95 μ M for the leaf extracted enzyme and 47 μ M for the recombinantly produced enzyme.

C. PPDK as a Rate-Limiting Enzyme of the C₄ Pathway

Under varying conditions of light and temperature, the rate of CO₂ assimilation by C₄ leaves can be limited by one or more enzymes in the pathway (Furbank et al., 1997; von Caemmerer and Furbank, 1999; Kubien et al., 2003). A number of earlier studies had implicated PPDK as a major rate-limiting enzyme of the C₄ pathway (Furbank et al., 1997 and references therein). These investigations arrived at this conclusion by showing how the level of PPDK enzyme activity, as measured in desalted crude C₄ leaf extracts, appeared to match the CO₂ assimilation rate of the corresponding intact parent leaf prior to extraction. In contrast, the similarly extracted activities of PEPc, NADP-ME and Rubisco were shown to be higher (and thus non rate-limiting) than the corresponding rate of intact leaf CO₂ assimilation. Given a plethora of variation in experimental conditions and imperfect extraction and assay techniques, such estimates were likely to be inaccurate. However, in the past decade, development of the transgenic C₄ *Flaveria* system and the subsequent production

of transgenic C₄ enzyme RNA-antisense lines has allowed a less problematic assessment of C₄ pathway enzyme-limitation points. This is well illustrated by a study that examined CO₂ assimilation as a function of antisense-reduced PPDK, Rubisco, and NADPH-MDH in *F. bidentis* transgenic lines (Furbank et al., 1997). This investigation implicated PPDK, along with Rubisco, as co-limiting activities with respect to whole leaf CO₂ assimilation. In a study that utilized an empirical multifactorial C₄ photosynthesis model (von Caemmerer and Furbank, 1999), the rate of PEP regeneration (i.e., PPDK activity) was predicted to limit C₄-leaf CO₂ assimilation at or above the thermal optimum of the C₄ photosynthesis process. Related evidence that PPDK activity can exert a limitation on C₄ leaf CO₂ assimilation comes from a pair of studies of the cool tolerant C₄-hybrid grass, *Miscanthus x giganteus* (Naidu and Long, 2004; Wang et al., 2008). Specifically, these studies demonstrated that maintenance of C₄ photosynthetic competence during plant growth at cool temperatures is highly correlated with an elevation in the amount of PPDK polypeptide, implying that the adaptation mechanism relies in part on the increased synthesis of PPDK enzyme in order to sustain flux into the C₄ pathway.

In summary, because PPDK is one of two enzymes demonstrated to co-limit C₄ leaf CO₂ assimilation, it represents a viable target for strategies aimed at the photosynthetic improvement of C₄ plant productivity via genetic engineering approaches.

II. Post-translational Regulation of C₄ PPDK

A. Light/Dark Regulation of C₄ PPDK Activity by Reversible Phosphorylation

1. Discovery of the PPDK Regulatory Protein, RP

As a potentially rate-limiting enzyme in the C₄ pathway, synchronization of PPDK activity with light availability in vivo is essential for efficient functioning of the C₄ cycle and its coordination with the C₃ pathway. This coordinate regulation of activity with light was demonstrated early on

by studies that showed PPDK extracted from dark-adapted maize leaves had negligible activity, while PPDK extracted from illuminated leaves contained highly active enzyme with maximal light activation state reached at irradiances of around one-half full sunlight (Edwards et al., 1985, and references therein). Further research demonstrated that light activation of the enzyme was specific to photosynthetically active radiation, i.e., activated solely by red and blue spectra. DCMU, an uncoupler of photophosphorylation (Yamamoto et al., 1974), was also shown to inhibit light activation of PPDK (Nakamoto and Edwards, 1986). These circumstantial observations alone implied that the activation could be due to physiological changes in the mesophyll-cell chloroplast stroma such as pH, redox state, or divalent cation level. However, a key observation that led to the elucidation of the causal agent of the activation process was that the inactivated PPDK in dark-adapted crude leaf extract could regain its activity simply by extended incubation of the extract at ambient temperatures (Edwards et al., 1985). Further pursuit of this phenomenon led to the finding that an enzyme activity was responsible for the PPDK activation effect. In subsequent investigations, this enzyme activity was shown to confer both dark-induced inactivation and light-induced activation of PPDK by catalyzing reversible phosphorylation of an active-site Thr residue (Thr-456 in maize) (Burnell and Hatch, 1983, 1985a; Ashton et al., 1984; Budde et al., 1985). Now named the PPDK Regulatory Protein (RP), it is a low abundance protein ($\leq 0.04\%$ of soluble maize leaf protein) specifically co-localized with PPDK in the stroma of mesophyll cell chloroplasts.

2. PPDK RP: Enzyme Properties

The collective enzyme properties of RP make this regulatory enzyme among the most unique of the many thousands of now classified regulatory protein kinases/protein phosphatases. These collective properties are: (i) its bifunctionality, catalyzing both PPDK phosphorylation and dephosphorylation. This is rare as most regulatory phosphorylation cycles have separate protein kinase and protein phosphatase enzymes; (ii) the use of ADP versus ATP (i.e., β -phosphate) as its phosphoryl substrate; and (iii) its utilization

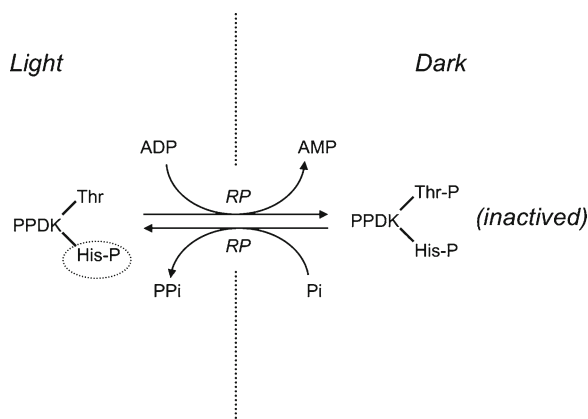


Fig. 2. Light/dark-mediated reversible phosphorylation of PPDK by RP. Dark induced inactivation of PPDK by RP proceeds by phosphorylation of a specific active-site Thr residue. Only the E-His-P intermediate enzyme form, as indicated by the encircled His-P residue, can undergo phosphorylation by RP. The catalytic His phosphate is removed in the dark by a yet-to-be identified mechanism (see section on “Putative Regulation by Adenylates” for a further discussion).

of a Pi-dependent, pyrophosphate forming dephosphorylation mechanism versus simple anhydride bond hydrolysis utilized by most protein phosphatases (Fig. 2) (Burnell and Hatch, 1983, 1985a; Roeske and Chollet, 1987; Chastain and Chollet, 2003).

More recent insights into the functional properties of C₄ RP have been gained by selective substitutions of the maize C₄ PPDK active-site His residue (His-458) and the proximal RP target Thr residue (Thr-456) (Chastain et al., 1997, 2000). The effect of these substitutions on RP catalyzed phosphorylation of the respective maize mutant PPDK enzymes are summarized in Fig. 3. Among the more informative of these were substitutions of the WT Thr-456 with Ser or Tyr. In vitro analysis of mutant enzyme showed that Ser was functionally interchangeable with Thr (i.e., phosphorylatable by RP) while Tyr was not (Fig. 3). The implication of this observation was that RP was mechanistically, and by inference, structurally related to the Ser/Thr super family of eukaryotic protein kinases (Hanks and Hunter, 1995; Hardie, 1999). Another informative substitution with respect to the RP catalytic mechanism was replacement of the catalytic His with Asn, a chemically related but nonphosphorylatable residue. As expected, this substitution produced a catalytically incompetent PPDK, but it also rendered the enzyme resistant

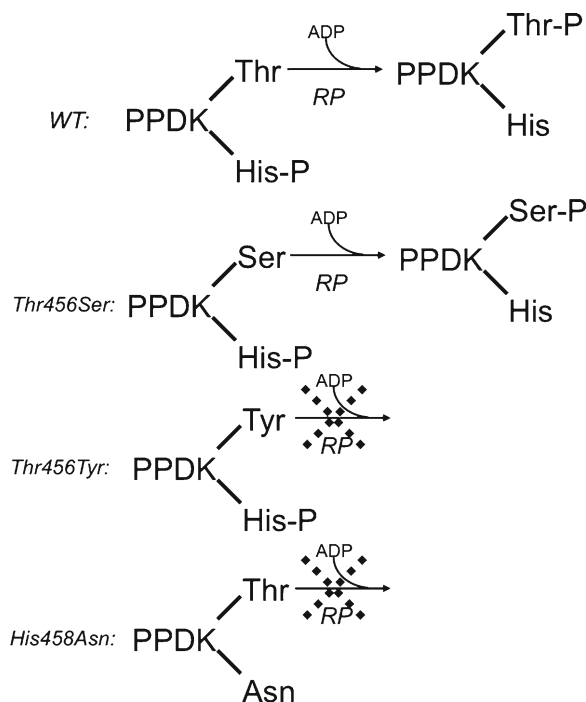


Fig. 3. Substitution experiments of the maize PPDK active-site Thr residue with the alternate protein kinase phosphorylation targets, Ser and Tyr. Serine can serve as an RP phosphorylation target but not Tyr. Insertion of the chemically related but nonphosphorylatable Asn in place of the catalytic His negates phosphorylation of the regulatory Thr residue.

to phosphorylation by exogenous RP, despite harboring the adjacent target Thr. The striking inability of this His458Asn mutant enzyme to undergo phosphorylation provided direct support for earlier biochemical studies which suggested that RP's protein kinase function has an absolute substrate requirement for the E-His-P form of the target enzyme (Fig. 2) (Burnell and Hatch, 1983; Burnell, 1984).

3. The PPDK Phosphoryl-Inactivation Mechanism

What is the mechanism by which the RP-catalyzed phosphorylation of a Thr residue converts active PPDK enzyme to inactive enzyme in a strict on/off fashion? A hypothesis that accounts for this on/off “switch” relates the di-anionic charge of the phosphate group to its placement on the regulatory Thr. Positioned in this manner, the electrostatic charge emanating from the central

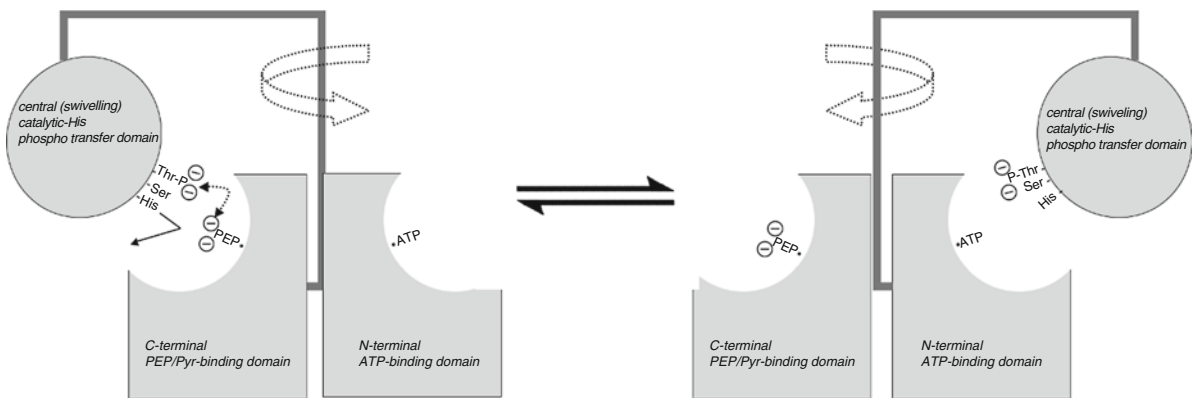


Fig. 4. Proposed PPDK phosphoryl-inactivation mechanism. By placement of a di-anionic phosphate group one residue removed from the central catalytic His, catalysis in the Pyr-PEP direction can be negated via electrostatic repulsion, as indicated by *double headed arrows*, of substrate bound at the C-terminal domain.

domain active-site would repulse the similarly charged Pyr or PEP bound to the C-terminal domain attempting to bind the substrate Pi at the adjacent His residue (Fig. 4) (note that the AMP to ATP partial reaction at the N-terminal domain is unaffected by regulatory Thr phosphorylation, (Burnell, 1984)). This hypothesis was tested by replacing the RP target Thr residue with mono-anionic charge bearing amino acids Glu or Asp (Chastain et al., 2000). These substitutions produced completely inactive enzyme, thus mimicking the effect of phosphorylation of the WT Thr residue at this same position. Replacement of the WT Thr residue with neutral Val or Ser resulted in PPDK with WT activity, demonstrating that amino acid replacement at this position per se does not lead to inactive enzyme. Hence, introduction of even a single anionic charge at this position without including the steric bulk of the larger phosphate is sufficient to abolish PPDK activity.

4. Regulation of RP's Opposing Activities

Putative Regulation by Adenylates: Stromal ADP as an Attenuator of RP Bifunctional Activity

Because of the bifunctional nature of RP, the opposing regulatory activities of the protein kinase and protein phosphatase must be finely controlled, so that PPDK activation state is correctly adjusted to match C₄ cycle activity (for example, in response to temperature fluctuations,

light variation). However, until the recent cloning of maize C₄ RP and its availability in stable recombinant form (Burnell and Chastain 2006), all previous biochemical studies of RP regulation have utilized partially purified preparations extracted from maize leaves. An impediment plaguing these studies is the extreme instability of RP activity once it is extracted from C₄ leaf tissue (Smith et al., 1994). This in turn placed limitations on the kind and veracity of in vitro experiments that could be used to assess RP regulation. Future studies using highly stable recombinant RP should overcome such limitations imposed on these past studies. Nevertheless, a plausible and simple “ADP-as-attenuator” model has emerged based on empirical evidence from past studies that can account for the strict regulatory requirements posed by RP.

As depicted in Figs. 5 and 6, the key component in governing the direction of RP catalysis is the stromal concentration of the RP protein kinase substrate ADP and its action as a potent competitive inhibitor of RP phosphatase activity (Table 2). Under this proposed scheme, stromal [ADP], which is a function of the stromal adenylate energy charge (AEC), exerts a default control as an attenuator on the opposing reactions as its level fluctuates in up/down fashion in parallel to the rate of photophosphorylation. For example, in the direction of decreasing illumination, the accompanying decrease in photophosphorylation transiently causes an elevation in stromal [ADP], tilting RP catalysis in the direction of PPDK phosphorylation (inactivation). Simi-

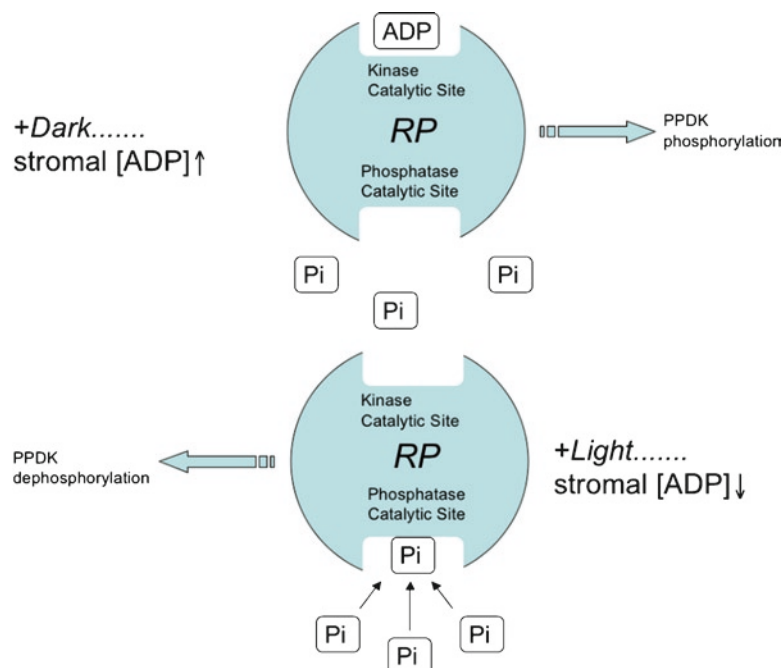


Fig. 5. Regulation of RP's opposing phosphorylation/dephosphorylation activities by stromal ADP level. As illustrated, the (proposed) separate protein kinase and protein phosphatase active-sites for RP allows for ADP to inhibit RP phosphatase activity in a competitive manner while also serving as substrate for PPK phosphorylation reaction (*top diagram*). In the light, active photophosphorylation causes an upward shift of stromal adenylate energy charge (AEC) and corresponding decline in stromal ADP, leading to dephosphorylation of phospho PPK (*bottom diagram*).

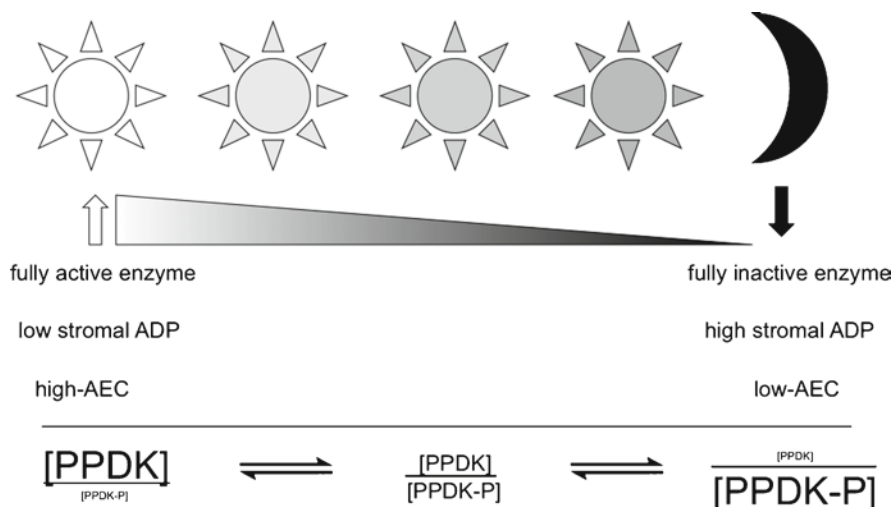


Fig. 6. Proposed model of ADP-as-attenuator of RP bidirectional activity. Depending on the prevailing light or dark conditions, the ratio of active, dephospho-PPDK to inactive, phospho-PPDK is carefully balanced to ensure that the rate of PEP regeneration catalyzed by PPK is synchronized with the available light energy incident on the C_4 leaf. This is accomplished by stromal ADP-level acting as a de facto sensor of photon flux density for attenuating PPK activity, rendering subtle up/down regulation in the overall pool of catalytically active PPK. PPK in C_4 leaves is fully active at approximately 1/2 full-sunlight ($\sim 1,000 \mu\text{mol photon m}^{-2}\text{s}^{-1}$). AEC, adenylate energy charge = $[\text{ATP}] + .5[\text{ADP}]/[\text{ATP} + \text{ADP} + \text{AMP}]$.

Table 2. Key Michaelis parameters of maize RP as measured in vitro.

Protein kinase		Protein phosphatase	
K _m ADP (μM)	50 ^a , 52 ^b	K _m Pi (μM)	700 ^a , 650 ^b
K _m PPDK-Thr (μM)	1.2 ^a	K _m PPDK-ThrP (μM)	0.7 ^a
		K _i ADP (μM)	84 ^a

^aBurnell and Hatch (1985a).^bRoeske and Chollet (1987).

larly, in the direction of increasing illumination and higher rates of photophosphorylation, stromal [ADP] declines, tilting the prevailing RP reaction towards dephosphorylation (activation) of inactive PPDK (Fig. 6). The key elements to this proposed mechanism are (i) ADP as a potent competitive inhibitor of the dephosphorylation reaction ($K_i = 84 \mu\text{M}$, Table 2) and (ii) light/dark induced changes in stromal [ADP]. Evidence supporting this working model comes from earlier studies that examined the effects of DCMU, a PSII electron-transport inhibitor, and CCCP, an uncoupler of photophosphorylation, on maize C₄-mesophyll protoplast and chloroplast PPDK activity (Nakamoto and Edwards, 1986; Nakamoto and Young, 1990). These findings showed that illumination of mesophyll cell preparations in the presence of DCMU or CCCP markedly inhibited light activation of PPDK, and this was correlated with lowered stromal ATP concentrations in the light. Moreover, in vitro evidence for physically separate active-sites for RP protein kinase and protein phosphatase catalysis lends credence to the proposal that ADP acts as both competitive inhibitor and substrate (Roeske and Chollet, 1987). Although this “ADP-as-attenuator” model appears to elegantly account for the apparent regulatory balancing of the opposing RP reactions, more accurate estimates of C₄-leaf mesophyll stromal adenylate concentrations are needed to fully validate it. For example, in a pair of studies that examined light/dark changes in in vivo PPDK activation state with respect to in vivo changes of mesophyll cell and chloroplast [ADP], the observed light-induced, ten-fold change in maize leaf PPDK activity was not highly correlated with the respective measured two-fold changes in [ADP] (Roeske and Chollet, 1989; Usuda 1988). However, this discrepancy may be an artifact incurred by the methods used in these reports for estimating in vivo stromal [ADP]. Such estimates

must take into account that ADP extracted and quantitated from chloroplasts is actually comprised of two fractions, a protein-bound fraction and a free fraction. Since only the latter form is available for RP regulation, the actual in situ free stromal [ADP] may be on par with those measured to inhibit RP phosphatase activity in vitro (Table 2). This seems plausible in light of studies that demonstrated the extensive and tight binding of stromal ADP to subunits of the abundant CF1 chloroplast ATP synthase (Hampp et al., 1982; Maylan and Allison, 2002).

Another potential factor that has bearing on the bidirectional regulation of RP relates to the fate of the phosphoryl group remaining on the PPDK catalytic His residue after the enzyme undergoes ADP-dependent inactivation to produce the E-HisP/ThrP PPDK [inactive] form, Figs. 2 and 3). In vitro, it has been shown that if this catalytic phosphate is not removed from inactivated PPDK, the rate of the Pi-dependent dephosphorylation/activation reaction is reduced by as much as fivefold (Burnell, 1984). Thus, if this slower activating PPDK enzyme-form were allowed to accumulate in dark adapted leaves, one could project a physiological scenario that negatively impacts the responsiveness of C₄ cycle activity. But such a scenario is never allowed to transpire in vivo since nearly all of the nascently inactivated and catalytic phosphorylated PPDK enzyme-form is known to be converted to the catalytically dephosphorylated state (E-His-ThrP) soon after the leaf has been dark adapted (Burnell and Hatch, 1985a, b). How this happens has yet to be resolved. One possibility is that the catalytic His-phosphate is removed from inactivated PPDK by the AMP+PPi to ATP + Pi back-reaction, thereby converting the enzyme to the preferred PPDK Thr-P dephosphorylation substrate. Confounding the plausibility of this mechanism is the abundant stromal pyrophosphatase activity known to occur in this

organelle that would function to keep stromal PPI at exceedingly low levels. Alternatively, the catalytic His-phosphate might be removed enzymatically, but at present a phosphatase that catalyzes this removal has yet to be identified.

Lack of Evidence for Post-translational Regulation of RP

Due to the instability of RP when isolated from C_4 leaves, a rigorous *in vitro* investigation of the enzyme for revealing potential post-translational regulation mechanisms has not been possible. Nevertheless, there is no indirect evidence to date to suggest that RP is post-translationally modified (for example, by reversible phosphorylation) or regulated by endogenous factors (for example, stromal pH). This view is supported by a study that examined RP activity after it was rapidly extracted from dark-adapted or illuminated maize leaves (Smith et al., 1994). In this investigation, RP activity from these leaves showed no preferential direction in catalysis, i.e., having equivalent relative competence in the *in vitro* phosphorylation or dephosphorylation of PPK, regardless of the light/dark pre-treatment of the parent leaves. Furthermore, the ratio of rapidly extracted, competing RP activities was also shown to be independent of pH utilized for extraction and assay. These sets of observations indicate that a post-translational regulatory mechanism, e.g., covalent modification, or changes in stromal pH, is not evident under conditions in which RP displays distinct *in vivo* regulation of its competing reactions. Likewise, stromal redox state, a well known regulatory mechanism for many stromal enzymes (via the ferredoxin/thioredoxin system) also has been shown to have no influence on RP regulation *in organello* or *in vitro* (Nakamoto and Young, 1990; Smith et al., 1994).

B. Other Post-translational Components Governing PPK Activity *In Vivo*

Unlike numerous metabolic enzymes (for example, PEPc), PPK activity is not subject to regulation by metabolite effectors. However, the direction of the reversible reaction catalyzed by PPK is significantly influenced by pH (Jenkins and Hatch, 1985). At alkaline pH ranges of >8.2 , the approximate stromal pH of illuminated chloroplasts, the

pyruvate to PEP reaction is strongly favored. At pH ranges of <7.0 , the approximate stromal pH of dark adapted chloroplasts, the PEP to pyruvate reaction is strongly favored. Enzyme activity is also stimulated several fold by NH_4^+ and K^+ (Jenkins and Hatch, 1985; Ashton et al., 1990).

III. Functional and Bioinformatic Analysis of Cloned Maize C_4 and *Arabidopsis* C_4 -Like PPK-Regulatory Protein

A. Cloning of RP from Maize and *Arabidopsis*

As mentioned above, one of the difficulties in biochemical characterization of RP is its low abundance and extreme instability upon extraction from C_4 leaves. This has prevented its purification to homogeneity (with the exception of a single report (Burnell and Hatch, 1983)) despite repeated attempts using more advanced purification schemes (Roeske and Chollet, 1987; Smith et al., 1994). Failure to isolate the RP polypeptide to a high level of purity has precluding its cloning by conventional means. In order to advance our understanding of this enigmatic enzyme, a cDNA clone was needed to elucidate its structure and enzyme mechanism. This clone was ultimately obtained by culling information from a proteomics study that profiled differential expression of soluble stromal polypeptides in isolated maize leaf mesophyll and bundle sheath cell chloroplasts (Majeran et al., 2005). In this report, a low abundance polypeptide of unknown function, specific to the mesophyll cell chloroplasts was identified (ZmGI Accession No. TC220929) and sub-cloned from a maize cDNA library. The encoded open reading frame (ORF) from this cDNA was functionally demonstrated *in vitro* to encode the elusive RP gene (Burnell and Chastain 2006). In parallel to the cloning of C_4 RP from maize, a similar effort cloned the C_4 -like RP from *Arabidopsis* (Accession No. At4g21210) (Chastain et al., 2008). A second RP-like gene was also discovered to be encoded by the *Arabidopsis* genome, but this cytoplasmic localized isoform appears to be of exclusive C_3 function and is not discussed further in this C_4 review.

B. Functional Properties of Recombinant Maize C₄- and Arabidopsis C₄-Like RP

In order to authenticate the cloned RP sequences, the respective recombinantly expressed proteins were functionally tested by previously established biochemical assays (Chastain et al., 2008; Ashton et al., 1990). The results of one of these assays, which is based on the immuno-detection of phosphorylated PPDK on western blots, is displayed in Fig. 7.

This specific test showed that the putative recombinant RP catalyzed the ADP-dependent, site-specific threonyl-phosphorylation of PPDK (Fig. 7a). Yet another critical biochemical test that confirmed RP specificity of the recombinant enzyme was the demonstration that ATP is required (along with ADP) in the assay mixture in order for the PPDK phosphorylation reaction to

take place. This is because RP is unable to phosphorylate PPDK at its target Thr unless the active-site catalytic His is also phosphorylated with the β -phosphate from ATP (e.g., the PPDK-His-P catalytic reaction intermediate, see Fig. 2). This RP property was aptly demonstrated when phosphorylation of PPDK was shown to be negated by the inclusion of pyruvate in the assay mixture (Fig. 7a). The addition of pyruvate to the phosphorylation reaction has the effect of scavenging phosphate from the catalytic PPDK-His-P reaction intermediate during its catalytic conversion to PEP (Fig. 7c) thereby “removing” the uniquely specific RP PPDK phosphorylation substrate from the reaction mix. Recombinant RP was also shown to catalyze the Pi-dependent dephosphorylation of phospho-PPDK (Fig. 7b). Although not displayed here, the results of these immuno-based assays were further corroborated by an analogous

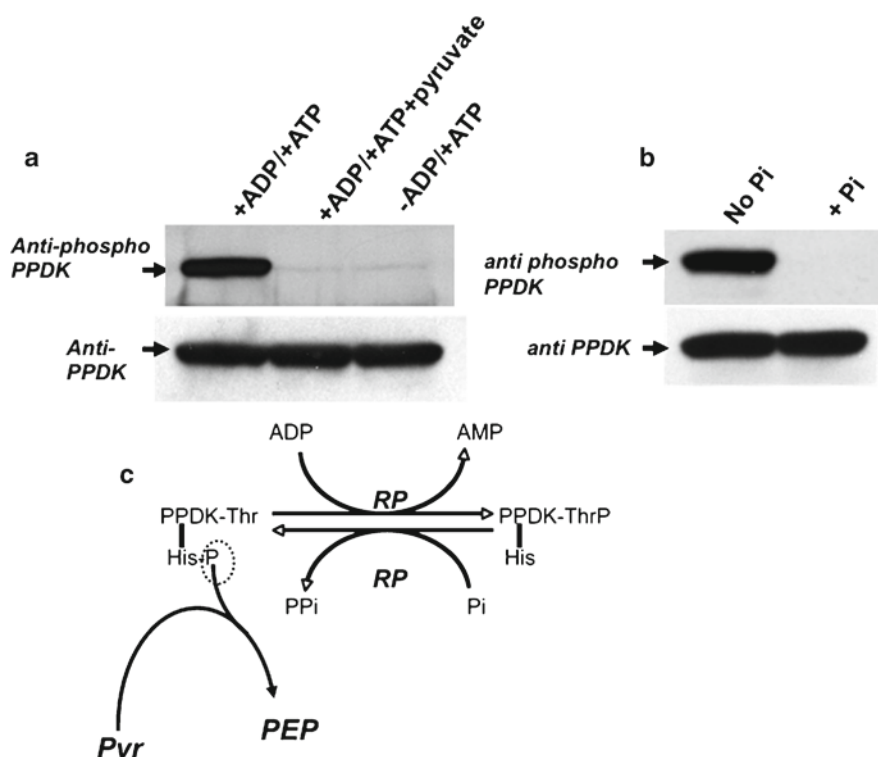


Fig. 7. Immuno-based in vitro assay of recombinantly produced *Arabidopsis* C₄-like RP. Western blots demonstrating the highly specific PPDK phosphorylating (protein kinase) (a) and PPDK-dephosphorylating (protein phosphatase) (b) activities of recombinantly produced enzyme. Shown are representative denaturing western blots of assay reaction aliquots probed with anti-PPDK-ThrP or anti-PPDK antibody as previously described (Chastain et al. 2008). Noted above each lane are variations in the standard reaction mixture: +ADP (1 mM), +ATP (0.2 mM); +pyruvate (2 mM); +Pi (2.5 mM). (c) Diagram illustrating the effect of added pyruvate to the RP protein kinase assay mixture (see text for a detailed explanation). The corresponding figure of this same assay performed with recombinant maize RP portrays the same result (as seen in Burnell and Chastain 2006).

spectrophotometer-based RP assay method (Burnell and Chastain 2006; Chastain et al., 2008). Thus, from these first experiments came confirming evidence that recombinantly produced RP possessed the requisite RP functional properties of (i) a protein kinase with strict substrate specificity (i.e., ADP as phosphoryl donor, PPK-His-P as phosphorylation target) and (ii) a Pi-dependent protein phosphatase (Table 2).

C. Bioinformatic Analysis of RP Primary Amino Acid Sequence

1. RP Is Highly Conserved in C₃ and C₄ Plants

A direct alignment of maize C₄ RP and *Arabidopsis* C₄-like RP (Fig. 8a) reveals a high degree of similarity between proteins with the most homologous region of the two polypeptides being a centrally positioned DUF 299 (Domain of Unknown Function) (Hulo et al., 2006). By definition, the DUF designation is assigned to conserved amino acid encoding sequences that are recurrent in various protein databases, but have no known functional precedent. In the representative maize and C₄-like *Arabidopsis* RP, this ~260-aa domain spans the central core of the 426- and 403-residue

maize and *Arabidopsis* full-length polypeptides, respectively (Fig. 8b). The only bioinformatics-deciphered motif structure within the RPs' DUF 299 is a centrally positioned, 8-residue ATP/GTP binding P-loop (Fig 8b). Interestingly, organisms possessing the DUF 299 domain are restricted phylogenetically to vascular plants, green algae, and a diverse group of PPK-encoding prokaryotes (Chastain et al., 2008). Both polypeptides are predicted to encode N-terminal chloroplast leader sequences (Table 3). Recent GFP-RP ORF fusion studies confirmed the chloroplast targeting of the *Arabidopsis* C₄-like RP. When the predicted N-terminal transit sequence was fused to the GFP ORF and transformed via microprojectile bombardment into *Arabidopsis* or tobacco leaves, accumulation of GFP was shown to be localized to the chloroplast stroma (Chastain et al., 2008).

2. RP Represents a Fundamentally New Structural Class of Regulatory Protein Kinase

As stated above, RP can phosphorylate Ser (but not Tyr) in place of the PPK wild-type Thr target residue. The implication of this observation was that RP was functionally and, by inference, structurally related to the Ser/Thr super family of

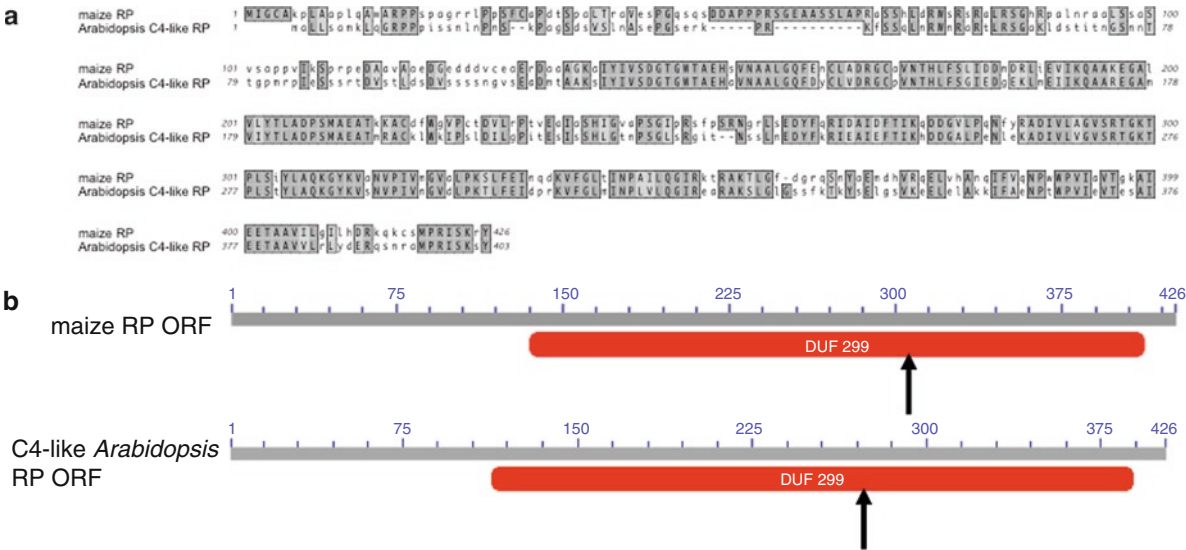


Fig. 8. Primary structure of maize C₄ RP and *Arabidopsis* C₄-like RP deduced amino acid sequence. (a) Direct alignment showing the high degree of RP primary structure similarity between the respective dicot and monocot species and (b) the position of the conserved DUF 299 region within the RP polypeptides with arrows indicating the bioinformatically identified 8-amino acid ATP/GTP binding P-loop motif.

Table 3. Summary of cloned recombinant RP properties.

	Maize C ₄ RP ^a	<i>Arabidopsis</i> C ₄ -like RP ^b
Length of encoded ORF	426 aa	402 aa
Predicted N-terminal organelle targeting transit peptide	Chloroplast-targeted	Chloroplast-targeted
ADP-dependent protein kinase function	Yes	Yes
Pi-dependent protein phosphatase function	Yes	Yes

^aBurnell and Chastain (2006).

^bChastain et al. (2008).

eukaryotic protein kinases (Hanks and Hunter, 1995; Hardie, 1999; Scheeff and Bourne, 2005). Thus, prior to its cloning it was anticipated that the primary structure of RP would encode the familial Ser/Thr protein kinase 12-subdomain structure. The premise for this is that *all* known eukaryotic Ser/Thr protein kinases share this highly conserved subdomain primary structure, all of which are requisite for enzymatic phosphorylation of target Ser/Thr substrate residues (Hanks and Hunter, 1995; Hardie, 1999). However, after its cloning, it was soon discovered that RP primary structure, either from maize or *Arabidopsis*, lacked even weakly facsimile eukaryotic or prokaryotic protein kinase subdomain structure. A more rigorous analysis using an algorithm-aided custom alignment also failed to locate any primary structure within the RP polypeptide or the internal ~260-residue DUF 299 domain that would correlate with the canonical subdomains I–XI inherent in all known eukaryotic Ser/Thr protein kinases, or the catalytically essential (and invariant) Ser/Thr protein kinase residues (Chastain et al., 2008). Lastly, an unrooted molecular phylogenetic analysis of full-length maize and *Arabidopsis* C₄-like RP amino-acid sequences with other vascular plant and green alga RPs and representative *Arabidopsis* Ser/Thr protein kinases and Ser/Thr protein phosphatases confirmed the related alignment analysis that RP, whether from plants or green algae, are unrelated to the canonically structured plant Ser/Thr protein kinases (Chastain et al., 2008). This tree analysis also demonstrated that the protein phosphatase function encoded in the RP primary structure is highly divergent from the ubiquitous Protein Phosphatase 1- and Protein Phosphatase 2A-catalytic subunits included in the tree analysis (Chastain et al., 2008).

IV. Future Directions

As discussed above, the gene for RP had proved to be recalcitrant to cloning efforts that were initiated soon after the enzyme was discovered in maize leaf extracts some two decades ago. Thus, many questions concerning this key regulator of the C₄ pathway had remained largely unapproachable. Its recent cloning therefore represents something of a watershed for revealing new insights on C₄ cycle regulation. Among the key questions that can now be addressed with the availability of an RP gene sequence include an unequivocal elucidation of how its opposing bidirectional activities are regulated. Additionally, the mechanism by which the catalytic His-phosphate is removed from nascently inactivated PPDK may also become more clear. Finally, assessment of photosynthetic regulation of transgenic C₄ plants with reduced RP levels (via RNAi or anti-sense technology) will undoubtedly provide the most revealing picture of how RP is integrated into the overall regulatory machinery of the C₄ pathway.

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Part IV

Diversity and Evolution

C₄ Photosynthesis Origins in the Monocots: A Review and Reanalysis

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Summary	319
I. Introduction	320
II. Alismatales	320
II. Cyperaceae	323
A. Rhynchosporae C ₄ Diversification	323
B. Abildgaardieae C ₄ Diversification	325
C. Eleocharidae C ₄ Diversification	325
D. Cyperaeae C ₄ Diversification	325
IV. Poaceae	326
A. Chloridoideae C ₄ Diversification	327
B. Panicoideae C ₄ Diversification	327
V. Conclusions	332
References	335

Summary

C₄ photosynthesis in the monocots occurs in species from three lineages: the Alismatales, Cyperaceae, and Poaceae. Previous estimates of C₄ origins in the monocots have suggested one origin of C₄ within the Alismatales, at least four origins in the Cyperaceae, and at least four (and likely more) origins in the Poaceae. The present Chapter explores the numbers of origins of C₄ in these three lineages further, summarizing the literature and reanalyzing the phylogenetic and photosynthetic pathway data for Panicoideae grasses, using Bayesian estimation of tree topologies and stochastic mapping of photosynthetic pathway characteristics. These results suggest that there have been a minimum of 24 separate C₄ origins in the monocots: 2 Alismatales, 5 Cyperaceae, and 17 Poaceae, and that transition frequencies and directions among photosynthetic pathway characteristics in the Panicoideae grasses are more complex than previously estimated. Further, the lack of species-level phylogenies and photosynthetic pathway characterizations in several lineages (particularly Cyperaceae and Chloridoideae grasses) preclude robust estimates of photosynthetic pathway origins and transitions. A concerted effort is needed to clarify these issues.

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I. Introduction

CO₂ concentrating mechanisms (CCMs) are prevalent throughout the monocots (Fig. 1; Givnish et al., 2006), and can be split between C₄ or C₄-like photosynthetic mechanisms and crassulacean acid metabolism (CAM; Sage et al., 1999; Smith and Winter, 1996). CAM photosynthesis is found in a number of monocot families, but is concentrating in the Orchidaceae and Bromeliaceae (Smith and Winter, 1996). The Orchidaceae and Bromeliaceae each likely include multiple origins of CAM (Avandhani et al., 1982; Crayn et al., 2004), but as this paper focuses on the origins of C₄ and C₄-like photosynthetic mechanisms, I will not discuss the non-C₄ CCMs further, except in passing. C₄ and C₄-like CCMs occur in three lineages: the Alismatales, Cyperaceae, and Poaceae (Kellogg, 1999). They are most notable among the grasses where C₄ enzyme pathways and their associated Kranz anatomical characteristics are important systematic characters (Avdulov, 1931; Brown, 1958; Hattersley, 1987; Prendergast et al., 1987; Kellogg, 2000), as well as their obvious impacts on ecological function and possible application towards crop improvement (Ku et al., 1999; Sukuki and Burnell, 2003; Sukuki et al., 2006). The Cyperaceae are also well noted for their CCM diversity (Bruhl and Wilson, 2007), but only a few species have been studied in detail (Agarie et al., 1997, 2002; Bruhl and Perry, 1995; Bruhl et al., 1987; Murphy et al., 2007; Uchino et al., 1995, 1998; Ueno, 1996a, b, 1998, 2001, 2004; Ueno and Samejima, 1989; Ueno and Wakayama, 2004; Ueno et al., 1986, 1988, 1989). Particularly, *Eleocharis vivipara* is a well-studied model species where carbon fixation method (C₃ or C₄) is variable with environment (terrestrial or submerged growth, respectively), and recent studies (Murphy et al., 2007) have shown the level and kind of CCM variability in *E. vivipara* depends on the genotype involved. While Haberlandt described Kranz anatomy in *Cyperus* in 1884, our understanding of Cyperaceae C₄ photosynthesis biochemistry and function is largely limited to *Eleocharis*.

Even less well understood are the CCMs present within the Alismatales. The CCM pathways within the Poaceae and Cyperaceae are primarily associated with C₄ photosynthetic systems with some anatomical restructuring (Kranz or Kranz-like anatomies; Carolin et al., 1977; Hattersley, 1987), while those within the Alismatales are more cryptic. While a few species are clearly facultatively C₄ (*Hydrilla verticillata* and *Egeria densa*), there is broad presence of HCO₃⁻ use or active H⁺ pumping of HCO₃⁻ into aquatic leaf cells; and clear, although weak, presence of CAM photosynthetic systems in some species (*Sagittaria subulata* and *Vallisneria spiralis*; Keeley, 1998).

Previous estimates of C₄ origins in the monocots have suggested that while three lineages (families) include all of the noted C₄ species (Kellogg, 1999; Soros and Bruhl, 2000), there have been multiple origins of C₄ within at least two of these (Cyperaceae and Poaceae). These studies, using the best phylogenetic estimates of the time, proposed one origin of C₄ within the Alismatales in the Hydrocharitaceae, at least four origins in the Cyperaceae, and at least four (and likely more) origins in the Poaceae.

Here I will further explore the numbers of origins of CCMs, particularly C₄ photosynthetic pathways, in these three lineages further. For most lineages, datasets (both phylogenetic and physiological) are lacking for detailed exploration of the evolutionary dynamics among photosynthetic pathway types. For these groups, I discuss the current state of knowledge. In the Panicoideae grasses, where our understanding of physiological diversity is more complete and large phylogenetic datasets are available, I apply a Bayesian estimation of tree topologies based on these previously published datasets and estimate the number of, direction of, and origins of the C₄ pathway and C₄ pathway characteristics in the lineage using stochastic mapping (Bollback, 2006). These hypotheses are then compared with previous estimates of physiological diversification in the panicoid grasses as a model for how other lineages might be studied and as a comparison to previous estimates based solely on parsimony methods.

II. Alismatales

Estimates of phylogenetic relationships among lineages within the Alismatales by Les et al. (1997) have strongly supported two clades (Fig. 2).

Abbreviations: CAM – Crassulacean acid metabolism; CCM – CO₂ concentrating mechanism; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PCK – Phosphoenolpyruvate carboxykinase; PEPC – Phosphoenolpyruvate carboxylase

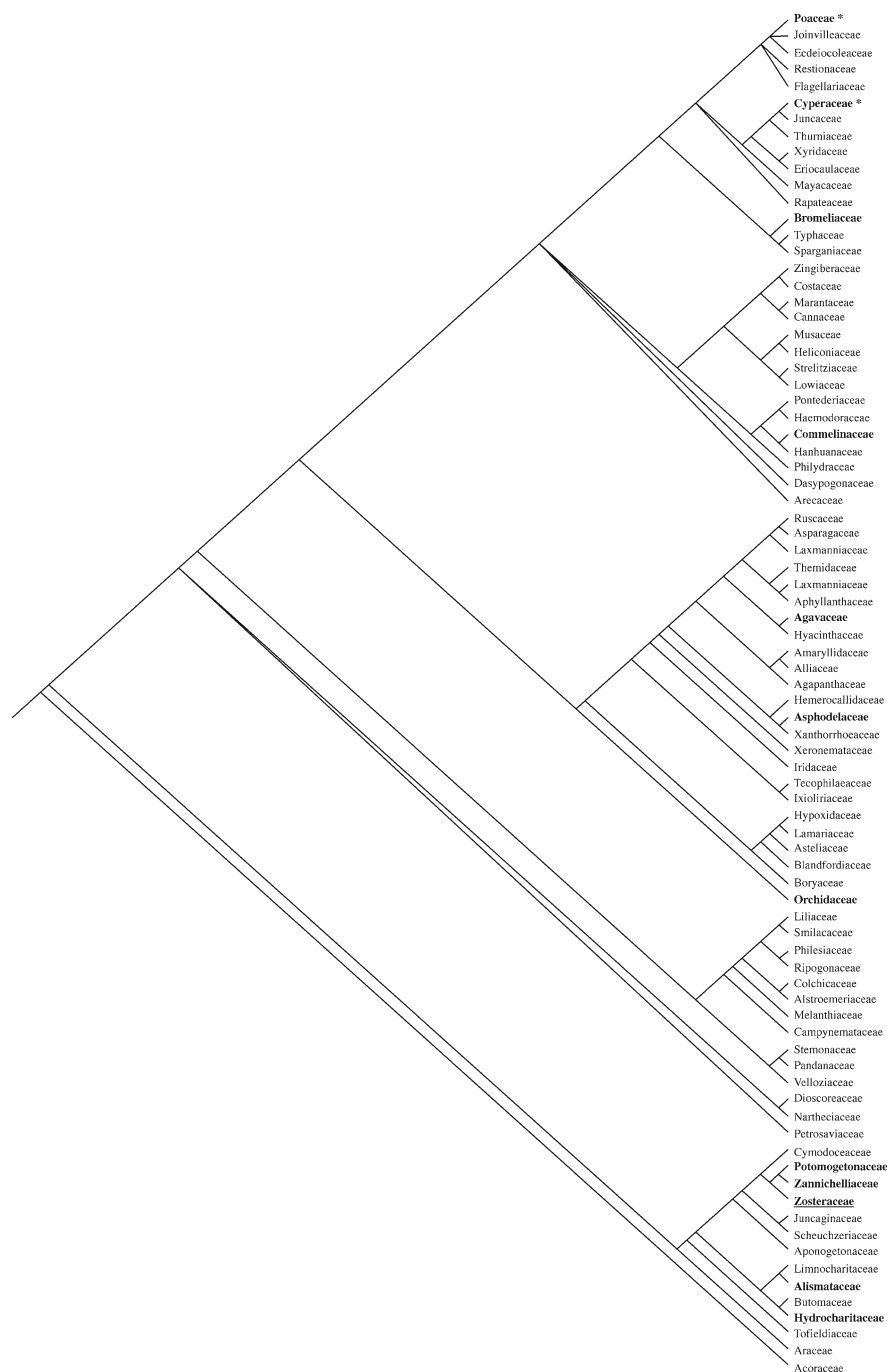


Fig. 1. Family-level phylogeny of monocot relationships modified from Givnish et al. (2006). The families Hydrocharitaceae, Potamogetonaceae, and Zannichelliaceae were added based on their placement in Les et al. (1997). **Bolded/underlined** families contain species with CCMs and those with asterices contain C₄ or C₄-like species. Dracenaceae (not shown) also has CAM species. C₄ and CAM designations are based on Kellogg (1999) and Smith and Winter (1996).

One of these clades includes the Alismataceae, Butomaceae, Hydrocharitaceae, Limnocharitaceae, and Najadaceae, and the other includes the Aponogetonaceae, Cymodoceaceae, Juncaginaceae,

Lilaeaceae, Posidoniaceae, Potamogetonaceae, Ruppiaceae, Scheuchzeriaceae, Zannichelliaceae, and Zosteraceae. Relationships within the Hydrocharitaceae are further supported by the analyses

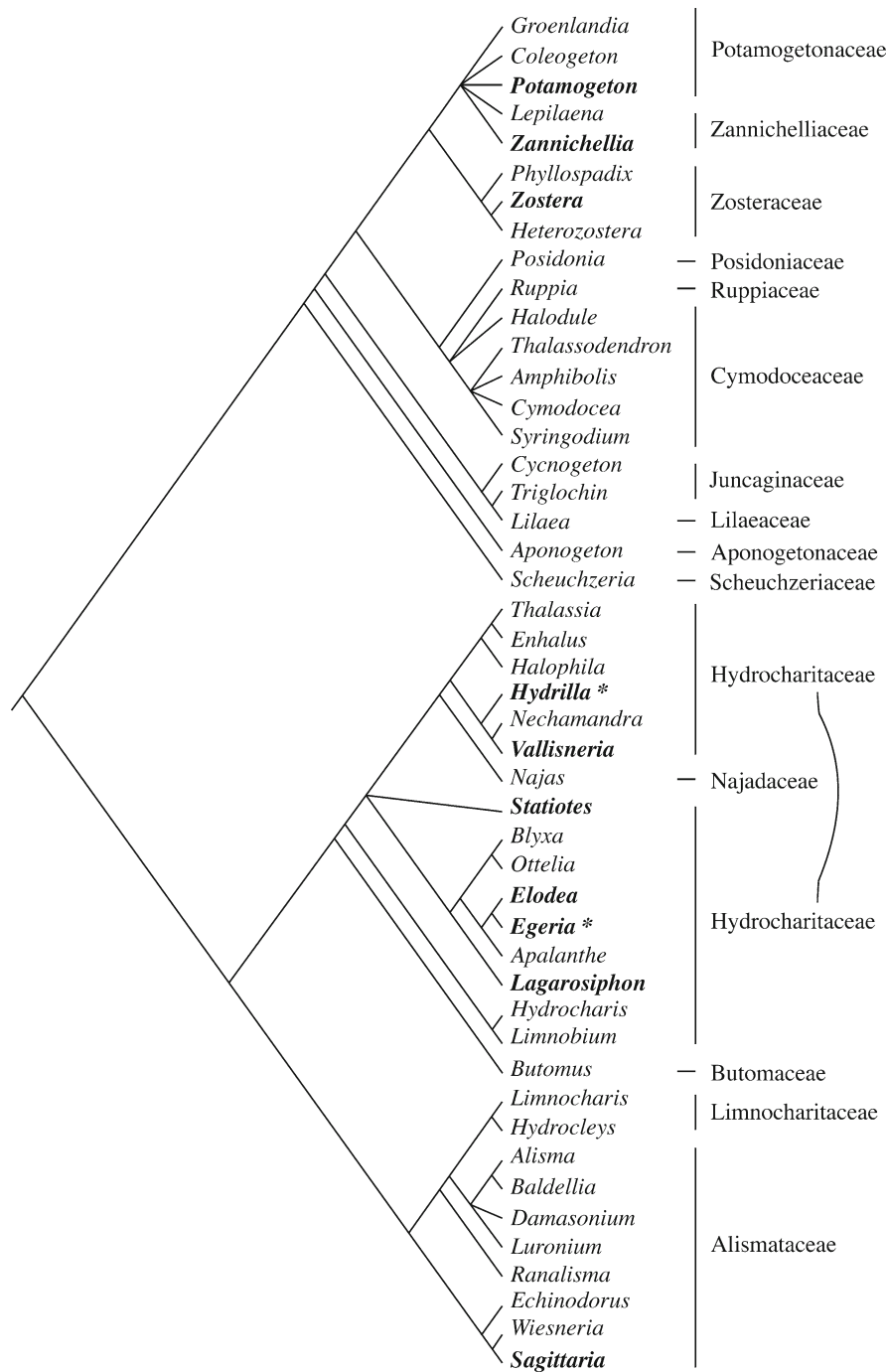


Fig. 2. Alismatales phylogeny modified from Les et al. (1997). *Bolded/underlined* genera contain species with CCMs and those with asterisks contain facultatively C_4 species.

of Tanaka et al. (1997). While there are still some issues of defining monophyletic clades within these lineages with strong statistical support, the phylogeny is sufficiently resolved to explore the origins of CCMs among these groups. There are

several different kinds of CCMs apparent across the Alismatales including two species with facultative C_4 CCM systems: *Hydrilla verticillata* (Bowes, 1985; Rao et al., 2006; Salvucci and Bowes, 1981; Van et al., 1976) and *Egeria densa*

(Casati et al., 2000, 2001; Lara et al., 2002). As these species are not closely related within the Hydrocharitaceae (Fig. 2; Les et al., 1997), this likely reflects two independent origins of the facultative C₄ system. While these parallel origins of C₄ photosynthesis in the Hydrocharitaceae are interesting, there is much more to CCM diversification within the Alismatales lineage.

Vallisneria spiralis (Hydrocharitaceae) is a close relative of *Hydrilla* and has been demonstrated to have high C₄ acid production and clear diurnal titratable acidity fluctuations reminiscent of CAM photosynthesis (Helder and Van Harmelen, 1982; Webb et al., 1988). Based on diurnal variation in titratable acidity, Keeley (1998) also proposed that CAM photosynthesis is present in several *Sagittaria* (Alismataceae) and *Vallisneria* species (Hydrocharitaceae). Van Ginkel et al. (2000) demonstrate a similar CO₂ concentrating mechanism (C₄-like) in *Elodea canadensis* as previously described in *Hydrilla*, while *Zostera marina* has a HCO₃⁻ pump (Beer et al., 2002). Beer et al. describe inorganic carbon (Ci) sensitivity in a number of genera, but do not equate this to C₄-like or CAM photosynthetic pathways. An overview of the various CCM in aquatic angiosperm species are given by Bowes et al. (2002) and Maberly and Madsen (2002). While most of these photosynthetic pathway variants are not C₄ in nature, the amount of variation in the physiological mechanisms of Ci concentration is notable. Looking at this variation in a rudimentary fashion, there seem to have been at least two C₄ origins within the Hydrocharitaceae, but an additional eight or more origins of other CCMs in the Alismatales lineage (Fig. 2). Only a subset of species within the subclass have been rigorously tested for deviation from a strict C₃ photosynthetic pathway, and the amount of variation found suggests that more intensive efforts in documenting variation in this lineage might uncover additional uncharacterized CCM variants, and these more comprehensive studies will be necessary to rigorously explore the number of origins of photosynthetic pathways in the group in a phylogenetic context.

II. Cyperaceae

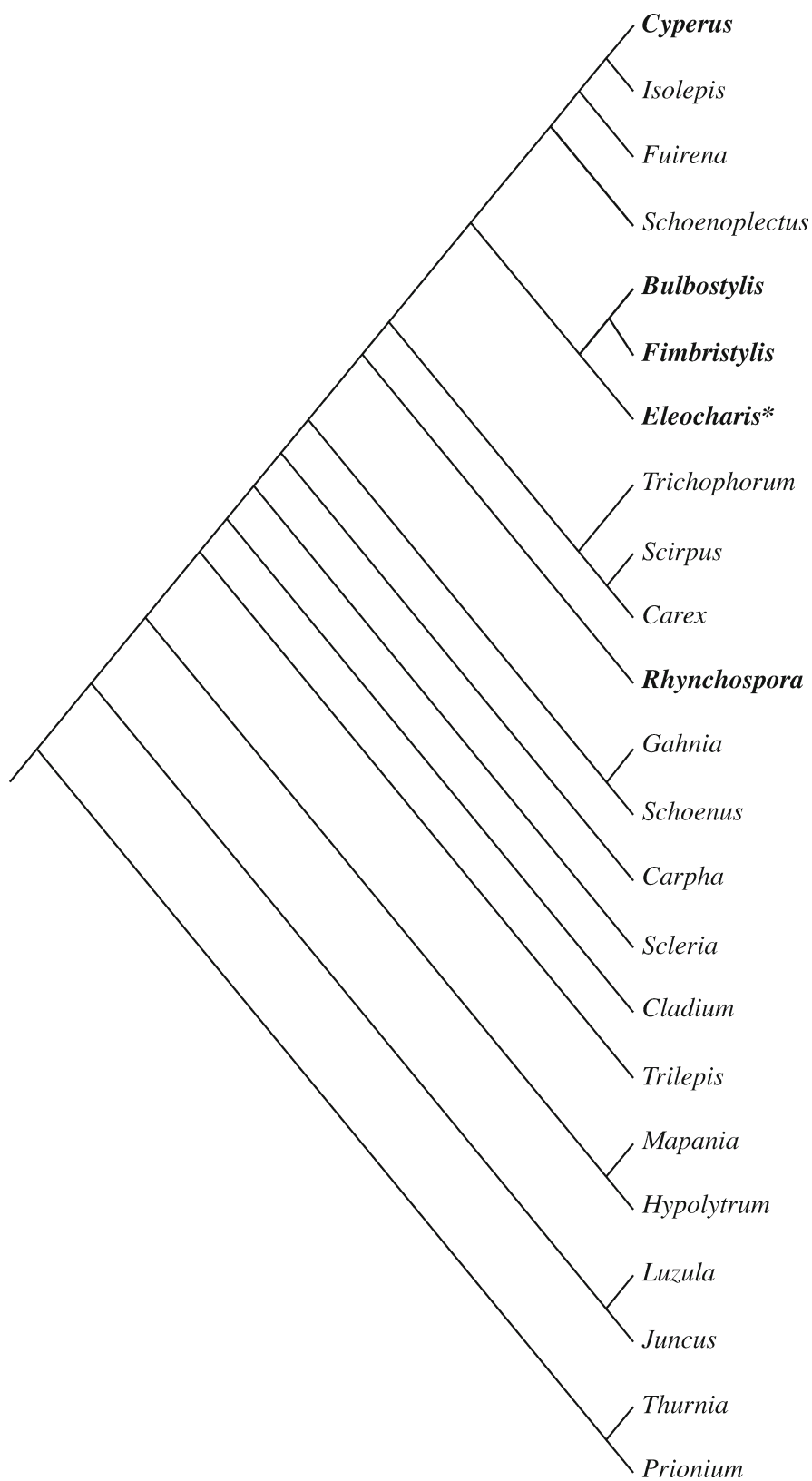
Among large lineages of angiosperms, the Cyperaceae phylogeny might be one of the least understood (Muasya et al., 1998, 2000, 2009a;

Simpson et al., 2007). Despite a large *rbcL* sequence dataset (Muasya et al., 2009a; Simpson et al., 2007), many of the interior nodes of the Cyperaceae phylogeny are not strongly supported. While efforts are underway to increase branch support among major clades of the family, much of the backbone of the phylogeny has yet to be resolved, leaving the monophyly of many generic and suprageneric classification units in question. Despite this, there is a clear indication of separate origins of the C₄ pathway in four lineages in the family: the Rhynchosporae, Abildgaardieae, Eleocharidae, and Cyperae tribes (Fig. 3; Bruhl and Wilson, 2007; Simpson et al., 2007; Soros and Bruhl, 2000).

Two of the three C₄ biochemical subtypes are present – NAD-malic enzyme (NAD-ME) and NADP-malic enzyme (NADP-ME) (Bruhl and Perry, 1995), as well as four C₄ anatomical types – rhynchosporoid, chlorocyperoid, fimbristylloid, and eleocharoid. However, there have been multiple origins of several anatomical types and the usefulness of the anatomical characterizations in determining C₄ species has been questioned (Bruhl and Wilson, 2007). Most of the C₄ biochemical work in Cyperaceae has focussed on *Eleocharis* and more studies of the other C₄ lineages are necessary.

A. Rhynchosporae C₄ Diversification

The Rhynchosporae includes the large genus *Rhynchospora* (~340 species) and *Pleurostachys* (~50 species). Ongoing studies (W.W. Thomas et al., personal communication) suggest that the C₃ *Pleurostachys* is derived from within *Rhynchospora*, which includes both C₃ and C₄ species (Bruhl and Wilson, 2007; Soros and Bruhl, 2000). The diversity of anatomical types within *Rhynchospora* has been used previously to suggest that there may be multiple origins of the C₄ pathway within *Rhynchospora* (Soros and Bruhl, 2000). Whether the *Pleurostachys* type represents a reversion from C₄ *Rhynchospora*, or is more related to C₃ *Rhynchospora* species is under evaluation, but will require more detailed sampling in the *Rhynchospora* phylogeny to clarify. Until we have a more detailed understanding of phylogenetic relationships in the Rhynchosporae, it is unclear whether one or more than one C₄ origin is present in the tribe.



B. *Abildgaardieae* C₄ Diversification

The *Abildgaardieae* includes several predominantly C₄ genera: *Abildgaardia*, *Bulbostylis*, *Crosslandia*, *Fimbristylis*, *Nelmesia*, and *Nemum* (Bruhl et al., 1992; Goetghebuer, 1998). This clade is likely sister to the *Eleocharidae* (Hinchliff et al., 2010), but relationships within the tribe are unclear. The few phylogenetic studies of the lineage (Ghamkhar et al., 2007; Yano and Hoshino, 2006), have sampled only a small number of the 500+ species, with a particular dearth of sampling within the large genera *Bulbostylis* (~200 species) and *Fimbristylis* (~300 species). Further, while these genera are predominantly C₄, at least based on the few anatomical and carbon isotope ratio studies conducted (Bruhl, 1990; Bruhl and Wilson, 2007; Lerman and Raynal, 1972; Metcalfe, 1971), the few C₃ species (including *Abildgaardia hygrophila* and *Fimbristylis variegata*) have not been included in any phylogenetic analyses. As their phylogenetic position relative to the C₄ species is unclear, whether they represent the ancestral condition of the tribe or are derived reversions to the C₃ pathway will require further study. Additional complications are created by the fact that most phylogenetic studies to date have suggested that the larger genera are not monophyletic as some of the smaller genera are nested within larger genera, and there is phylogenetic entanglement of the large genera (Ghamkhar et al., 2007). This clade needs to be studied much more thoroughly in terms of both phylogeny and physiology in order to understand the number of transitions among physiological types in the clade.

C. *Eleocharidae* C₄ Diversification

Among sedges, *Eleocharis* is the most understood in terms of the physiological mechanisms and anatomical structures associated with the C₄ pathway (Agarie et al., 1997, 2002; Bruhl and Perry, 1995; Bruhl et al., 1987; Murphy et al., 2007; Uchino et al., 1995, 1998; Ueno, 1996a, b, 1998,

2001, 2004; Ueno and Samejima, 1989; Ueno and Wakayama, 2004; Ueno et al., 1986, 1988, 1989). Studies have suggested that there have been two separate origins of the C₄ pathway in the lineage, once in *Eleocharis vivipara*, and the other in a clade including *E. baldwinii* and its relatives (Roalson and Friar, 2000; Roalson and Hinchliff, 2007). These two C₄ origins are relatively distant in the *Eleocharis* phylogeny, and are apparently of different ages, with *E. vivipara* nested within a clade of C₃ species, and the *E. baldwinii* clade including much deeper branches associated with species spread across the New and Old World tropics (Roalson and Hinchliff, 2007). The fact that there is a clade of several C₄ species of broad distribution suggests the C₄ pathway predated the divergence of these species, however, these species are not well understood and the absolute number of species in this clade is unclear. The different characteristics of C₄ photosynthesis among these species is also of interest to understanding the origins of C₄ – *E. vivipara* is variable for C₄ under different growing conditions (Agarie et al., 1997, 2002; Murphy et al., 2007; Uchino et al., 1998; Ueno, 1996a, b, 1998, 2001; Ueno et al., 1988), while the *Eleocharis* species in the *E. baldwinii* clade appear to have a much more stable photosynthetic pathway (Uchino et al., 1995; Ueno, 2004; Ueno and Wakayama, 2004). Further characterizations of *Eleocharis* species under different growing conditions will be necessary to understand the different possible functions of these C₄ types.

D. *Cypereae* C₄ Diversification

The *Cypereae* are a diverse lineage including C₃ and C₄ species that has strong support for its monophyly (Muasya et al., 2002, 2009b; Simpson et al., 2007), however, generic boundaries within the tribe remain unresolved. Studies have indicated that the C₃ *Cyperus* species are closely related to *Courtoisina*, *Kyllingiella*, and *Oxycaryum* (all C₃), while C₄ *Cyperus* species are related to *Alinula*, *Ascolepis*, *Kyllinga*,

Fig. 3. Cyperaceae phylogeny modified from Simpson et al. (2007) and Hinchliff et al. (2010) with representatives of Thurniaceae (*Thurnia* and *Prionium*) and Juncaceae (*Juncus* and *Luzula*) as outgroups. Branch names listed reflect either the dominant or best known genus of that lineage. Bolded genera contain C₄ or C₄-like species and asterices represent expected multiple origins of C₄ within that clade.

Lipocarpha, *Pycreus*, *Remirea*, and *Sphaerocyperus* (all C_4 ; Muasya et al., 2002, 2009b). Additionally, within each of these clades, *Cyperus* appears paraphyletic in relation to all of these other genera, and most of the genera are not clearly monophyletic. Many of the nodes within each of these clades are not strongly supported, however, so generic boundaries remain unclear. As all C_4 species sampled to date (regardless of generic placement) fall within one clade to the exclusion of the C_3 species, it seems likely that there has been a single origin of the C_4 pathway in the Cyperaceae.

IV. Poaceae

The origins of C_4 photosynthesis are perhaps the most clearly understood in the grass family (Christin et al., 2007, 2008; Giussani et al., 2001; Kellogg, 2000, 2001; Sinha and Kellogg, 1996; Vicentini et al., 2008). Early estimates (Kellogg, 1999, 2000) suggested at least four separate origins in the family, all within the large PACCMAD (Panicoideae/Arundinoideae/Chloridoideae/Centothecoideae/Micrairoideae/Aristidoideae/Danthonioideae) clade. These estimates noted that given the uncertain monophyly of the large genus *Panicum*, the Paniceae clade likely included more than one C_3 – C_4 transition, making the total number of transitions in the family more than this minimum of four. More recent studies have suggested from eight (Christin et al., 2007) to 12–17 (Christin et al., 2008) C_4 origins in the grasses. It should be noted, however, that the C_3 – C_4 transition is not the only photosynthetic pathway modification of importance. Within the C_4 lineages, there are also transitions among C_4 biochemical types (NAD-ME, NADP-ME, and phosphoenolpyruvate carboxykinase, PCK), and these may be as important to the diversification of these lineages as the gain of C_4 .

Selective pressures driving photosynthetic pathway changes and the timing of the origins of C_4 lineages have recently seen a flurry of research activity (Christin et al., 2007, 2008; Roalson, 2007, 2008; Vicentini et al., 2008). One study of note indicated that strong selective pressures at particular amino acid positions in the phosphoenolpyruvate carboxylase (PEPC) gene family favored parallel amino acid substitutions numerous times

in distinct C_4 grass lineages (Christin et al., 2007); this pattern needs to be more thoroughly explored in other lineages (Roalson, 2007). Using a phylogenetically-based molecular dating approach, Christin et al. (2008) and Vicentini et al. (2008) both estimated the late-Oligocene epoch (about 30 million years ago) as the most likely time for the earliest C_4 origin in the grasses. Atmospheric CO_2 levels declined to below current levels by the late-Oligocene (Pagani and Tippie, 2005), and the correspondence between the estimated first origin of C_4 grasses and CO_2 reduction supports the hypothesis that CO_2 reduction created (in part) the selection pressure for the evolution of C_4 photosynthesis (Ehleringer et al., 1991; Sage, 2004). Further, some analyses suggest a significant clustering of C_3 to C_4 and C_4 to C_3 transitions (Vicentini et al., 2008), but without a clear indication as to the selection pressures driving these different clusters of transitions. Testing of the influences of various climatic and geographical factors on origins of C_4 photosynthesis is still in its infancy and requires further integration of climate modeling, ancestral state reconstruction, and phylogenetic analysis methods (Roalson, 2008).

The five grass subfamilies/clades with C_4 species are the Aristidoideae, Centothecoideae, Chloridoideae, Micrairoideae, and Panicoideae (Kellogg, 2001; Sánchez-Ken et al., 2007); however, most of the C_4 species diversity in the Poaceae is concentrating within the Chloridoideae and Panicoideae lineages. Studies within the Aristidoideae (Cerro-Tlatilpa and Columbus, pers. comm. 2007) suggest that there were likely two transitions to C_4 within the subfamily with *Aristida longifolia*, the only known C_3 *Aristida* species, sister to the rest of the genus, and the C_3 genus *Sartidia* sister to the C_4 *Stipagrostis*. The exact nature of the transitions from C_3 to C_4 in the subfamily are being explored (Cerro-Tlatilpa and Columbus, pers. comm. 2007). In the Centothecoideae, the C_4 *Danthoniopsis* and *Tristachya* form a clade apparently derived from C_3 ancestors (Sánchez-Ken et al., 2007) and in the Micrairoideae the C_4 *Eriachne* and *Pheidochloa* are apparently derived from a C_3 ancestor (Sánchez-Ken and Clark, 2007; Sánchez-Ken et al., 2007). Therefore there appear to be five C_4 origins outside of the Chloridoideae and Panicoideae lineages.

A. Chloridoideae C₄ Diversification

The Chloridoideae are a strongly-supported monophyletic lineage by numerous analyses (see GPWG 2000, and references cited therein), however, relationships within the subfamily are not well resolved (Columbus et al., 2007; Hilu and Alice, 2001). Given the predominance of C₄ photosynthesis in the subfamily, it seems likely that most of the Chloridoideae diversity is associated with a single origin of C₄. However, recent analyses suggest that the *Centropodia*/*Merxmuellera* clade is sister to the rest of the chloridoids (Christin et al., 2008), and the C₄ PEPC gene of *Centropodia* appears to be of a separate origin from the rest of the C₄ chloridoids (Christin et al., 2007). This strongly supports two origins of C₄ in the Chloridoideae, one in *Centropodia* and the other at the base of the rest of the chloridoids. Additionally, at least one species of *Eragrostis* (*E. walteri*) appears to have reverted to C₃ (Ellis, 1984; Kellogg, 1999) as all indications suggest that it is not sister to the rest of the chloridoids (Columbus et al., 2007; Hilu and Alice, 2001; Van den Borre and Watson, 1994). There is also significant variation in the C₄ subtype in the subfamily with several genera (*Bouteloua*, *Leptochloa*, *Sporobolus*, *Chloris*, *Hilaria*, *Muhlenbergia*, *Spartina*, and *Zoysia*) having the PCK subtype rather than the more common NAD-ME C₄ decarboxylating enzyme (Sage et al., 1999). Three of these genera (*Bouteloua*, *Leptochloa*, and *Sporobolus*) show variation in C₄ type with some species with the NAD-ME and others with the PCK subtypes (Hattersley, 1987). Unfortunately, our understanding of diversification in the chloridoids has lagged behind that for other grass lineages. This lack of understanding is problematic at three particular levels: (1) the two largest phylogenies (Columbus et al., 2007; Hilu and Alice, 2001) of the subfamily do not provide strong support of relationships among lineages; (2) several genera are clearly para- or polyphyletic (including but not limited to *Chloris*, *Eragrostis*, *Leptochloa*, *Muhlenbergia*, and *Sporobolus*) and detailed studies to resolve these issues are not yet complete or have limited sampling; and (3) only a small subset of species in the Chloridoideae have been biochemically typed, which is necessary to determine the C₄ pathway subtype functioning

(Hattersley, 1987). Given these limitations, however, it seems very likely that there have been at least four transitions from NAD-ME to PCK biochemical pathways. Further, if *Zoysia* is sister to the *Sporobolus*/*Spartina* clade (Columbus et al., 2007), there is the possibility that the NAD-ME *Sporobolus* species could be a reversion from a PCK subtype ancestor. Clarification of the patterns of photosynthetic pathway diversification in the Chloridoideae will require a better understanding of phylogenetic relationships and species-level biochemical subtyping.

B. Panicoideae C₄ Diversification

In the grasses, C₄ diversification has been studied the most in the Panicoideae (Aliscioni et al., 2003; Giussani et al., 2001; Vicentini et al., 2008). Giussani et al. (2001) demonstrated multiple origins of C₄ in the clade, and the incorporation of anatomical and biochemical studies in combination with phylogenetic hypotheses have identified many of the patterns of photosynthesis diversity in the lineage. However, these previous studies have only reconstructed patterns of photosynthetic pathway diversification in a parsimony framework (Giussani et al., 2001), or a likelihood framework where photosynthetic type was coded as a single multistate character (Vicentini et al., 2008). Here I will further explore these patterns in a Bayesian inference framework to more explicitly estimate the probabilities of different kinds of character transitions associated with photosynthetic pathways. These methods will allow for more explicit testing of character change probabilities, providing insight into not only the number of origins of particular characters, but also the patterns of co-occurrence of characters that combined are used to designate photosynthetic pathway type.

cpDNA *ndhF* DNA sequence and photosynthetic pathway data of 107 species of Panicoideae grasses and outgroups are taken from Giussani et al. (2001). Bayesian inference analyses were run using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001). Priors for the molecular dataset included a model with six substitution types, rates following a gamma distribution (four categories), and allowing a proportion of invariant sites, based on results from DT_ModSel (Minin et al., 2003) analysis of the data. DT_ModSel examines the

fit of various substitution models to the data set using the Bayesian information criterion and additionally incorporates relative branch-length error estimates in a decision theory framework (Minin et al., 2003). Two Bayesian analyses were run in parallel to test for convergence and mixing, each with ten million generations (sampling every 1,000 generations, four chains), with the first two million generations excluded as burnin.

Character reconstruction analyses were performed using SIMMAP 1.0 (Bollback, 2006). SIMMAP uses a stochastic model of character state change to reconstruct character histories across trees sampled from the posterior distribution of trees from Bayesian analyses (Huelsenbeck et al., 2003; Nielsen, 2002). SIMMAP has two primary advantages over parsimony methods: (1) character reconstruction probabilities take into account uncertainty in tree topology by estimating ancestral state reconstructions across a distribution of probable trees, and (2) branch lengths are used to make decisions about the likelihood of the number of state changes on a branch, allowing for multiple state changes on a single branch (Huelsenbeck et al., 2003). A detailed comparison of advantages and disadvantages of different character reconstruction methods is beyond the scope of this paper, but recent studies have supported the applicability of stochastic mapping methods to reconstructing character histories (Gueidan et al., 2007; Raffiudin and Crozier, 2007; Renner et al., 2007).

Character states for photosynthetic pathway data are listed in Table 1. Character states for each taxon were derived from character distributions reported by Giussani et al. (2001), as compiled from the literature (see Giussani et al., 2001 references for original citations). Six characters were coded (Table 1), with character six a representation of all unique combinations of the other five character states. The only deviation from this was for taxa with a question or polymorphism for decarboxylating enzyme (either NAD-ME or PCK), and these were included within the PCK coded combination in order to keep within the maximum number of character states allowed by SIMMAP.

Bayesian analyses resulted in two sets of 8,000 trees each (post-burnin), which both converged on the same posterior probability estimates, suggesting that stationarity was reached and convergence and mixing among chains occurred efficiently. The

results of one of these analyses is represented in Fig. 4, showing the 50% majority rule of all post-burnin trees. This consensus topology is congruent with previous phylogenetic hypotheses of Panicoideae relationships based on maximum parsimony analyses (Giussani et al., 2001). The Panicoideae are split into three large clades, one representing $x = 9$ Paniceae, a second representing $x = 10$ Paniceae, and the third including Andropogoneae, as previously found (Giussani et al., 2001). There is continued weak support for a paraphyletic Paniceae in relation to the Andropogoneae.

SIMMAP character state reconstruction results are summarized in Table 2. Results include estimates of: (1) bias, the directional bias of transitions; (2) rate, the overall transition rate; (3) transitions, the average number of transitions; and (4) individual average transition rates among each pair of states. All C_4 characters show a large number of average transitions within each character, as estimated across the 80,000 replicated reconstructions. These 80,000 reconstructions are the result of ten independent reconstructions on each of the 8,000 fully-resolved Bayesian trees that resulted from the initial Bayesian inference analysis. Averaging the number of transitions in total and across individual states across all 80,000 reconstructions thus allows branch length and topological uncertainty to be integrated into the estimates of character state change. Analysis of transitions with the taxa coded simply as C_3 or C_4 resulted in, on average, 11.8333 transitions between these states, but maybe more importantly, the average transitions from C_3 to C_4 ($0 \rightarrow 1$) was 11.4119 while the transitions from C_4 to C_3 was only 0.4214. This suggests that reversions from C_4 to C_3 in the Panicoideae have been extremely uncommon, while parallel origins of C_4 has occurred quite frequently (Table 2). This inference of multiple origins of C_4 rather than reversions to C_3 is also supported by the parallel origins of particular amino acids in the PEPC gene in these lineages (Christin et al., 2007).

When photosynthetic pathway character transitions are compared in more detail by looking at estimates of the transition frequency among all of the biochemical types, the picture is even more complex. The average number of transitions among these seven states is 18.4102 (Table 2), suggesting that in addition to multiple transitions between C_3 and C_4 , there have been multiple transitions in

Table 1. Physiological characters and character states used for character state reconstructions in SIMMAP.

Character	State 0	State 1	State 2	State 3	State 4	State 5	State 6
1: C ₃ /C ₄	C ₃	C ₄	—	—	—	—	—
2: Decarboxylating enzyme	N/A	NADP-ME	PCK	NAD-ME	intermediate C ₃ /C ₄	PS-PCK	PCK or NAD-ME
3: Chloroplast position	Absent	Centrifugal	Centripetal	—	—	—	—
4: Chloroplast structure	Granal	Agranal	—	—	—	—	—
5: Number of bundle sheathes	One	Two	—	—	—	—	—
6: Unique character combinations (char. 1/2/3/4/5)	00001	11110	12101	13201	11111	15???	04201

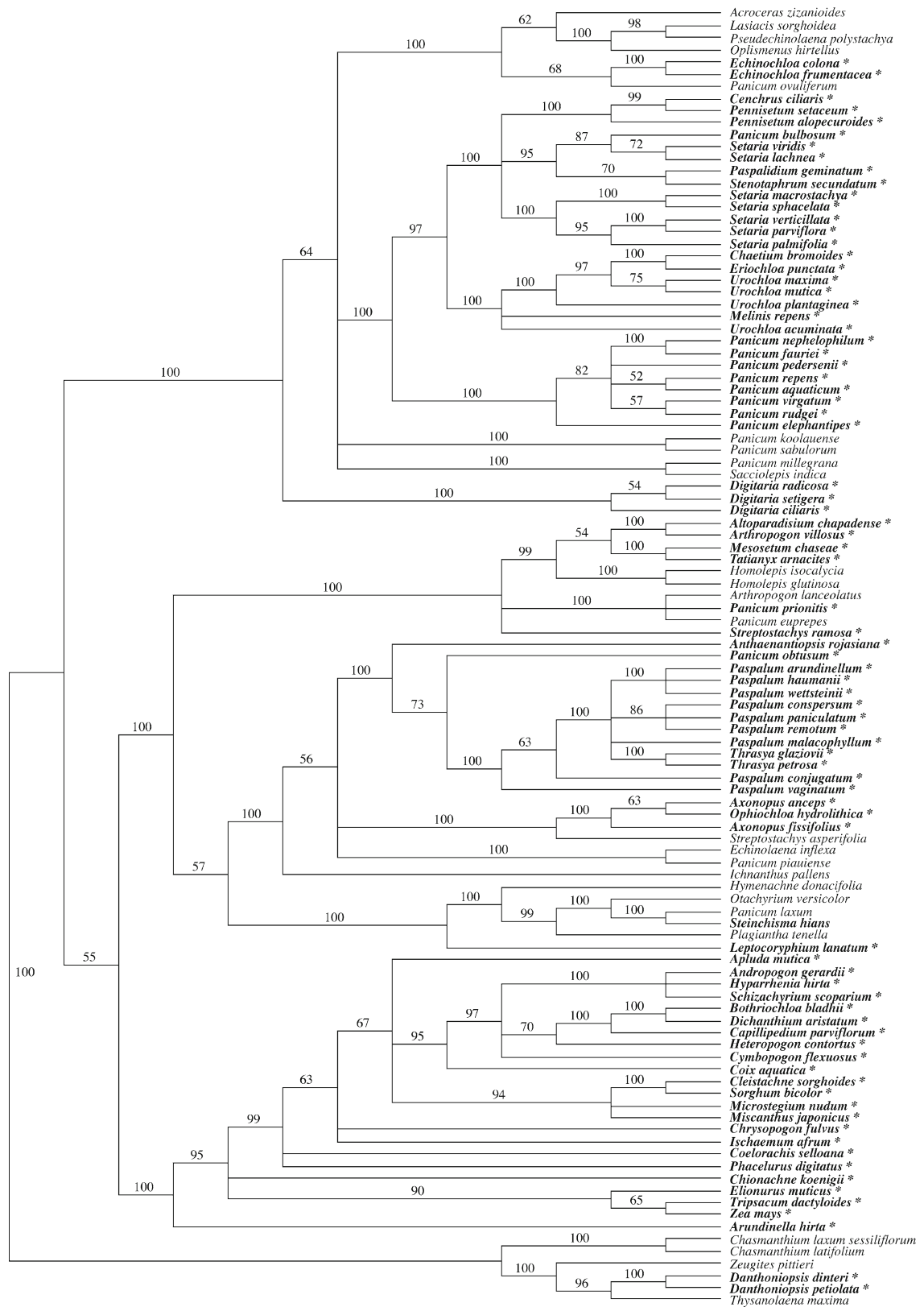


Fig. 4. Panicoideae Bayesian inference majority rule consensus tree. Numbers above branches refer to Bayesian posterior probability values, represented as the percentage of post-burn-in trees including the noted branch. Bolded species have some type of CCM and those with asterices are C_4 species.

Table 2. SIMMAP estimated character state transition rates. All analyses are based on 80,000 replicates.

Character 1	Bias	Rate	Trans.	0 $\Rightarrow$ 1	1 $\Rightarrow$ 0	0 $\Rightarrow$ 3	0 $\Rightarrow$ 4	0 $\Rightarrow$ 5	0 $\Rightarrow$ 6	1 $\Rightarrow$ 0
C ₃ /C ₄	0.4971	1.7635	11.8333	11.4119	0.4214	0 $\Rightarrow$ 2	0 $\Rightarrow$ 3	0 $\Rightarrow$ 4	0 $\Rightarrow$ 5	0 $\Rightarrow$ 6
Character 2	Bias	Rate	Trans.	0 $\Rightarrow$ 1	0 $\Rightarrow$ 2	0.3532	0.0483	1.0433	0.0526	3.5312
D. E.	0.1429	4.3942	18.4102	7.4859	0.3532	1 $\Rightarrow$ 3	1 $\Rightarrow$ 4	1 $\Rightarrow$ 5	1 $\Rightarrow$ 6	2 $\Rightarrow$ 1
				1 $\Rightarrow$ 2	1 $\Rightarrow$ 3	0.0621	0.0584	0.0665	0.4469	0.5858
				0.5898	0.0621	2 $\Rightarrow$ 4	2 $\Rightarrow$ 5	2 $\Rightarrow$ 6	3 $\Rightarrow$ 0	3 $\Rightarrow$ 2
				2 $\Rightarrow$ 3	2 $\Rightarrow$ 4	0.0163	0.9809	0.3941	0.0321	0.0248
				0.176	0.0163	3 $\Rightarrow$ 5	3 $\Rightarrow$ 6	4 $\Rightarrow$ 0	0.0524	4 $\Rightarrow$ 3
				3 $\Rightarrow$ 4	3 $\Rightarrow$ 5	0.0226	0.0954	0.0268	0.0376	0.0085
				0.0226	0.0278	4 $\Rightarrow$ 6	5 $\Rightarrow$ 0	5 $\Rightarrow$ 1	5 $\Rightarrow$ 2	5 $\Rightarrow$ 4
				4 $\Rightarrow$ 5	4 $\Rightarrow$ 6	0.0134	0.0167	0.0381	0.0105	0.0083
				0.0134	0.0045	6 $\Rightarrow$ 1	6 $\Rightarrow$ 2	6 $\Rightarrow$ 3	6 $\Rightarrow$ 4	6 $\Rightarrow$ 5
				5 $\Rightarrow$ 6	6 $\Rightarrow$ 0	0.0046	0.1192	0.0078	0.0025	0.0060
				0.0046	0.1192	0 $\Rightarrow$ 1	0 $\Rightarrow$ 2	1 $\Rightarrow$ 0	2 $\Rightarrow$ 0	
Character 3	Bias	Rate	Trans.	8.5105	1.1125	3.7493	1.3755	0.0768	2.1829	
CP Position	0.3333	4.3886	17.0074	0 $\Rightarrow$ 1	1 $\Rightarrow$ 0					
Character 4	Bias	Rate	Trans.	0 $\Rightarrow$ 1	1 $\Rightarrow$ 0					
CP Struct.	0.4908	1.6082	12.2085	11.7727	0.4357					
Character 5	Bias	Rate	Trans.	0 $\Rightarrow$ 1	1 $\Rightarrow$ 0					
# bund. sh.	0.7268	1.6153	11.1953	0.4570	10.7382					
Character 6	Bias	Rate	Trans.	0 $\Rightarrow$ 1	0 $\Rightarrow$ 2	0 $\Rightarrow$ 3	0 $\Rightarrow$ 4	0 $\Rightarrow$ 5	0 $\Rightarrow$ 6	1 $\Rightarrow$ 0
Combined	0.1429	4.435	18.7187	7.6688	0.8542	0.0685	1.0052	0.0415	1.0309	2.0511
				1 $\Rightarrow$ 2	1 $\Rightarrow$ 3	1 $\Rightarrow$ 4	1 $\Rightarrow$ 5	1 $\Rightarrow$ 6	2 $\Rightarrow$ 0	2 $\Rightarrow$ 1
				0.1900	0.0619	0.0255	0.0116	0.0067	0.0505	1.0465
				2 $\Rightarrow$ 3	2 $\Rightarrow$ 4	2 $\Rightarrow$ 5	2 $\Rightarrow$ 6	3 $\Rightarrow$ 0	3 $\Rightarrow$ 1	3 $\Rightarrow$ 2
				1.2170	0.0120	0.9754	0.0111	0.0133	0.0559	2.0737
				3 $\Rightarrow$ 4	3 $\Rightarrow$ 5	3 $\Rightarrow$ 6	4 $\Rightarrow$ 0	4 $\Rightarrow$ 1	4 $\Rightarrow$ 2	4 $\Rightarrow$ 3
				0.0086	0.0123	0.0075	0.0447	0.0354	0.0078	0.0024
				4 $\Rightarrow$ 5	4 $\Rightarrow$ 6	5 $\Rightarrow$ 0	5 $\Rightarrow$ 1	5 $\Rightarrow$ 2	5 $\Rightarrow$ 3	5 $\Rightarrow$ 4
				0.0059	0.0009	0.0054	0.0346	0.0082	0.0022	0.0027
				5 $\Rightarrow$ 6	6 $\Rightarrow$ 0	6 $\Rightarrow$ 1	6 $\Rightarrow$ 2	6 $\Rightarrow$ 3	6 $\Rightarrow$ 4	6 $\Rightarrow$ 5
				0.0007	0.0157	0.0342	0.2631	1.1170	0.1039	0.1084

Trans. transitions; D.E. decarboxylating enzyme; CP chloroplast; struct. structure; # bund. sh. number of bundle sheaths

decarboxylating enzyme within the C_4 lineages. The most frequent transition types were between C_3 and NADP-ME, C_3 and intermediate C_3/C_4 , NADP-ME and PCK, and PCK and PS-PCK.

Comparison of these results with previous estimates of photosynthetic pathway origins using maximum parsimony suggests more transitions in photosynthetic pathway types than previously inferred, with (on average) 11.8333 transitions, compared to the eight or nine inferred by parsimony (Giussani et al., 2001). Further, the transition types and frequency of some of the C_4 associated characters, such as chloroplast position in the bundle sheathes, appear much more homoplastic when estimated using stochastic mapping than parsimony (Table 2; Giussani et al., 2001). When the unique combination of characters is reconstructed as a single character (character 6; Tables 1 and 2), even more total C_4 -associated transitions are inferred – 18.7187, on average – suggesting that this system is much more dynamic than just transitions between C_3 and C_4 , or even among the biochemical types.

Two C_3 - C_4 intermediates (*Neurachne munroi* [not sampled here] and *Steinchisma hians*) appear to have separate origins in the Panicoideae and are each independently derived from C_3 lineages (Christin et al., 2008). Whether these species represent transitional forms between strict C_3 and C_4 pathways is unclear and needs further study. *Alloteropsis* (not sampled here), a genus of seven species, is primarily C_4 , but includes *A. semialata* subsp. *eckloniana*, a C_3 taxon that is apparently a revertant from a C_4 ancestor (Ibrahim et al., 2008). This would suggest both a gain and loss of C_4 associated with the *Alloteropsis* lineage.

These results suggest that when photosynthetic pathway evolution is explored within a framework that explicitly incorporates branching uncertainty and branch lengths, and averaged across replicates and topologies, there is an increased number of inferred origins/transitions among C_4 -associated characteristics. Additionally, transition frequencies and the directionality of different character state changes can be directly assessed using this approach. Further refinement of the phylogenetic hypotheses in the Panicoideae and other monocot lineages, and the implementation of these more statistically robust techniques, should allow researchers to better explore origins of photosynthetic pathway (and other) characteristics of interest.

V. Conclusions

As a whole, monocots show a remarkable diversity, not only in carbon fixation methods, but also the diversity of transition types involved in arriving at similar physiological processes. Alismatids show remarkable diversity in CCMs that are generally not associated with a full C_4 biochemical/anatomical system, but rather inducible biochemical pathways (Bowes, 1985; Casati et al., 2000, 2001; Lara et al., 2002; Rao, et al., 2006; Salvucci and Bowes, 1981; Van et al., 1976). Sedges show anatomical and biochemical diversity in photosynthetic pathway, particularly in *Eleocharis*, and between five and seven independent origins of C_4 . Not enough is known about the distribution of C_4 subtypes in the family to have a clear idea of the patterns of change among subtypes within the C_4 lineages. Grasses show numerous origins of C_4 types, and within C_4 clades, apparent multiple transitions between C_4 subtypes. While recent studies of phylogeny and C_4 physiology and biochemistry have greatly increased our knowledge of the evolution of C_4 photosynthesis, there are still major holes in our knowledge associated with some lineages (Cyperaceae outside of *Eleocharis*; Chloridoideae grasses) as well as the selective advantages or disadvantages of different C_3 and C_4 systems under different environmental conditions. Recent studies have suggested that there has been strong selection on the PEPC gene products associated with C_4 photosynthesis in grasses driving multiple origins of the pathway (Christin et al., 2007), further supporting the inferences here of a large number of parallel origins of C_4 lineages with few reversions to C_3 . The implications of possible strong selective pressure at particular amino acid sites in C_4 -associated genes has yet to be explored broadly (but see Gowik et al., 2006), and further studies will be necessary to see how often the patterns found by Christin et al., are applicable. More detailed studies integrating phylogeny, biochemistry, molecular evolution, and gene and promotor function will be necessary to take this next step forward in our understanding of the process of diversification of photosynthetic pathways. As of now, however, it appears that there have been a minimum of 24 separate C_4 origins in the monocots (Table 3; 2 Alismatales; 5 Cyperaceae; and 17 Poaceae).

Table 3. Known distribution of C₄ lineages in the monocots. If the *Axonopus/Ophiochloa* clade, *Paspalum* clade, and *Streptostachys* are inferred to together represent one gain with subsequent loss of C₄ in parts of *Streptostachys*, then there are an estimated 24 origins of C₄ in the monocots.

Classification unit (Family, etc.)	Lineage	C ₄ genera/species	C ₄ subtype	Representative references
Hydrocharitaceae	<i>Egeria densa</i> <i>Hydrilla verticillata</i>	One species	Facultative NADP-ME	Casati et al., 2000
Cyperaceae	Abildgaardieae	One species <i>Abildgaardia</i> , <i>Bulbostylis</i> , <i>Crosslandia</i> , <i>Fimbristylis</i> , <i>Nelmesia</i> , <i>Nemum</i> (500+ species)	Facultative NADP-ME NADP-ME	Rao et al., 2006 Bruhl and Wilson, 2007
	Cypereae C ₄ clade	<i>Alinula</i> , <i>Ascolepis</i> , <i>Cyperus</i> in part, <i>Kyllinga</i> , <i>Lipocarpus</i> , <i>Pycneus</i> , <i>Remirea</i> , <i>Sphaerocyperus</i> (~750 species)	NADP-ME	Bruhl and Wilson, 2007
	<i>Eleocharis</i> section <i>Tenuissimae</i> s.s.	<i>Eleocharis</i> in part (~15 of ~250 species)	NAD-ME	Bruhl and Wilson, 2007
	<i>Eleocharis vivipara</i>	One species	NAD-ME	Murphy et al., 2007
	Rhynchosporae	<i>Rhynchospora</i> in part (~21 of 350 species)	NADP-ME	Bruhl and Wilson, 2007
Poaceae – Andropogoneae	Andropogoneae	85 genera and ~1,000 species	NADP-ME	Mathews et al., 2002
Poaceae – Aristidoideae	<i>Aristida</i>	<i>Aristida</i> (all but one of ~330 species)	NADP-ME	Christin et al., 2008
	<i>Stipagrostis</i>	<i>Stipagrostis</i> (50 species)	NAD-ME or PCK	Christin et al., 2008
Poaceae – Centothecoideae	<i>Danthoniopsis</i> , <i>Loudetia</i> , + <i>Tristachya</i> clade	<i>Danthoniopsis</i> , <i>Loudetia</i> , <i>Tristachya</i> (66 species)	NADP-ME	Christin et al., 2008
Poaceae – Chloridoideae	Chloridoideae clade minus <i>Centropodia</i> and <i>Merxmüllera</i>	~166 genera and ~1,500 species	NAD-ME, PCK	Columbus et al., 2007
Poaceae – Chloridoideae	<i>Centropodia</i>	Four species	NAD-ME	Christin et al., 2007, 2008
Poaceae – Micrairoideae	<i>Eriachne</i> + <i>Pheidochloa</i> clade	<i>Eriachne</i> , <i>Pheidochloa</i> (~43 species)	NADP-ME	Sanchez-Ken et al., 2007
Poaceae – Panicoideae – Paniceae (x = 9)	<i>Alloteropsis</i>	Seven species (one subsp. of <i>A. semialata</i> is C ₃ and apparently a revertant from C ₄)	NADP-ME, PCK	Christin et al., 2008; Ibrahim et al., 2008
	<i>Digitaria</i> clade	<i>Digitaria</i> (220 species)	NADP-ME	Sage et al., 1999
	<i>Echinochloa</i> clade	<i>Echinochloa</i> (35 species)	NADP-ME	Sage et al., 1999
	<i>Neurachne minor</i>	One species	NADP-ME	Christin et al., 2008
	<i>Panicum/Pennisetum/Setaria</i> clade	<i>Brachiaria</i> , <i>Cenchrus</i> , <i>Chaetium</i> , <i>Eriochloa</i> , <i>Melinis</i> , <i>Panicum</i> in part, <i>Paspalidium</i> , <i>Pennisetum</i> , <i>Setaria</i> , <i>Spinifex</i> , <i>Stenotaphrum</i> , <i>Urochloa</i> , <i>Zuloagaea</i> (~515 species)	NADP-ME, PCK, NAD-ME	Christin et al., 2008; Vicentini et al., 2008
Poaceae – Panicoideae – Paniceae (x = 10)	<i>Altoparadisium/Tatianyx</i> clade	40+ species	NADP-ME (possibly others?)	Christin et al., 2008
	<i>Axonopus/Ophiochloa</i> clade*	~115 species	NADP-ME	Sage et al., 1999

(continued)

Table 3. (continued)

Classification unit (Family, etc.)	Lineage	C ₄ genera/species	C ₄ subtype	Representative references
	<i>Leptocoryphium lanatum</i>	One species	NADP-ME	Christin et al., 2008
	<i>Panicum prionitis</i> clade	Two species	NADP-ME	Christin et al., 2008
	<i>Paspalum</i> clade*	~355 species	NADP-ME	Sage et al., 1999
	<i>Steinchisma hians</i>	One species	C ₃ /C ₄ NADP-ME	Sage et al., 1999
	<i>Streptostachys ramosa</i> ^a	One species (<i>Streptostachys macrantha</i> is also C ₄ , but has yet to be included in any phylogenetic analyses)	NADP-ME	Vicentini et al., 2008

^a These three origins possibly represent one gain with subsequent loss of C₄ in parts of *Streptostachys* (Christin et al., 2008)

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The Geologic History of C₄ Plants

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Summary	339
I. Introduction.....	340
II. Geologic Evidence	340
A. Geochemical Approach and Interpretation.....	340
B. Geochemical Signals in the Fossil Record	342
C. Macrofossils.....	343
D. Microfossils	343
III. Origin of C ₄ Photosynthesis.....	345
A. Oligocene	345
B. Earlier Origins for C ₄ Photosynthesis?.....	346
IV. Expansion of C ₄ Grasslands.....	347
A. CO ₂ Starvation Hypothesis	347
B. Palaeoclimate Hypotheses	348
C. Grass-grazer Co-evolution Hypothesis	350
D. Fire Hypothesis.....	351
E. Fire-Climate Feedbacks.....	351
F. An Evolutionary Link Between Fire and Grazing?	352
V. Conclusions.....	353
Acknowledgments	354
References	354

Summary

Our understanding of C₄ plant history has been revolutionized by the use of carbon isotopes to construct geologic records of photosynthetic pathway. Through isotopic analyses of fossil teeth and soils, geochemists have discovered that the dominance of low latitude ecosystems by C₄ species is a relatively recent phenomenon. A major expansion of C₄ grasslands occurred across four continents only during the Late Miocene and Pliocene (2–8 Myr ago, Ma), with intriguing evidence suggesting a presence of C₄ plants at low abundance for at least 10 Myr before this event. Analysis of calibrated molecular phylogenies for the grasses indicates that declining atmospheric CO₂ began to select for C₄ photosynthesis during the Oligocene (25–30 Ma), but there remains an important gap in the geochemical data between this event and Miocene evidence of the pathway. A similar atmospheric selection pressure may have operated during the Permo-Carboniferous (270–330 Ma), but isotope surveys have so far failed to detect any direct evidence of C₄ species. Understanding when C₄ plants first originated, and why they remained sub-dominant components of ecosystems for so long, therefore remain important unresolved problems in this field. However, the worldwide expansion of C₄ grasslands is better understood. A range

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of complementary geologic data now indicate that increasing climatic seasonality or aridity caused a retraction of woodland vegetation and allowed the incursion of C_4 grasses. Abrupt increases in charcoal abundance in the Late Miocene and analogies with modern fire-maintained mesic grasslands indicate an important additional role for fire in this vegetation change. However, significant uncertainties remain, especially in explaining why earlier seasonal climates did not promote C_4 grassland expansion, and what drove this event in North America, where there is no evidence of abrupt climate change. I propose that the evolution of grazing resistance in C_4 grasses could have promoted fires, providing a mechanism for vegetation change without the need to evoke paleoclimate change.

I. Introduction

Investigations of the geologic history of C_4 photosynthesis were revolutionized by the discovery of a carbon isotope signature that can be transmitted through tropic pathways and preserved in geologic sediments. Following this advance, geologists began to trace C_4 plants through Earth history and uncovered a surprise; the current dominance of savanna ecosystems by C_4 species arose only 2–8 Myr ago (Ma) in the Late Miocene and Pliocene (Fig. 1) when C_4 grasslands expanded across four continents. This major geologic event demands explanation, and has inspired alternative hypotheses about the selection pressures for C_4 photosynthesis and causes of C_4 plant dominance.

The subject was reviewed comprehensively a decade ago (Cerling et al., 1997; Cerling, 1999), and the reader is referred to this work for an expert account of earlier research. Here, I focus on the developments that have taken place in this field within the last decade. Our understanding of the palaeo-environmental background to C_4 plant evolution has improved significantly in this time, provoked by the need to understand past climates in the context of anthropogenic global change. Progress in reconstructing the ‘tree of life’ using phylogenetic techniques has further illuminated the evolutionary history of C_4 plants, and aided interpretation of the geologic record. Yet significant uncertainties remain and debate continues, with attention principally focused on two key questions:

- When did C_4 photosynthesis evolve and what were the principal selection pressures?
- What caused the Miocene-Pliocene expansion of ecosystems dominated by C_4 plants?

Abbreviations: Ma – Myr ago Rubisco – Ribulose-1,5-bisphosphate Carboxylase/oxygenase CA – Carbonic Anhydrase PEPC – PhosphoenolpyruvateCarboxylase

In this chapter, I address these questions in three sections. First, I provide an overview of the geologic record of C_4 plants, outlining the main approaches, detailing the principal findings, and highlighting new methodological developments. Secondly, I examine the origins of C_4 photosynthesis by focusing primarily on phylogenetic and model data. Finally, I consider the hypotheses that have been advanced to explain the Miocene-Pliocene rise of C_4 -dominated ecosystems, and evaluate each using data from the fossil record. Throughout I highlight key areas where new advances are likely to resolve important uncertainties.

II. Geologic Evidence

A. Geochemical Approach and Interpretation

The most significant advances in our understanding of C_4 plant evolutionary history have come from the measurement of stable carbon isotope

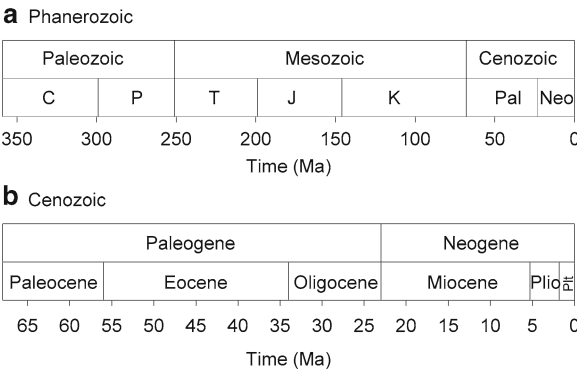


Fig. 1. Geologic time scale. (a) Phanerozoic (from 360 Myr ago, Ma), showing subdivision into: C, Carboniferous; P, Permian; T, Triassic; J, Jurassic; K, Cretaceous; Pal, Paleogene; and Neo, Neogene Periods. (b) Cenozoic, showing Epochs (Plio, Pliocene; Plt, Pleistocene). Time is shown as millions of years ago (Ma) (Gradstein et al., 2004).

ratios in geologic materials. Carbon fixation by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C₃ photosynthesis discriminates strongly against the heavy isotope of carbon (¹³C) relative to its more abundant form (¹²C) (Farquhar et al., 1982). In contrast, the coupled carbon-fixation reactions of the C₄ pathway carried out by carbonic anhydrase (CA) and phosphoenolpyruvate carboxylase (PEPC) slightly favour ¹²C over ¹³C (Farquhar, 1983). Subsequent discrimination by Rubisco in the bundle sheath cells represents the surplus of C₄ cycle activity over Rubisco capacity, and has only a minor overall effect on leaf discrimination against ¹³CO₂ (Farquhar, 1983). As a consequence, the stable carbon

isotope composition ($\delta^{13}\text{C}$) of C₃ and C₄ plant tissues differs on average by ~14‰, a difference large enough to identify the photosynthetic pathway from plant remains with a high degree of confidence (Fig. 2).

Crucially for geochemical analyses, this isotopic signature of the photosynthetic pathway persists through trophic pathways, despite further fractionation steps (Fig. 2; Cerling, 1999). The $\delta^{13}\text{C}$ signature of C₄ photosynthesis has now been recovered from carbonized plant macrofossils (Nambudiri et al., 1978), the carbon residue from smoke and charcoal deposition in the deep ocean (Bird and Cali, 1998), the organic matter and carbonates preserved in fossil soils (paleosols;

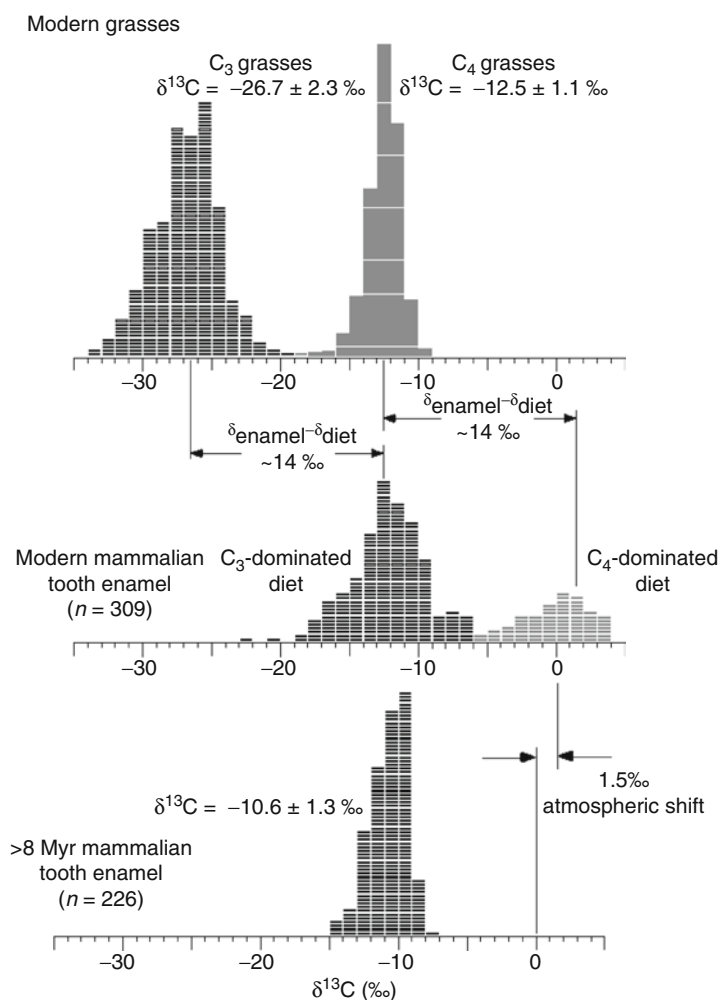


Fig. 2. Frequency histograms of $\delta^{13}\text{C}$ for modern C₃ and C₄ grasses and the grazers that feed upon them, showing the direct translation of an isotope signal from C₄ plants into herbivore tooth enamel. The data are from Cerling et al. (1997), and the $\delta^{13}\text{C}$ axis of fossil tooth enamel is shifted to account for the change in $\delta^{13}\text{C}$ of atmospheric CO₂ due to fossil fuel combustion (Reproduced from Cerling et al., 1997. With permission).

Cerling et al., 1989; Quade et al., 1989; Fox and Koch, 2003), the fossilized tooth enamel of mammalian herbivores (Lee-Thorp and van der Merwe, 1987; Lee-Thorp et al., 1989; Cerling et al., 1997), and the fossil egg shells of flightless birds (Stern et al., 1994; Ségalen et al., 2006). Further 'C₄-like' $\delta^{13}\text{C}$ signals have been inferred from long-chain *n*-alkanes recovered from paleosols and marine sediments, thought to derive from the leaf cuticular waxes of terrestrial plants (Freeman and Colarusso, 2001; Tipple and Pagani, 2007). Carbon originating from smoke, soils or animals carries a signal reflecting the proportion of C₄ plant biomass in the ecosystem or diet. Using this information, it is therefore possible to reconstruct C₄ plant abundance in extinct plant communities using linear mixing models, which interpolate between estimated 'pure C₃' and 'pure C₄' end-member values of $\delta^{13}\text{C}$.

The mixing model approach to interpreting $\delta^{13}\text{C}$ signals is subject to three key uncertainties. First, the $\delta^{13}\text{C}$ of plants depends directly on the $\delta^{13}\text{C}$ value of atmospheric CO₂, in addition to discrimination by physical and metabolic processes within the plant. Atmospheric $\delta^{13}\text{C}$ values vary significantly on geologic timescales, and introduce an uncertainty of ~2‰ into Cenozoic records (Passey et al., 2002). Secondly, the 'pure' C₃ and C₄ end-members are not fixed values, varying among C₄ species with the extent of bundle sheath leakage (Farquhar, 1983), and in C₃ species according to the supply of CO₂ via stomatal conductance relative to photosynthetic demand (Farquhar et al., 1982). The latter varies significantly along environmental gradients of water availability (Stewart et al., 1995). A third issue is the problem of interpretation because, in isolation from other data, a geochemical signal will inform only about the photosynthetic pathway, and not the taxonomic identity or ecology of the plants producing them. Further issues surrounding the interpretation of $\delta^{13}\text{C}$ data are discussed by Tipple and Pagani (2007).

B. Geochemical Signals in the Fossil Record

Analyses of $\delta^{13}\text{C}$ reveal a striking rise of C₄ plants to dominance in terrestrial ecosystems from the Late Miocene into the Pliocene (2–8 Ma; Fig. 3a–c). The isotope shift is found in multiple independent

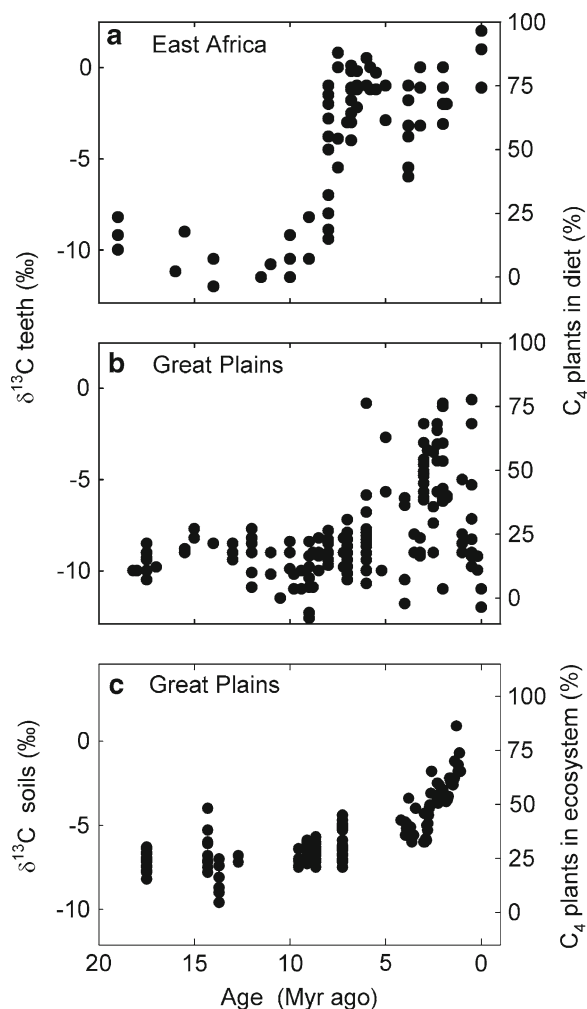


Fig. 3. Examples of the shifts in stable carbon isotope ratio ($\delta^{13}\text{C}$) characterizing the Miocene rise of C₄ plants in (a) East Africa (Cerling et al., 1997) and (b) and (c), the northern Great Plains of North America (>37 °N) from (b) tooth enamel (Passey et al., 2002) and (c) paleosol carbonates (Fox and Koch 2003). The proportion of biomass contributed by C₄ plants to the diet of animals or total plant biomass was estimated following Passey et al. (2002) for teeth and Fox and Koch (2003).

geologic materials, and has so far been detected in localities encompassing tropical to warm temperate climates across four continents: the US Great Plains, Argentina, Bolivia, India, Pakistan, Nepal, Kenya, Ethiopia, Chad and China (reviewed by Cerling et al., 1997; Latorre et al., 1997; Cerling, 1999; Ding and Yang, 2000; Zazzo et al., 2000; Levin et al., 2004). Three lines of evidence suggest that this major evolutionary event represents a worldwide expansion of grasses, rather than

other C₄ groups. First, the isotope shift is accompanied by massive increases in the abundance of grass cuticle fragments and pollen (Morley and Richards, 1993; Jacobs et al., 1999; Hoorn et al., 2000). Secondly, fossil faunas show a transformation at the same time, from forest-dwelling taxa such as mouse-deer and loris, to groups more typical of open habitats, such as giraffe, ungulates and hippopotamus (reviewed Cerling et al., 1998). Critically, many of the ungulates have high-crowned teeth, which are considered adaptations to the dental wear caused by grazing (Strömberg, 2006). Finally, the calibration of molecular phylogenies against fossil dates suggest that C₄ photosynthesis originated in the grasses during the Oligocene (Christin et al., 2008; Vicentini et al., 2008), although a molecular clock approach suggests that C₄ members of the Chenopodiaceae may have also evolved by the Late Miocene (Kadereit et al., 2003).

The earlier history of C₄ photosynthesis is less clear. Using the mixing model approach to interpreting $\delta^{13}\text{C}$ data, it is possible to infer the contributions that C₄ plants made to the diet of herbivores (tooth enamel/egg shells) or the biomass of vegetation (paleosols). Recent interpretations of the geologic data using this approach suggest that C₄ plants were an important, but subdominant, constituent of ecosystems for at least 10 Myr before the Miocene-Pliocene expansion of C₄ grasslands. The $\delta^{13}\text{C}$ signature of Early and Middle Miocene paleosol carbonates and *n*-alkanes indicates that C₄ species contributed up to 25–30% of the total plant biomass, but is close to the range of uncertainty introduced by the isotopic composition of atmospheric CO₂ and C₃/C₄ end-members (Fox and Koch, 2003; Tipple and Pagani, 2007). Although the $\delta^{13}\text{C}$ of herbivore teeth is generally interpreted as an exclusively C₃ diet throughout the Early and Middle Miocene (Cerling et al., 1997), relaxation of the assumptions about the C₃ end member value is consistent with a small (<30%) contribution from C₄ plants (Tipple and Pagani, 2007).

In summary, the origins and early history of C₄ photosynthesis remain unclear, because low C₄ plant biomass tends to fall within the range of uncertainty for $\delta^{13}\text{C}$ mixing models. The obvious way of avoiding this issue is to directly measure the $\delta^{13}\text{C}$ of plant materials, which carry a ‘pure’ signal of photosynthetic pathway. However, the

recovery and identification of plant macrofossils raises a new set of problems.

C. Macrofossils

Unequivocal identification of the C₄ pathway in the fossilized remains of plants is not straightforward, because it cannot be detected from gross morphology alone. However, Kranz anatomy and the $\delta^{13}\text{C}$ signature of C₄ photosynthesis are recognized in petrified grass leaves from the Ricardo Formation of California, dating to the Late Miocene (12.5 Ma) (Nambudiri et al., 1978). Kranz anatomy is also identified unequivocally in a much younger silicified grass from the Ogallala Formation of Kansas, dated to the latest Miocene, 5–7 Ma (Thomasson et al., 1986). Older geologic sediments (14 Ma) at the Fort Ternan locality in Kenya contain plants that have been classified on the basis of morphological characters (Dugas and Retallack, 1993). Although some of the genera assigned to these plants are comprised entirely of C₄ species in the modern world, one is C₃ and, since direct anatomical or isotopic evidence of C₄ photosynthesis is lacking in the fossils, the pathway can only be inferred (Cerling, 1999).

The macrofossil record of C₄ plants is therefore extremely impoverished and, without the richer geochemical record of C₄ photosynthesis, our knowledge of their evolutionary history would be extremely thin. However, fossils do offer two crucial pieces of information that geochemistry cannot currently provide; direct evidence of C₄ plants, and the taxonomic identity of these extinct species. The latter information is important for linking patterns in the geologic record with ecological processes and evolutionary patterns inferred from molecular phylogenies. New analytical techniques for the investigation of microfossils are beginning to offer a way to bridge this critical gap, allowing the identification of taxonomic affinity and photosynthetic pathway within the same microscopic samples.

D. Microfossils

Microfossils encompass a diversity of plant remains, including pollen, phytoliths and cuticle fragments. Each can be classified into taxonomic groupings with a varying degree of precision but, until recently, there has been no means of matching

this identification with information about the photosynthetic pathway. In recent years, however, new techniques have been developed to address this issue.

Pollen is abundant throughout the geologic record, because its sporopollenin wall is highly resistant to decay and, especially in wind pollinated groups like the grasses, it is produced in large quantities and dispersed over long distances. This microfossil type provides a varying degree of taxonomic information depending on the plant group, with identification possible to the genus or species level in some cases, but only the family level in others such as the grasses. Grass pollen has been identified in Maastrichtian (Late Cretaceous, 65–70 Ma) sediments, and is common from the Paleocene onwards (Fig. 1; Jacobs et al., 1999), but the photosynthetic pathway of parent plants is unknown. New techniques for analyzing the $\delta^{13}\text{C}$ of this material could therefore open a rich seam of paleobotanical information about the early history of C_4 photosynthesis in the grasses.

Isotopic measurements of bulk pollen samples have utilized conventional analytical techniques, and require relatively large samples of 10^2 – 10^3 pollen grains (Amundson et al., 1997; Jahren, 2004). The measurements demonstrate the potential to recover a signal of photosynthetic pathway from this material, and offer the possibility of extending plant isotope records into the Paleogene. However, new technical advances for measuring $\delta^{13}\text{C}$ in individual pollen grains offer these same possibilities without the need to laboriously pool samples of pollen by hand. Crucially, they eliminate the requirement for data interpretation using a mixing model, allowing direct measurements of the plant $\delta^{13}\text{C}$ value.

The spooling wire combustion device allows tiny samples to be completely oxidized for analysis using carbon isotope mass spectrometry (Sessions et al., 2005; Eek et al., 2007). Development of this technique for application to individual pollen grains is on-going, and currently allows the photosynthetic pathway of parent plants to be identified with >85% reliability (Nelson et al., 2007). Using this method, Nelson et al. (2008) have demonstrated that the $\delta^{13}\text{C}$ distributions of pollen samples recovered from the surface sediments of lakes in the Great Plains correlate well with the photosynthetic pathway used by

neighboring grassland vegetation. Ultimately, the approach may allow the identification of rare C_4 plants within a predominantly C_3 community, and has obvious applications in tracing the earliest geologic history of C_4 photosynthesis.

Phytoliths are microscopic silica bodies that form in all groups of living vascular plants, but are especially abundant in grasses, and are preserved in abundance in geologic sediments as biopal (Strömberg, 2004). The assignment of fossil phytoliths to particular phylogenetic groups is complicated by multiplicity, where each species produces more than one morphotype, and redundancy, where different species produce the same morphotypes (Piperno, 1988). However, recent developments in sampling protocols, expansion of reference collections, and the development of objective techniques for categorizing morphotypes, now allow robust statistical inferences about the grass phylogenetic groups that contributed to fossil phytolith assemblages (Strömberg, 2004, 2005). Using this approach, Strömberg (2005) has identified grasses belonging to the sub-family Chloridoideae in Great Plains sediments dating to the Early Miocene, and Prasad et al. (2005) have inferred members of the PACCMAD crown group from fossilized dinosaur dung in India dating to the Late Cretaceous. The PACCMAD clade encompasses all of today's C_4 grass species, including the predominantly C_4 Chloridoideae, but the dates when the C_4 pathway originated in these groups are not currently known.

As with pollen, the analysis of occluded organic matter in comparatively large samples (~50 mg) of phytoliths gives the potential to recover a $\delta^{13}\text{C}$ signal (Smith and White, 2004). This technique is problematic, because of differences in the biochemistry of organic matter occluded by C_3 and C_4 species, and interspecific variation. However, preliminary work suggests that Great Plains grass communities were comprised of around 50% C_4 species by 12 Ma (Smith, 2002), approximately 8 Myr before the major expansion of C_4 grasslands in this region (Fox and Koch, 2003). Further developments are clearly required, but the coupling of phytolith classification with isotopic analyses offers a potentially powerful, and presently unexploited, approach for establishing the taxonomic identity and photosynthetic pathway of early grasses.

III. Origin of C₄ Photosynthesis

A. Oligocene

Geochemical analyses trace an isotopic signal of the C₄ pathway back to 16–18 Ma in the Early Miocene (reviewed in Tipple and Pagani, 2007), and here the trail runs cold (Section 17.2.2). The incomplete nature of the fossil record raises the possibility that C₄ plants had appeared at least by the end-Oligocene, and new techniques for analyzing the $\delta^{13}\text{C}$ of individual pollen grains (Section 17.2.4) may ultimately be able to test this idea by identifying rare C₄ plants among more numerous C₃ species. However, a ~15% error rate in the technique currently precludes this application (Nelson et al., 2007).

Our current best information on the origins of C₄ photosynthesis comes from two molecular phylogenies for the grasses, calibrated using fossil dates based on macrofossil and phytolith material. Both were based upon sequence data for the chloroplast marker *ndhF* assembled by Giussani et al. (2001), and used previously to make inferences about photosynthetic pathway evolution in the grass sub-family Panicoideae. The first extended this survey of the *ndhF* marker to capture the phylogenetic diversity of C₄ grasses (Christin et al., 2008). The second retained a focus on the Panicoideae, but included data on the nuclear gene *phyB* as an additional line of evidence about the true species phylogeny (Vicentini et al., 2008). The results of both studies are consistent with the hypothesis that C₄ photosynthesis first originated in the grasses during the Oligocene, at ~30 Ma (Christin et al., 2008; Vicentini et al., 2008). However, alternative fossil dating scenarios tested by Vicentini et al. (2008) raise the possibility of dates as early as the Palaeocene-Eocene.

An Oligocene origin for C₄ photosynthesis in the grasses is consistent with the current leading evolutionary hypothesis, which proposes that declining atmospheric CO₂ concentrations selected for the C₄ pathway as a mechanism for minimizing the metabolic costs of photorespiration (Ehleringer et al., 1991). The hypothesis is grounded in physiological measurements of photosynthetic energy requirements, which demonstrate that the energetic costs of photorespiration in a C₃ leaf at 20–25 °C exceed the costs of

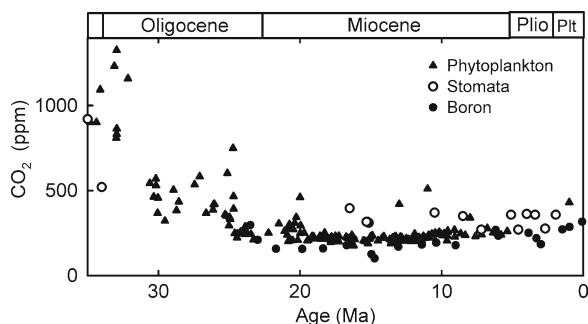


Fig. 4. Reconstructions of atmospheric CO₂ concentrations from the Oligocene to the present based on the $\delta^{13}\text{C}$ of marine phytoplankton, the stomatal densities of fossil leaves, and the boron isotope ratios of planktonic foraminifera. These CO₂ proxy data were compiled by Royer (2006), but paleosol proxy data are not shown, because the uncertainty in this technique (± 500 ppm) precludes meaningful estimation of low CO₂ levels. The geologic timescale shows ages in Myr ago (Ma); Plio = Pliocene; Plt = Pleistocene.

running a C₄ cycle at CO₂ concentrations below ~500 ppm (Ehleringer and Björkman, 1977). Proxy data for atmospheric CO₂ indicate that this critical threshold was crossed during the Oligocene, as CO₂ plummeted from values in excess of three-times pre-industrial, to somewhere close to the pre-industrial level (Fig. 4; Pagani et al., 1999, 2005; Pearson and Palmer, 2000; Royer et al., 2001, 2005). Using a likelihood modeling analysis of their phylogenetic data, Christin et al. (2008) showed that the likelihood of evolving C₄ photosynthesis increased significantly in the interval from 28 Ma to present. This date corresponds remarkably well with CO₂ decline in the Oligocene and, on the basis of current evidence, the CO₂-hypothesis for C₄ origins has therefore become extremely compelling.

However, only the earliest of C₄ evolutionary origins inferred from these calibrated phylogenetic trees correlate directly with the Oligocene decline in atmospheric CO₂, and later evolutionary origins of the pathway are dated to the Miocene and Pliocene (Christin et al., 2008; Vicentini et al., 2008). In addition, Vicentini et al. (2008) infer a number of reversals from C₄ to C₃ photosynthesis, and show that both C₃ to C₄ transitions (origins) and C₄–C₃ transitions (reversals) are highly clustered at different points in time. These results indicate the likely involvement of cyclical variations in CO₂, climatic norms or seasonality in the evolutionary process (Vicentini et al., 2008).

Geologic proxies show clearly that global climate fluctuated strongly during the Miocene and Pliocene, with an interval of warmth during the Mid-Miocene Climatic Optimum at 15–17 Ma, and a period of significant cooling and aridification accompanying successive periods of ice sheet expansion in Northern and Southern Hemispheres (Zachos et al., 2001; Dupont-Nivet et al., 2007). However, the extent to which these climatic changes were coupled with variation in atmospheric CO₂ remains controversial. Most proxy evidence indicates constant, low concentrations of CO₂ throughout periods of major climatic change in the Miocene (e.g. Pagani et al., 1999; Royer et al., 2001), while other data suggest a closer coupling between CO₂ and climate (Kürchner et al., 2008). Therefore, although declining CO₂ seems a likely selection pressure for the evolution of C₄ photosynthesis, it is unlikely to have been the only factor at work (Roalson, 2008). The resolution of these issues will require more precise dating of phylogenetic trees (i.e. more fossil data points) and a more in-depth understanding of the palaeoclimatic changes occurring during this crucial interval of grass evolutionary history.

B. Earlier Origins for C₄ Photosynthesis?

If C₄ photosynthesis evolved as an adaptation to atmospheric conditions favoring photorespiration, then it could potentially have origins during earlier episodes of low atmospheric CO₂ or elevated O₂. To identify these, Osborne and Beerling (2006) used a modeling approach to calculate the energetic costs of C₃ and C₄ photosynthesis through geologic time.

The analysis begins with a consideration of atmospheric CO₂ and O₂ variation through the past 500 Myr of the Phanerozoic (Fig. 5a), calculated using models of the geologic carbon and oxygen cycles (Berner, 2005). The reconstructions of CO₂ are consistent with independent geologic evidence derived from multiple proxies, based on the $\delta^{13}\text{C}$ of palaeosols, the stomatal density of fossil leaves, the $\delta^{13}\text{C}$ of phytoplankton, and the $\delta^{11}\text{B}$ of planktonic foraminifera (data collated by Royer et al., 2005; Royer, 2006). The reconstructions for O₂ are reproduced by independent isotopic evidence (Berner et al., 2000). These show a major decline in atmospheric CO₂ and a rise in O₂ during the Late Paleozoic (~350 Ma),

corresponding to the rise of vascular land plants and expansion of forest vegetation. The marked decrease in atmospheric CO₂ is thought to have been caused by silicate rock weathering, enhanced by the activity of plant roots (Algeo and Scheckler, 1998). Silicates react with CO₂ in the soil solution to produce bicarbonate ions that are carried to the oceans. Here, further reactions create carbonates that are deposited in sedimentary rocks such as limestone, and reduce atmospheric CO₂ on geological timescales (Berner, 2005). Plant roots directly accelerate the initial weathering reaction through the release of CO₂ from respiration and acidification of the soil medium via organic acid exudation. A coincident increase in organic carbon burial is considered a further key carbon sink but, more critically, would have unbalanced the O₂ cycle by sequestering a large reservoir of reduced carbon, leaving a large quantity of O₂ in the atmosphere (Berner, 2005). Together, these changes in atmospheric composition during the Permo-Carboniferous are thought to have caused a massive decline in the CO₂/O₂ mixing ratio (Figs. 1 and 5a; Beerling, 2005).

The energetic costs of a low CO₂/O₂ mixing ratio can be assessed by modeling the quantum yield, the maximum energetic efficiency of photosynthesis under limiting light conditions, which depends on the ratio of photosynthesis to photorespiration. It therefore also acts as an important indicator of the relative inhibition of photosynthesis by photorespiration under high light (Sage and Kubien, 2003). The quantum yield of C₄ photosynthesis is insensitive to variation in the atmospheric CO₂ and O₂ composition and, had this pathway originated early in the Phanerozoic, is expected to have been invariant over the past 500 Myr (Fig. 5b). In contrast, the quantum yield of C₃ photosynthesis depends directly on the CO₂/O₂ mixing ratio, and modeled values show decreases during the Permo-Carboniferous and late Cenozoic due to massive increases in the calculated rate of photorespiration (Fig. 4b).

However, the quantum yield in C₃ plants also depends on temperature, which tracks variation in atmospheric CO₂ via the greenhouse effect. To account for this influence on leaf physiology, Fig. 5b shows quantum yield values calculated on the basis of global mean temperature, derived from a simple planetary energy balance model, and tropical temperatures, obtained from general

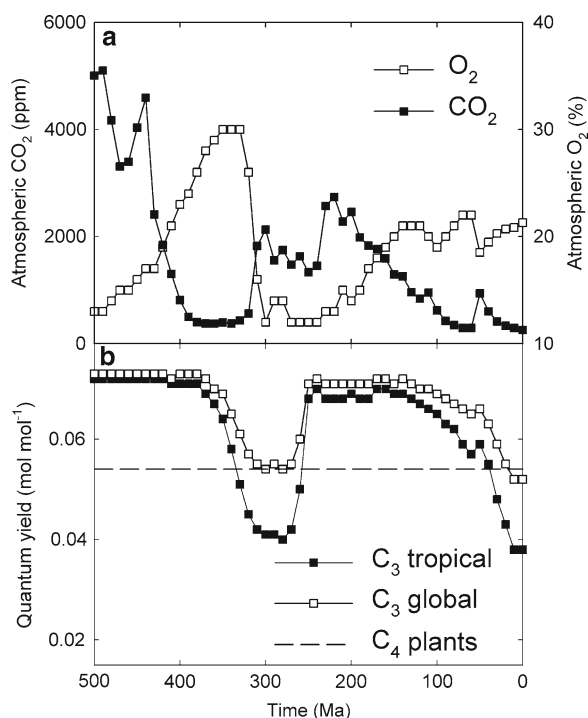


Fig. 5. (a) Atmospheric CO₂ and O₂, and (b) modeled quantum yield of C₃ and C₄ plants during the past 500 Myr of the Phanerozoic (Redrawn from Osborne and Beerling, 2006). The values for C₃ plants were calculated using estimates of either the global mean temperature (calculated for each time-point using a zero-dimensional energy balance model) or tropical mean surface temperature (based on a correlation with global temperature, developed using general circulation model simulations of paleoclimate) details are provided by Osborne and Beerling (2006).

circulation model (GCM) simulations of past climates (Osborne and Beerling, 2006). Model calculations show that decreases in photorespiration during cool intervals are insufficient to offset the direct effect of low atmospheric CO₂. The quantum yield of C₃ plants in tropical climates therefore falls below the theoretical C₄ value during the Permo-Carboniferous and late Cenozoic (Fig. 5b), indicating a greater efficiency for C₄ photosynthesis over the C₃ type. Because C₄ photosynthesis did originate during the latter interval, this result suggests the Permo-Carboniferous as a time when atmospheric conditions would have selected strongly for the pathway.

To test the hypothesis of an early origin for C₄ plants, Beerling (2005) and Osborne and Beerling (2006) surveyed the stable carbon isotope values of plant fossils dating to the late Carboniferous

and early Permian. A global dynamic vegetation model was run using a GCM climate for the Carboniferous, to identify the geographical regions most likely to support C₄ plants. However, none of the tested plant samples carried an isotopic signal of C₄ photosynthesis (Beerling, 2005; Osborne and Beerling, 2006). At present, there is therefore no direct evidence of the pathway during the Permo-Carboniferous, although anomalous isotopic values obtained from charcoal samples may be explained as C₄ plants (Jones, 1994).

Anomalous carbon isotope values have also been measured in *n*-alkanes isolated from Late Cretaceous marine sediments, and interpreted as a signal of C₄ plants growing in Africa (Kuypers et al., 1999). This intriguing result would push the date for C₄ plant origins back to ~90 Ma, and deserves further investigation.

IV. Expansion of C₄ Grasslands

Hypotheses about the Cenozoic history of C₄ grasslands must be able to explain both the Miocene-Pliocene expansion of this ecosystem and the earlier presence of C₄ grasses as minor components of plant communities; i.e. what caused the rise of C₄ grasses, and what had previously held them in check? In the remainder of this chapter, I outline the hypotheses advanced to explain these features of the geologic record, and the evidence on which each is based. Many of the hypotheses are not mutually exclusive, offering complementary mechanisms, and the relative importance of each may vary significantly among geographical regions.

A. CO₂ Starvation Hypothesis

The leading hypothesis during the 1990s proposed that atmospheric CO₂ declined below a critical threshold in the Late Miocene, giving a significant energetic, and therefore competitive, advantage to C₄ species over their C₃ counterparts (Ehleringer et al., 1991, 1997; Cerling et al., 1997). However, three independent geologic CO₂ proxies have now indicated that CO₂ levels were stable and below the threshold concentration for >10 Myr before C₄ plant expansion (Fig. 4). Although one proxy record does indicate some variation in CO₂ during the Early to Mid Miocene, it provides no

data for the crucial period from the Late Miocene to Pliocene (Kürchner et al., 2008). Based on current evidence, alternative trigger mechanisms therefore seem more likely, and declining CO_2 is now implicated in the origin of C_4 photosynthesis, rather than the Miocene expansion of C_4 grasslands (Ehleringer et al., 1991; Pagani et al., 2005). These arguments are presented earlier in this chapter (Section 17.3.1), and are elaborated elsewhere (Pagani et al., 1999; Sage, 2001, 2004; Keeley and Rundel, 2005; Osborne and Beerling, 2006; Osborne, 2008; Christin et al., 2008; Vicentini et al., 2008).

B. Palaeoclimate Hypotheses

Climate change has long been favored as an alternative explanation for the rapid pace of Late Miocene vegetation change, particularly in South Asia (Quade et al., 1989). In this region, a coherent story has emerged from complementary geologic sources (Osborne and Beerling, 2006). The story begins with the $\delta^{13}\text{C}$ of palaeosol carbonates and herbivore tooth enamel, which show parallel shifts from pure C_3 to pure C_4 vegetation between 7.7 and 5.5 Ma (Fig. 6a–b). A climatic driver of this turnover in plant community composition is suggested by the stable oxygen isotope ratio ($\delta^{18}\text{O}$) of palaeosol carbonates, which increases 0.5–1.0 Myr ahead of the $\delta^{13}\text{C}$ shift (Fig. 6c). Rising $\delta^{18}\text{O}$ implies a warmer climate, an increasing proportion of summer rainfall, and / or a warmer source of rainwater, each of which are consistent with intensification of the Indian Monsoon (Quade and Cerling, 1995). This idea is supported by an increase in the abundance of the planktonic foraminifer *Globigerina bulloides* in the Arabian Sea over the same period (Fig. 6d), indicating greater monsoon-driven upwelling. Intensification of the Indian Monsoon is, in turn, attributed to uplift of the Tibetan Plateau, which exaggerates the differential heating of sea and land that drives the monsoon system (Wang et al., 2005). According to the leading palaeoclimate hypothesis for this region, the development of a strongly seasonal climate killed C_3 trees via a greater frequency and intensity of drought events, allowing C_4 grasses to invade and produce savanna or grassland vegetation. Both pollen and faunal evidence are consistent with this interpretation (Fig. 6e; Cerling et al., 1998; Hoorn et al., 2000).

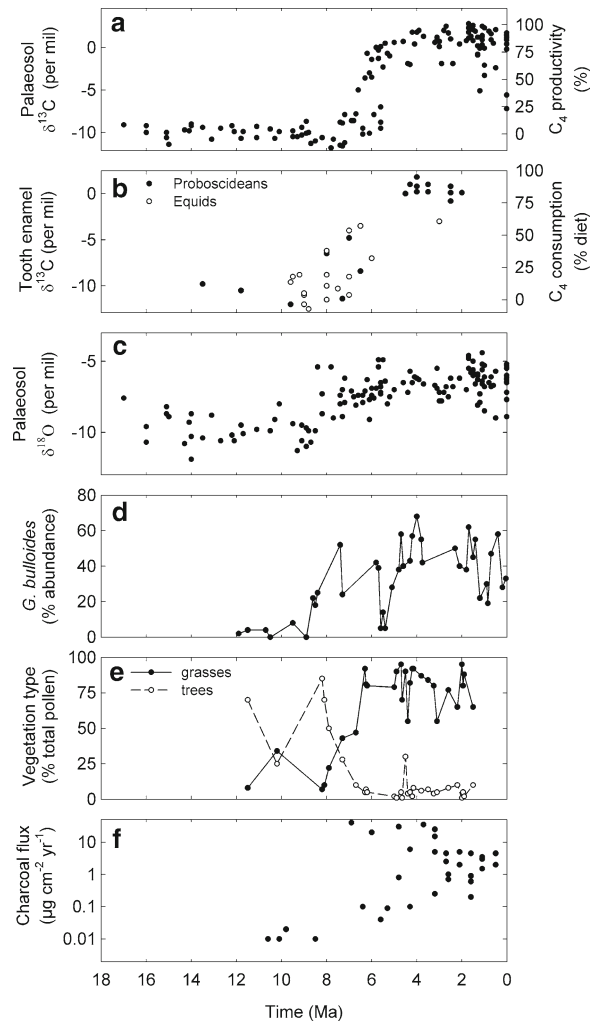


Fig. 6. Geologic evidence of ecosystem dynamics and climate change during the Miocene and Pliocene in South Asia (northern Pakistan and Nepal): (a) $\delta^{13}\text{C}$ of palaeosol carbonates (data from Quade and Cerling, 1995) and inferred C_4 plant productivity (following Fox and Koch, 2003); (b) $\delta^{13}\text{C}$ of tooth enamel from Equids (horses) and Proboscideans (elephant-like mammals) (Data from Quade and Cerling, 1995) and the inferred proportion of diet comprised of C_4 plant biomass (following Passey et al., 2002); (c) $\delta^{18}\text{O}$ of palaeosol carbonates (data source as a); (d) Abundance of *Globigerina bulloides* in the Arabian Sea (Data from Zhisheng et al., 2001); (e) Vegetation type inferred from pollen abundance (Data from Hoorn et al., 2000); (f) Charcoal flux to North Pacific sediments (Data from Keeley and Rundel, 2003) (Reproduced from Osborne and Beerling, 2006. With permission).

However, questions have been raised over the precise mechanism. Seasonal variation in the $\delta^{18}\text{O}$ recorded in the shell growth of freshwater bivalves suggests no change in monsoon

intensity from 10.7 Ma in Nepal (Dettman et al., 2001). Instead, these data point to a decrease in the total amount of rainfall at 8 Ma, with no alteration of seasonality patterns. The timing of Tibetan uplift is also debated, and several recent authors place this event significantly earlier than the Late Miocene (Coleman and Hodges, 2002; Spicer et al., 2003; Rowley and Currie, 2006; DeCelles et al., 2007). The linkage between tectonic events in the Himalayas and major re-organization of the Asian climate system is therefore appealing, but by no means proven (Molnar, 2005), and evidence suggests that a strong Indian Monsoon system could have been in place much earlier than previously recognized.

Intriguing further evidence suggests that strong monsoonal climates may have existed from at least the Early Miocene (>15 Ma) in other regions. General circulation model (GCM) simulations indicate intense monsoon activity in the Early Miocene paleoclimate of East Asia, driven by retreat of the Paratethys Sea (Ramstein et al., 1997; Fluteau et al., 1999; Zhongshi et al., 2007). Geologic records of wind-blown dust and lake sediments in China are consistent with these simulations, indicating a transition from arid to seasonally wet monsoonal conditions during the Early Miocene (Chenggao and Renaut, 1994; Guo et al., 2002; Wang et al., 2005). However, $\delta^{13}\text{C}$ data demonstrate that C₄ plants were at best a minor component of vegetation during this interval (Jia et al., 2003), and pollen assemblages from southern China show that monsoon intensification drove a change from C₃ steppe to C₃ forests (Sun and Wang, 2005).

GCM simulations also indicate that the region affected by the African Monsoon was larger than at present during the Oligocene and Early Miocene, due to the more southerly position of the African continent (Fluteau et al., 1999). Paleoclimate proxy data from Africa are scarce and not securely dated (Fluteau et al., 1999). However, paleobotanical evidence from the Early Miocene suggests a gradient of vegetation in the West and East African sub-tropics ranging from humid forest through woodland to deserts, but no grassland (Jacobs et al., 1999; Jacobs, 2004), although there is some evidence of C₃ tree-grass savannas by the Middle Miocene (Retallack, 2001a). Further model simulations for the Late Miocene suggest

that East African C₄ grassland expansion may have occurred in response to a general decline in rainfall, without major changes in seasonality, driven by uplift of the East African Rift System (Sepulchre et al., 2006).

Lunt et al. (2007) have provided a further dimension to this picture, with their vegetation modelling based on Late Oligocene and pre-Industrial GCM simulations. Intriguingly, their model simulated large areas of C₄ grassland for the Late Oligocene, a result that is clearly at odds with palaeovegetation reconstructions for this interval. Three explanations were advanced by the authors of this study: first, that the vegetation model failed to capture key processes, especially the dynamics of fire; secondly, that an evolutionary niche existed for C₄ grasses in the Oligocene but was not filled; and thirdly, that the GCM failed to accurately simulate the Oligocene climate (Lunt et al., 2007).

Clearly, further sedimentary indicators of palaeoclimate are required for the Late Oligocene, Early and Middle Miocene of Asia and Africa. The limited data currently available suggest that monsoonal climates during this interval may have favored C₃ woodlands rather than C₄ grasslands, implying that climatic seasonality alone may have been insufficient to drive C₄ grassland expansion. However, major climatic shifts clearly occurred during the Late Miocene and Pliocene; measurements of $\delta^{18}\text{O}$ from paleosol carbonates show positive excursions in Pakistan (Quade and Cerling, 1995), Nepal (Quade et al., 1995), South America (Latorre et al., 1997) and East Africa (Levin et al., 2004). Similar patterns are also observed in Greece and Turkey (Quade et al., 1994), where vegetation remained dominated by C₃ trees through the Pliocene and Pleistocene to the present day. An important exception to this global trend is the Great Plains, where $\delta^{18}\text{O}$ shows little change during the crucial transition from C₃ to C₄ vegetation (Fox and Koch, 2004). Osborne and Beerling (2006) note that regional stasis in the $\delta^{18}\text{O}$ signal against a background of global cooling (Zachos et al., 2001) may represent regional climatic changes associated with the initiation of Atlantic Ocean circulation following closure of the Panamanian Seaway. An alternative is that expansion of C₄ grasslands across the Great Plains was not triggered directly by climate change.

C. Grass-grazer Co-evolution Hypothesis

An alternative hypothesis is suggested by inferences of vegetation type based on interpretation of paleosols (Retallack, 2001a). Mollic palaeosols develop under sod-forming grasslands, and are characterized by a dark layer of organic crumbs, whilst alternate morphological types indicate woody vegetation or desert bunchgrass communities (Retallack, 2001a). In combination with the use of the calcic horizon depth as a palaeo-precipitation proxy, these allow reconstructions of vegetation-climate relationships in the geologic past (Retallack, 2001b).

Early and Middle Miocene reconstructions using this technique for South Asia, East Africa and North America suggest that regional rainfall gradients caused vegetation to vary from woodland through to desert (Fig. 7). Crucially, mollic palaeosols are found only in semi-arid areas during this period (Fig. 7), indicating the persistence of steppe-like grasslands in dry regions only. Direct paleobotanical evidence provides some support for these inferences based on paleosols, demonstrating the presence of woodland and savanna ecosystems in East Africa during the Early and Middle Miocene (Jacobs et al., 1999). However, macrofossil, phytoliths and pollen evidence recovered from the US Great Plains is inconsistent with the inference of dry climates at this time, instead suggesting a productive woodland or savanna vegetation with a grass layer dominated by C_3 species (Jacobs et al., 1999; Strömberg 2004, 2005). The presence of plant and animal species which are today confined to humid climates, suggests that the Great Plains climate was relatively wet, rather than arid (Hutchinson, 1982; Axelrod, 1985; Strömberg, 2004).

Paleosol data also suggest an important change in the grassland-climate relationship during the Late Miocene, showing grass colonization of wetter habitats, and a shift in the grassland-woodland boundary (ecotone) to more mesic areas (Fig. 7). The obvious inference from these patterns is that expansion of C_4 grasslands occurred through the displacement of mesic C_3 woodland by C_4 grasses. Retallack (2001b) proposes that this climatic shift in the grassland-woodland ecotone was driven by evolutionary innovations in the grasses, caused by co-evolution with grazing mammals. This idea is developed further in Section 17.4.6.

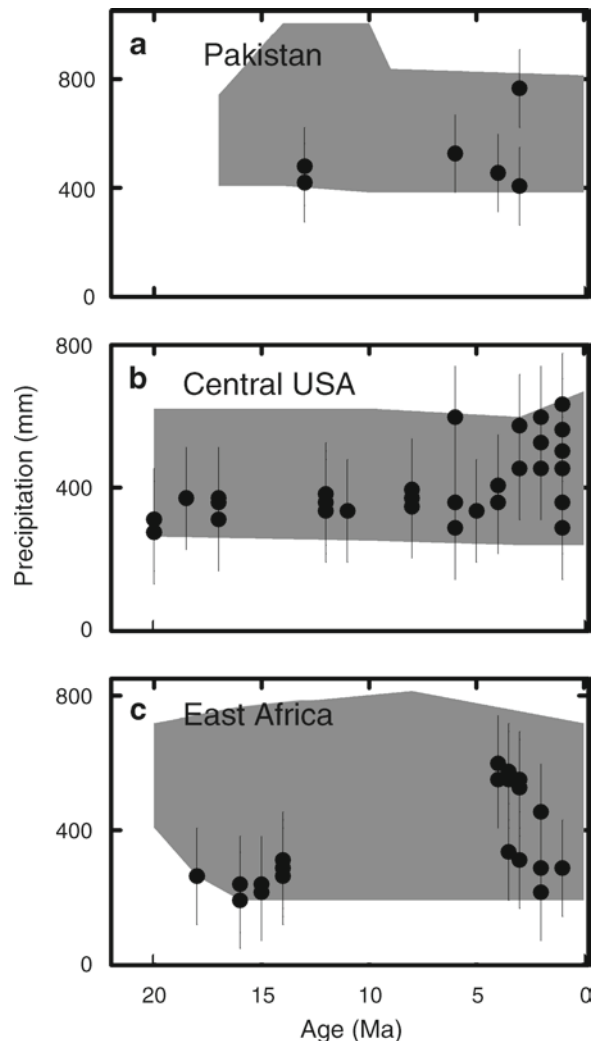


Fig. 7. Paleo-precipitation based on paleosol morphology from (a) Pakistan, (b) the central USA, and (c) East Africa (Retallack, 2001b). Gray shading delimits the range of values obtained across paleosols developed beneath all vegetation types, with the symbols showing values for mollic soils, which develop under sod-forming grasslands (Retallack, 2001b) (Reproduced from Osborne, 2008. With permission).

An alternative explanation for the ecotone shift is that increased disturbance intensity / frequency from megaherbivores or fire caused a decline in woody plant cover. The role of fire is discussed in Sections 17.4.4 and 17.4.5. However, megaherbivores such as elephants can also be tremendously destructive, with significant effects on the density of trees in modern savannas (Sankaran et al., 2008). The Order Proboscidea, which contains the elephants, originated at least 55 Ma (Gheerbrant et al., 1996), and underwent a major

adaptive radiation ~27 Ma during the late Oligocene (Kappelman et al., 2003). Some groups evolved grazing adaptations during C₄ grassland expansion (Cerling et al., 1998), whilst others such as the Gomphotheres remained primarily browsers (Fox and Fisher, 2004). Increasing megaherbivore browsing pressure in response to a shortening growing season during the Pliocene has therefore been proposed as a mechanism which pushed the tree-grass balance in favor of C₄ grasses (Fox and Koch, 2004).

D. Fire Hypothesis

A further interpretation is prompted by evidence from modern ecosystems, which suggests that a significant fraction of the world's mesic C₄ grasslands are sustained by fire. Attempts to explain global vegetation in terms of climate fail across large areas of the temperate and sub-tropical regions, because similar climatic conditions may support forests, woodlands, shrublands or grasslands (Bond, 2005). This observation indicates that woody plant biomass is reduced significantly from its climatic potential by fire or herbivory (Bond, 2005), an idea that is supported by correlations between: (a) woody plant cover and the time interval between fires in mesic regions of Africa (Sankaran et al., 2005); and (b) the total burned area (or fire risk) and extent of savanna (wooded grassland) at the global scale (Mouillot and Field, 2005; Riaño et al., 2007). Experiments within C₄ grasslands demonstrate that fire-sensitive tree species establish at mesic sites when protected from fires (reviewed Bond et al., 2003; 2005). In fact, simulations with a vegetation model indicate that the global forest area could double in the complete absence of fires (Bond et al., 2005).

Keeley and Rundel (2003, 2005) propose that the mechanisms maintaining C₄ grasslands and savannas in the modern world also drove the Miocene-Pliocene expansion of C₄ grasslands via increased fire occurrence. The hypothesis is supported by geologic evidence of fires, preserved as charcoal and soot particles ('black carbon') deposited in deep ocean sediments by offshore winds. Black carbon recovered from the north-west Pacific Ocean and South China Sea shows a five- to hundred-fold increase at the Miocene-Pliocene boundary, coinciding with the expansion of C₄ grasslands on neighboring continents

(Fig. 6f; Herring, 1985; Jia et al., 2003; Keeley and Rundel, 2003). Furthermore, the incidence of charred grass cuticle recovered from Atlantic sediments off the coast of West Africa increases dramatically during the same time interval (Morley and Richards, 1993).

The way in which fires can drive the replacement of forest vegetation by grasslands is illustrated by the recent transformation of Hawaiian ecosystems by invasive grasses. Prior to grass invasion, Hawaiian submontane woodland consisted of trees with a shrub understorey, with a very minor grass component and low fire frequency (D'Antonio and Vitousek, 1992). Invasion during the 1960s by the North American C₄ grass *Schizachyrium condensatum* increased fire frequency by a factor of five, and the area burned in each fire by a factor of four. A single fire was sufficient to kill most native species, and these new fire-prone grasslands were subsequently invaded by the highly flammable African C₄ grass species *Melinis minutiflora*, which requires open habitats to establish. Grasses may therefore promote the occurrence of fires by providing a high flammable fuel load during the dry season, and entrain a strong positive feedback because each fire prevents the establishment of fire-sensitive woody plants (Fig. 8a). Once the cover of woody species is lost, the fire-adapted grass canopies dry more rapidly under direct irradiation from the sun, soil N is lost through volatilization during fires, and the forest seed bank is lost; all factors which make forest regeneration significantly less likely, and entrain a positive feedback that pushes the system from C₃ forests to C₄ grasslands (reviewed Sage and Kubien, 2003). The net effect of these processes is that anthropogenic fires in the modern world typically drive the replacement of woody vegetation by C₄ grasslands (reviewed Sage and Kubien, 2003). Although the extent to which some of these grasslands are an anthropogenic or a natural 'fire climax' ecosystem remains controversial, convincing evidence has been assembled for the latter in South Africa and Madagascar (Bond et al., 2003; 2008).

E. Fire-Climate Feedbacks

Fire occurrence shows an important interaction with climate. The moisture content of biomass is too high to carry fire in wet, aseasonal

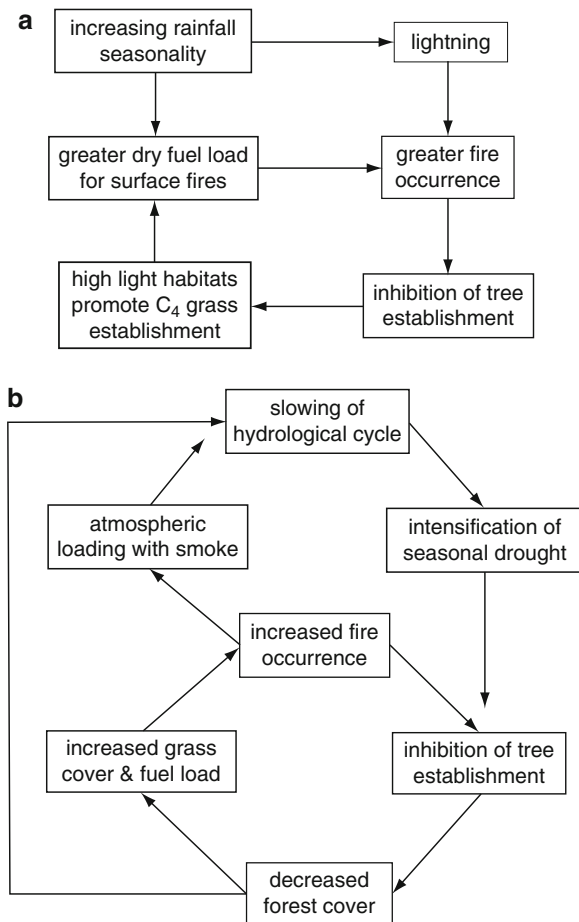


Fig. 8. Schematic showing the principal vegetation-fire interactions involved in (a) the fire feedback hypothesis (Beerling and Osborne, 2006), and (b) the fire climate feedback hypothesis (Keeley and Rundel, 2003; 2005).

environments, but low productivity in dry regions means that too little fuel is produced for large fires (Keeley and Rundel, 2005). Fire climates are therefore characterized by a warm wet season which supports rapid plant growth, interspersed by periods of dry weather sufficient to lower fuel moisture content. An ignition source, via lightning strikes, is also required during this key dry period. Keeley and Rundel (2003, 2005) hypothesize that the spatial extent of such climates increased dramatically during the Late Miocene as seasonality intensified (Fig. 8a). Paleoclimate change therefore forms the basis for vegetation change, and the dominance of ecosystems by C_4 grasses depends on a suitable fire climate.

The fire-driven replacement of C_3 forests by C_4 grasslands may also be linked with climate through other mechanisms. Qualitative analysis suggests

that tree mortality and atmospheric loading by smoke aerosols could entrain climatic feedbacks (Fig. 8b), by suppressing evapotranspiration and therefore precipitation (Beerling and Osborne, 2006). These vegetation-climate and fire-climate feedbacks are positive because decreasing precipitation intensifies drought events, causing further tree mortality and greater fire risk (Fig. 8b). Once initiated by external forcing (e.g. from climate change), feedbacks in the fire-vegetation-climate system should therefore cause the rapid replacement of trees by grasses (Beerling and Osborne, 2006; Osborne and Beerling, 2006). These proposed feedbacks provide plausible links between ecosystems and regional climate, but their quantitative significance remains unexplored.

Further links between the climate system and ecosystem fire regime are indicated by the geologic record of rising dust deposition in deep sea sediments during the Late Miocene (Rea, 1994; Rea et al., 1998). A number of interpretations of these data are possible, including higher wind intensity and greater continental aridity, both of which could serve to increase fire occurrence by impacting on its frequency and extent (Tippie and Pagani, 2007). A final possibility is that lightning frequency during the critical interval of dry weather could have changed in fire-prone climates, but this proposition is difficult to test using geologic data. A critical requirement for the fire mechanism is that a source of ignition (lightning) must coincide with the window of time when fuel (plant biomass) is dry enough to burn. In the absence of an ignition source, the process of ecological succession would have pushed the undisturbed vegetation towards seasonally dry forest.

F. An Evolutionary Link Between Fire and Grazing?

Fire is clearly capable of converting woody vegetation to C_4 grassland and sustaining this new biome, even in regions with the climatic potential to support forests (Section IV.D; Bond et al., 2005). However, the application of this mechanism to the origin of C_4 ecosystems raises two key questions. First, how were radical changes in the fire regime triggered during the Late Miocene and, crucially, why did they not occur earlier in geologic history? Climates capable of supporting frequent fires may have been absent during the Early and Middle Miocene (Keeley and Rundel, 2005), but emerging

palaeoclimate data indicate that strong monsoonal systems and continental aridity may have significantly pre-dated the expansion of C₄ ecosystems in Africa and Asia (Section 17.3.2). Alternative mechanisms for increased fire occurrence are therefore possible, and I here outline a scenario where evolutionary responses to grazing select for fire-promoting traits in C₄ grasses (Osborne, 2008). This hypothesis is not linked directly with the photosynthetic pathway of these grasses, and could apply equally to C₃ clades in regions such as the Mediterranean where the climate favors fires (e.g. Grigulis et al., 2005).

Ecological theory predicts that grazing pressure in nutrient-deficient habitats will select for leaf defences against herbivores (Grime, 2001). A growing body of empirical evidence suggests that evolution in response to such selection pressure leads to herbivore defence syndromes, characterised by the simultaneous deployment of multiple, covarying traits (Agrawal and Fishbein, 2006; Agrawal, 2007). An important syndrome developing on nutrient-poor soils is the 'resistance strategy', which deters herbivores and minimise the rewards of consumption (Agrawal and Fishbein, 2006). Grazing-resistant grasses are characterised by a low foliar nutrient content, high fibre and silica content, high leaf toughness, and the accumulation of chemical deterrents to herbivory, such as condensed tannins (Ellis, 1990; Vicari and Bazely, 1993; Massey et al., 2007). Such traits are well-known in southern African 'sourveld' grassland communities, growing on the highly leached soils typical of mesic sub-tropical climates (Ellis, 1990; Scholes, 1992; Bond et al., 2003). The palatability and nutritional value of foliage in these ecosystems declines significantly from the summer to the winter due to the accumulation of chemical defences (Huntley, 1984). Grazing pressure is therefore strongly seasonal, allowing the build-up of biomass fuel during the dry season.

The leaf litter produced by grazing-resistant plants is typically recalcitrant because chemical defences and high fibre to nutrient ratios retard decomposition (Horner et al., 1988; Cornelissen et al., 1999). Slow decomposition causes the build-up of leaf litter at the soil surface and persistence of standing dead plant material, increasing the fuel load for surface fires, and therefore fire frequency (Bond et al., 2003). Through these feedbacks, nutrient-poor, mesic grasslands typically have a fire return interval short enough to exclude

most woody plants, but allow the persistence of resprouting perennial grasses, or annuals with heat tolerant seeds and a persistent seedbank.

I therefore proposed a scenario whereby increasing grazing pressure in Miocene grassland or woody savanna communities selected for grazing resistance / fire promoting traits in grasses (Osborne, 2008). It is important to recognize that this mechanism is not associated directly with photosynthetic pathway, but instead linked to the occurrence of C₄ grasses in heavily grazed and fire-prone grasslands, savannas or woodlands. Is there any evidence that grazing pressure on C₄ grasses increased during the Late Miocene? Some support comes from the contrast in $\delta^{13}\text{C}$ patterns of herbivore teeth and paleosols in the northern Great Plains, which suggests that selective feeding on C₄ grasses began prior to C₄ grassland expansion (Fig. 3b–c). Mesic grasslands are today maintained in this region by fires, and fire prevention measures allow the incursion of woody plants (Bragg and Hulbert, 1976). Clearly, the grazing resistance hypothesis is still in its infancy, and requires further investigation, and the picture is complicated by the direct effects of grazers on grass-woody plant dynamics (Beerling and Osborne, 2006). Nevertheless, the hypothesis provides a biotic mechanism that links geologic inferences of an ecotone shift (Section 17.4.3) with those of greater fire occurrence at the Miocene-Pliocene boundary (Section 17.4.4), without the need to evoke climate change.

V. Conclusions

The past decade has brought significant advances in our understanding of the geologic history of C₄ plants. A major expansion of C₄-dominated ecosystems occurred across four continents during the late Miocene and Pliocene (2–8 Myr ago), and the balance of evidence now suggests that changes in climate and fire regime could have driven this event. The earlier history of C₄ plants is less clear, but intriguing evidence suggests that they were present at low abundance for at least 10 Myr before rising to ecosystem dominance, and points to declining atmospheric CO₂ as a key selection pressure for the pathway. Understanding when C₄ plants first originated, why they remained sub-dominant components of ecosystems for so long, and why they rose to dominance in the late

Miocene, remain important unresolved problems in this field. However, recent technical advances in the analysis and interpretation of plant microfossils promise to offer further insight into these issues.

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Part V

C₄ Engineering and Bioenergy

Hurdles to Engineering Greater Photosynthetic Rates in Crop Plants: C₄ Rice

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Summary	361
I. Introduction.....	362
II. Why Try to Engineer a C ₄ Crop Plant?.....	362
III. How Can Crop Productivity Be Increased by C ₄ Photosynthesis?	363
IV. The Requirements for C ₄ Photosynthesis.....	363
V. Which Plant Should We Transform?	366
VI. Which Mechanism of C ₄ Photosynthesis Should Be Used and Why?	367
A. The Single-Cell Model.....	367
B. The Two-Cell Model	368
C. How Many Changes Are Required in the Two-Cell Model?	369
VII. Early Attempts at Transferring C ₄ -Traits into C ₃ Plants	369
VIII. Alternate Approaches to Improving Photosynthetic Rates	372
A. Recycling Photorespiratory Products.....	372
B. Introduction of an Alternate Carbon Concentrating Mechanism	372
IX. Hurdles to Engineering C ₄ Crops.....	373
X. Assessment of C ₄ -ness	374
XI. Conclusions	374
Acknowledgment	375
References	375
Patents Related to C ₄ Rice	378

Summary

It is now 20 years since a suggestion was first made of introducing a C₄ photosynthetic pathway into rice plants to increase rice productivity. With the amount of arable land decreasing, the human population increasing and rice productivity plateauing there is an urgent need to significantly increase rice productivity. One way of increasing rice productivity is by introducing the C₄ photosynthetic pathway into rice by genetic manipulation. Over the past decade, a number of attempts have been made to modify the photosynthetic pathways present in C₃ plants by introducing genes encoding enzymes associated with C₄ photosynthesis. These efforts have increased the expression of specific enzymes such as pyruvate, orthophosphate dikinase, phosphoenolpyruvate carboxylase and NADP-malic enzyme in a range of plants, notably rice, tobacco, and potato. Claims and counter claims have been made regarding the success of attempts to construct a C₄ rice plant, but to date, no functional C₄ pathway has been engineered into C₃

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species. Novel approaches are now required to introduce a functional C_4 photosynthetic pathway into rice and this may require a change to the molecular architecture of photosynthetic tissue (introduction of Kranz anatomy), the coordinated co-expression of enzyme regulatory mechanisms and the expression of specific transporters in rice leaves.

I. Introduction

In taking on the challenge of contributing a chapter on *Hurdles in the Engineering of C_4 Crops* I recognize that, over the last 6 or 7 years, a number of reviews have been published that address related topics; these include Dunwell (2000), Matsuoka et al. (2001), Edwards et al. (2001), Häusler et al. (2002) and Leegood (2002). In the following chapter I address the topic from a different perspective and highlight issues that have been overlooked in previous treatments. I also identify gaps in our knowledge that require filling before we can construct plants that use the C_4 pathway to significantly enhance photosynthesis and plant productivity. The need to significantly increase plant productivity has taken on renewed urgency following the rapid rise in the price of oil and the diversion of food crops to the production of ethanol and bio-fuels; this has led to a shortage of food, increased food prices and social unrest in some countries.

II. Why Try to Engineer a C_4 Crop Plant?

Land available for cultivation is decreasing while the human population is increasing, leading to predictions that the world will face severe food crises by 2050 (Dawe, 2000; Sheehy et al., 2007a). Because the vast majority of all energy for food ultimately comes from the sun via plants, it follows that increasing the food supplies for future human needs will require increased plant productivity.

This can be achieved in a number of ways. Selective breeding can be used to maximize the conversion of light energy to chemical energy, and the storage of that chemical energy in forms fit for human consumption (for example, in edible grain, or animals). It is generally agreed that breeding efforts to date have raised potential plant productivity to the maximum levels the environment can sustain. Plant productivity can also be maximized by provision of abundant supplies of water and nutrients; however, yield enhancements due to addition of fertilizers and irrigation are approaching the maximum in much of the world (Dawe, 2000). Minimizing insect and disease losses, and eliminating competition from weeds can also maximize plant productivity. Again, genetic modification and use of pesticides have greatly reduced these limitations, often with substantial environmental consequences (Dawe, 2007).

To boost yields by the magnitude required to keep pace with the growth of the human food and energy demands by 2050, alternate approaches to increase plant productivity will be required. A number of approaches to achieve this goal are under consideration (Long et al., 2006). A leading proposal under consideration is to engineer C_4 photosynthesis into major C_3 crops (Sheehy et al., 2007a). This is largely because C_3 photosynthesis is inherently less efficient than C_4 photosynthesis in the climatic conditions where high crop yields are possible (Long, 1999). The major contributor to the lower photosynthetic efficiency observed in C_3 plants is the high levels of photorespiration present in C_3 plants at warmer temperatures. Photorespiration is a result of the loose specificity of Rubisco for CO_2 . In addition to catalyzing the carboxylation of RuBP, Rubisco also catalyzes its oxygenation. With atmospheric concentrations of CO_2 at about 0.04% and oxygen concentrations almost 500 times higher, even though Rubisco has a higher affinity for CO_2 compared to O_2 ($K_m [CO_2] = 10 \mu M$ versus $K_m [O_2] = 200 \mu M$) it catalyzes the oxygenation of RuBP. Instead of producing two molecules of 3-phosphoglycerate (3-PGA), the oxygenation reaction produces one

Abbreviations: BSC – Bundle sheath cell CA – Carbonic anhydrase cDNA – Complementary DNA BicA – Inorganic carbon (bicarbonate) transporter NAD-ME – NAD-malic enzyme NADP-ME – NADP-malic enzyme OAA – Oxaloacetate PCR – Photosynthetic carbon reduction PEP – Phosphoenolpyruvate PEPC – PEP carboxylase PEPCCK – PEP carboxykinase PEPS – PEP synthetase PDRP – Pyruvate, orthophosphate dikinase regulatory protein PDK – Pyruvate, orthophosphate dikinase PGA – Phosphoglycerate RuBP – Ribulose-1,5-bisphosphate Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase

molecule of 3-PGA and one molecule of phosphoglycolate. Photorespiration is the term given to the pathway present in C₃ plants (like rice and wheat) to recycle the glycolate produced during the oxygenation of Rubisco (Tolbert, 1997; Leegood et al., 1995). In recovering the carbon in phosphoglycolate, plants expend energy and release previously fixed CO₂, leading to a loss of photosynthetic capacity, and a decrease in the efficiency of water, light and nitrogen use (Long, 1999).

Efforts to increase the specificity of Rubisco for CO₂ in order to decrease photorespiration have usually decreased catalytic turnover rates of Rubisco, and impaired photosynthesis (see Parry et al., 2003; Zhu et al., 2004). A glimmer of hope in the quest to overcome the low substrate specificity of Rubisco was provided by the identification of an algal and higher plant Rubiscos with greater specificity for CO₂ (Whitney et al., 2001; Read and Tabita, 1994). Attempts to increase photosynthetic efficiency of C₃ plants by the introduction of the type II Rubisco have been frustrated by failure of the foreign Rubiscos to assemble into an active protein in transformed plants (Parry et al., 2003). The prospects of increasing the catalytic efficiency of Rubisco by genetic manipulation have been outlined previously (Spreitzer and Salvucci, 2002) and will not be discussed further.

It is generally recognized that C₄ crops (notably maize and sugarcane) have a higher photosynthetic capacity than their C₃ counterparts and this is possibly due to the very low rates of photorespiration present in C₄ plants (Hatch, 1987). Furthermore, given suitable climatic conditions, it is generally recognized that C₄ plants are more productive than their C₃ counterparts. Therefore it is logical to suggest that plant productivity might be increased by increasing the photosynthetic yield of existing crop species and prospective future crops that might be used as bioenergy feedstocks. Introducing C₄ photosynthesis into C₃ crops is the most obvious means to realize spectacular gains in photosynthetic productivity.

III. How Can Crop Productivity Be Increased by C₄ Photosynthesis?

Two alternate approaches are available to increase food productivity based on recognition of the photosynthetic benefits of C₄ photosynthesis. Firstly,

a non-domesticated C₄ plant could be genetically manipulated to produce a new crop product. For example, the weed rice mimic (*Echinochloa crusgalli*) could be bred to produce rice-like grain (Sage, 2000). Alternatively, a C₃ crop could be genetically manipulated to carry out C₄ photosynthesis. C₄ plants have evolved from C₃ ancestors on more than 50 occasions in total, and almost 20 occasions in the grasses (Sage, 2004 and Chapter 16, this volume), indicating the latter approach would be feasible. At present there are four major C₄ crops that produce food directly consumed by humans (maize, sugarcane, sorghum and millet, where “millet” collectively describes a number of plant species). With the exception of maize, none of these crops produces a grain acceptable to most people, and therefore have limited utility in most cultures. Except for maize and possibly sorghum, the major grains directly consumed by humans are all C₃ crops, most notably rice, wheat, barley, oats and pulses (beans and peas) (Brown, 1999). Therefore, without radical shifts in diet, improvements in the human food supply will require large yield improvements in C₃ crop productivity. Because a large proportion of the human population obtains their daily caloric intake from rice, it is logical to suggest the most immediate means of dramatically increasing food yield for the people that need it the most is to improve the productivity of rice by introducing the C₄ pathway.

IV. The Requirements for C₄ Photosynthesis

Is the development of a C₄ rice plant a feasible objective or an impossible dream? To answer this, we need to understand the operation of C₄ photosynthesis in its various guises. C₄ photosynthesis exists in three biochemical subtypes (Fig. 1). These are the NADP-malic enzyme subtype, the NAD-malic enzyme subtype and the PEP carboxykinase subtype. In land plants, C₄ photosynthesis also required structural changes to separate the C₄ metabolic cycle and the C₃ cycle [the photosynthetic carbon reduction (PCR) cycle] into distinct compartments. This is usually, but not necessarily, associated with a specific type of leaf anatomy, Kranz anatomy. Kranz anatomy is characterized by an outer layer of mesophyll cells surrounding an inner ring of larger bundle sheath

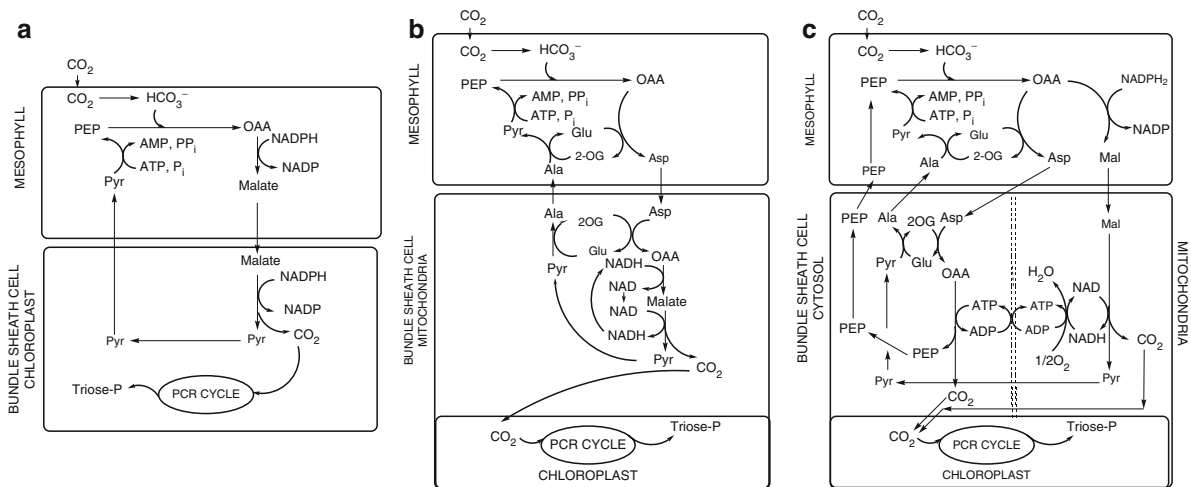


Fig. 1. The three C₄ photosynthetic pathways. (a) The NADP-malic enzyme type pathway in which C₄ acid decarboxylation occurs in the BSC chloroplasts. (b) The NAD-malic enzyme type pathway in which C₄ acid decarboxylation occurs in the BSC mitochondria. (c) The PEPCK type pathway in which C₄ acid decarboxylation occurs in both the BSC cytosol and mitochondria. Abbreviations: Ala, alanine; Asp, aspartate; Glu, glutamate; 2-OG, 2-oxoglutarate; OAA, oxaloacetate; Pyr, pyruvate; PEP, phosphoenolpyruvate; Mal, malate.

cells that, in turn, surround the vascular tissue. Importantly, the mesophyll and the bundle sheath cells are in close contact, minimizing the inter-cellular diffusion distance (Dengler and Nelson, 1999). Biochemically the two photosynthetic tissue types are distinguished by the presence of PEP carboxylase in the mesophyll cells and Rubisco in the bundle sheath, and the absence of Rubisco in the mesophyll cells (see Kanai and Edwards, 1999). Unlike the biochemical subtypes, there is much greater variation in the anatomical forms associated with C₄ photosynthesis. Over 14 specific anatomical types have been identified in monocots and dicots (Dengler and Nelson, 1999). Most exhibit some version of Kranz anatomy, but in two evolutionary lineages, Kranz anatomy is not apparent; instead, C₄ photosynthesis operates within single cells (Voznesenskaya et al., 2001, 2002 and see Chapter 4, this volume).

All three C₄ biochemical subtypes possess carbonic anhydrase and PEP carboxylase in the cytosol of the mesophyll cells. Here inorganic carbon, in the form of CO₂ is converted to bicarbonate. The conversion of CO₂ to bicarbonate is significant for two reasons; first, by converting CO₂ to bicarbonate, the cytosolic concentration of CO₂ is decreased, thereby increasing the concentration gradient across the cell membrane and drawing more CO₂ into the cell. Second, bicarbonate is the inorganic carbon substrate for PEP

carboxylase and is a form of inorganic carbon that differs in shape to oxygen and does not compete with O₂ in the active site of PEP carboxylase. Therefore, PEP carboxylase can efficiently catalyze the carboxylation of PEP to oxaloacetate (OAA) despite the presence of high levels of O₂.

The least complex C₄ subtype is the NADP-malic enzyme type. This pathway is characterized by the presence of NADP-malic enzyme in the chloroplasts of the bundle sheath cells (Hatch et al., 1975). PEP carboxylase catalyzes the carboxylation of PEP to form OAA that is then reduced to malate in an NADPH-dependent reaction utilizing one of the products of the light reactions of photosynthesis. The malate diffuses through numerous plasmodesmata located in the cell walls separating the mesophyll and the bundle sheath cells and is transported into the bundle sheath chloroplasts (Boag and Jenkins, 1986) where it is decarboxylated to pyruvate and CO₂ and NADP⁺ is reduced. The released pyruvate is transported back to the mesophyll chloroplasts where it is converted to PEP via pyruvate, orthophosphate dikinase (PPDK). The CO₂ released in the BSC is then refixed by Rubisco and reduced by NADPH via the PCR cycle to triose phosphates, and eventually glucose and sucrose.

A notable feature of NADP-ME subtypes is the ultrastructure of the bundle sheath chloroplasts are generally altered to accommodate the

C₄ biochemical cycle. The reduction of OAA to malate in the mesophyll cell chloroplasts and the subsequent oxidative decarboxylation of malate to pyruvate in the bundle sheath cells imports reducing equivalents into the bundle sheath cells, thereby decreasing the need to produce NADPH in the BSC. As a result, bundle sheath chloroplasts have little photosystem II expression and water splitting, minimizing the O₂ production in the BSC. The photorespiratory potential is greatly reduced by the combination of low O₂ and higher CO₂ in the BSC, enabling the NADP-ME subtype to be the most efficient version of C₄ photosynthesis (Kanai and Edwards, 1999). This greater efficiency justifies considering the NADP-ME type as the subtype to engineer into C₃ crops; however, to realize this efficiency, it will be necessary to work on the less obvious changes arising in NADP-ME C₄ species, such as the ultrastructure and stoichiometry of electron transport chains in the BSC chloroplasts.

In NAD-malic enzyme type C₄ plants, OAA formed in the mesophyll cytosol by PEPCK is transaminated to aspartate. Aspartate diffuses down its concentration gradient into the BSCs and is transported into the mitochondria. The aspartate is converted to OAA by transamination, the OAA reduced to malate, which is decarboxylated via NAD-ME, releasing pyruvate and CO₂ (Hatch and Kagawa, 1974). The released pyruvate is then phosphorylated to PEP as in NADP-ME type plants. In NAD-ME types, the BSC chloroplasts are usually localized into the inner region of the bundle sheath cells along with the mitochondria and peroxisomes. This forces the CO₂ escaping the mitochondria to diffuse through the chloroplasts, where much of it can be refixed by Rubisco. The large vacuole between the organelles and the cell wall also slows CO₂ escape, allowing for high levels of refixation. As with NADP-ME changes, changes in cellular ultrastructure are also required for efficient C₄ function, and would have to be planned for in any engineering attempt. However, much less reducing power is shuttled into the BSC of NAD-ME subtypes, such that linear electron transport is fully required to generate the NADPH required for carbon reduction to glucose. The presence of linear electron transport and water splitting leads to a build up of BSC O₂ and a lower efficiency in NAD-ME compared to NADP-ME.

The third C₄ pathway is the most complex with two decarboxylases implicated in the BSCs (Burnell and Hatch, 1988a). As in the two ME-type C₄ plants, PEP is carboxylated to OAA, however, the OAA is converted to either malate or aspartate, both of which diffuse into the bundle sheath cells (Weiner et al., 1988). In the BSCs the aspartate is transaminated to release OAA, which is decarboxylated to PEP, and CO₂ in an ATP-dependent reaction catalyzed by cytosolic PEP carboxykinase. Simultaneously, the malate is transported into the BSC mitochondria where it is oxidatively decarboxylated by NAD-ME releasing pyruvate, CO₂ and NADH. The NADH is oxidized via the mitochondrial electron transport chain producing the ATP required in the PEP carboxykinase-catalyzed reaction. The PEP released via PEPCK diffuses back to the mesophyll cytosol where it is carboxylated to OAA. The pyruvate released via mitochondrial NAD-ME returns to the mesophyll chloroplasts where it is converted to PEP via PPDK. Significantly, the release of PEP during the PEPCK-catalyzed reaction and its recycling to the mesophyll cells requires less PPDK in the mesophyll cells and this is supported by the detection of lower PPDK levels in PEPCK-type C₄ plants (Burnell and Hatch, 1988b).

In comparing the three decarboxylation pathways of C₄ photosynthesis it becomes apparent that C₄ photosynthesis requires multiple major changes in the structure and function of leaves. The following summarizes the major requirements for an efficient C₄ pathway involving two cellular compartments:

- CO₂ must be converted to bicarbonate in the mesophyll cells.
- PEP carboxylase activity is high but is restricted to the mesophyll cells.
- Rapid PEP regeneration is required for the incorporation of bicarbonate into organic acids.
- OAA is converted to either malate and/or aspartate prior to its diffusion into the bundle sheath cells.
- Either or both malate and aspartate must diffuse rapidly from the mesophyll cells to the bundle sheath cells.
- C₄ acid decarboxylation must occur in the chloroplasts, mitochondria or the cytosol of the bundle sheath cell.
- Rubisco and the PCR cycle is restricted to, and is active in, the chloroplasts of bundle sheath cells.
- The three carbon compound released during decarboxylation in the bundle sheath cells must be

returned to the mesophyll cells where, if required, it must be converted to PEP prior to being carboxylated to OAA.

These changes allow the C_4 pathway to act as a biochemical pump that concentrates CO_2 in the BSC. This increases the concentration of CO_2 (relative to the concentration of oxygen) in the vicinity of Rubisco, minimizing the rate of photorespiration while allowing Rubisco to function close to CO_2 saturation (Hatch, 1987). In C_3 plants, the carboxylation efficiency of Rubisco is low and C_3 plants compensate for the low substrate specificity by synthesizing large amounts of the enzyme (Dey et al., 1997). Rubisco can constitute up to 30% of the total protein present in C_3 leaves (Sage et al., 1987). Since C_4 plants are able to incorporate CO_2 much more efficiently than C_3 plants, they do not need to synthesize as much Rubisco and they invest their nitrogen resources in proteins other than Rubisco. This property makes C_4 plants more nitrogen use efficient (Brown, 1999; Long, 1999). In addition, because C_4 plants convert their CO_2 to bicarbonate and then fix the bicarbonate via PEP carboxylase (which is not affected by oxygen concentrations) C_4 plants are able to survive at lower CO_2 concentrations and can survive with less open stomata relative to C_3 plants. This lowers the amount of water lost through stomata per unit carbon fixed, making C_4 plants more water use efficient (Hatch, 1987).

With this understanding of the differences between C_3 and C_4 photosynthesis, and recognizing that it is now common to transform plants with foreign genes, it is reasonable to suggest that introducing genes from C_4 plants into C_3 plants could increase photosynthetic rates of C_3 plants. But a number of questions instantly arise:

1. Which C_3 plant(s) should be transformed?
2. Which C_4 photosynthetic mechanism should be introduced into the C_3 plant?
3. Which genes should be transferred and where should they be expressed?

I will address each of these questions in order.

V. Which Plant Should We Transform?

Of the major food crops grown worldwide rice, maize, sorghum, wheat, barley and the other major crop grasses from the family Poaceae

(Graminaeae) are mankind's most important sources of calories. The argument for initially targeting rice as the first plant species to convert to C_4 photosynthesis include the high dependence on rice for caloric intake and poverty alleviation in many Asian and African countries. In some countries in Asia, rice makes up more than 70% of the food consumed by many people, and due to rapid economic and population growth, the food needs of Asia are growing more rapidly than any other region (Dawe, 2000). As a grass from low latitudes, rice grows in warm to hot conditions where photorespiration is substantial. Thus, of all the major C_3 food crops, rice would show the greatest yield enhancement from introducing C_4 photosynthesis.

Other crops should also be considered. Wheat is the other leading grain directly consumed by humans, and thus might provide marked benefits to food stocks if C_4 photosynthesis were introduced in wheat lines. Wheat, however, is a crop of cool climates, and thus does not show the level of photorespiratory inhibition as would be observed in a warm-climate crop such as rice. This is also true of most other C_3 grains such as oats, rye, and barley. Dicot crops also should be considered. Dicots might be easier to engineer, because their genetics may more closely resemble the model plant *Arabidopsis* (Brown et al., 2005), and there are many more evolutionary lineages with C_3 – C_4 intermediates to examine for critical genetic changes during C_4 evolution (Sage et al., 1999). *Flaveria* in the Asteraceae, for example, has served as an important model for dissecting the molecular level changes associated with the evolution of the genes for PEP carboxylase and carbonic anhydrase expression and regulation in C_4 plants (Chapter 13, this volume). *Flaveria*, however, is a difficult model as transformation is problematic, the genome is relatively large, and the plants cannot be rapidly grown to seed as with *Arabidopsis*. *Cleome gynandra* has been suggested as an alternative dicot to examine the genetics of C_4 evolution, given its close taxonomic affinity to *Arabidopsis* (Brown et al., 2005). Of the dicot crops, there are a number of C_3 Brassicaceae species relatively close to *Arabidopsis* (such as rape-seed); however, these are all cold climate crops that would probably be inhibited by the C_4 pathway. The most important warm season C_3 dicots are legumes, sweet potato and manihot.

All would benefit from having C₄ photosynthesis but none more so than legumes such as cowpea and soybean, because the legumes could use the additional carbon and energy from C₄ photosynthesis to support nitrogen fixation from their root nodules. This would simultaneously support increased yield for the plant from increased photosynthesis, and increased N fertility. For these reasons, discussions of engineering C₄ photosynthesis into C₃ species have focused on rice and soybean.

VI. Which Mechanism of C₄ Photosynthesis Should Be Used and Why?

In July 2006, a meeting was held at the Philippine headquarters of IRRI (International Rice Research Institute) to discuss potential pathways to C₄ rice (Sheehy et al., 2007a). Following 3 days of discussions, two potential pathways to developing a C₄ rice plant were proposed; these were identified as the *single-cell model* and the *two-cell model*.

A. The Single-Cell Model

The single cell model was first suggested in 1990 (Fig. 2; Burnell, 1990). In the single cell model, the cytosol and the chloroplast compartments of C₃ plants would be used to mimic the mesophyll and bundle sheath cells, respectively, of C₄ plants; CA and PEPC would be targeted to the cytosol, and PPDK and a C₄ acid decarboxylating enzyme would be targeted to the chloroplasts. The attraction of this model lies in the inherent simplicity of the design relative to a two-celled model that requires Kranz anatomy. The anatomy of the rice leaf does not require significant alteration, which eliminates the need to engineer Kranz anatomy, mesophyll to BSC transport systems, and ultrastructural changes in the BSC. Since the genetic controls over the anatomical, transport and ultrastructural changes are not known, yet are considered to be complex, the single-cell model may be the only feasible approach for some time. The feasibility of the single-celled approach is supported by the discovery in the last decade of three terrestrial plant species, and numerous algae and aquatic macrophytes that perform single-celled C₄ photosynthesis (see Holaday and

Bowes, 1980; Magnin et al., 1997; Salvucci and Bowes, 1981; Bowes et al., 2002; Chapters 4 and 5, this volume). A notable difference between the terrestrial single-celled C₄ species (*Suaeda aralocapsica* and *Bienertia cycloptera*) and the aquatic species (*Hydrilla* and *Egretta* spp.) is the terrestrial species exhibit spatial separation of Rubisco and PEP carboxylation; PEP carboxylation is located around the outer cell perimeter while Rubisco is located in a ball of chloroplasts in the middle of the cell as in *Bienertia* spp., or on the inner pole of the cell (as in *Suaeda aralocapsica*). In both cases, a large vacuole separates the regions where PEP carboxylation and Rubisco occur, and presumably acts as a diffusive barrier to slow CO₂ escape from the inner compartment following decarboxylation (Edwards et al., 2007). Plant species exhibiting single-celled C₄ photosynthesis exhibit low growth rates relative to crops, and in terrestrial settings, they are only known from highly stressed sites with drought and high salinity (Bowes et al., 2007). Hence, the yield enhancements from a single cell system may be limited.

The first design proposed to guide a C₄ engineering project in rice was a single-celled design (Fig. 2, Burnell, 1990). Inorganic carbon in the form of CO₂ would diffuse from the air spaces into the cytosol where, in the presence of a cytosolically expressed carbonic anhydrase, it would be converted to bicarbonate. PEPC would be expressed in the cytosol. The remainder of the C₄ acid cycle introduced into rice was targeted to the chloroplast, the site of C₄ acid decarboxylation

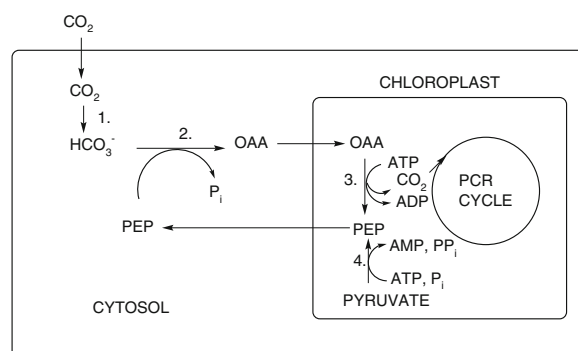


Fig. 2. Single celled C₄ photosynthesis. A summary of the location and expression in rice of four C₄ enzymes in an attempt to increase photosynthetic rates (From Burnell, 1990). Enzymes: 1 – spinach carbonic anhydrase; 2 – maize leaf PEP carboxylase; 3 – *Urochloa panicoides* PEP carboxykinase; 4 – maize leaf PPDK.

and the incorporation of the released CO_2 into sugars via the PCR cycle. The separation of the two carboxylating mechanisms (PEPC and Rubisco) was seen as crucial for the successful operation of the single-celled model of C_4 rice as it was anticipated that this segregation would generate a higher CO_2 concentration in the chloroplasts compared to the unmodified plant. This segregation or compartmentalization necessitated the operation of an efficient oxaloacetate transport mechanism across the chloroplast membrane. Highly specific oxaloacetate transporters are present in spinach chloroplasts (Hatch et al., 1984). The efficient operation of a single-celled C_4 system would also require the efficient transport of PEP across the chloroplast membrane into the cytosol; phosphoenolpyruvate/phosphate antiporters had been reported in a variety of plants that could be exploited once their genes have been identified (Neuhaus et al., 1988).

The model proposed that an enzyme capable of decarboxylating OAA would be expressed in the chloroplast stroma. PEPCK was chosen as the decarboxylating enzyme because OAA decarboxylation would directly yield PEP and only ATP would be required as the second substrate for the decarboxylation reaction. This ATP could be produced via cyclic photophosphorylation without causing concomitant release of oxygen that could stimulate photorespiration. While PEP regeneration would largely be conducted by PEPCK, there would still be an initial requirement to synthesize PEP (to start the C_4 acid cycle) and a need for additional PEP synthesis as it may be removed for the biosynthesis of other compounds such as aromatic amino acids. Therefore PPDK would need to be expressed with a transit peptide targeting it to the chloroplasts.

In addition it was considered important that the levels of chloroplast carbonic anhydrase should be decreased; 98% of CA activity of C_3 plants is located in the chloroplast (Reed and Graham, 1981; Fett and Coleman, 1984; Kachru and Anderson, 1974). This could be achieved by antisense technology.

This rather simple model for single-celled C_4 photosynthesis was based on the premise that the fixation of CO_2 into a C_4 acid, its transport into the chloroplast via a C_4 acid and its subsequent release from the C_4 acid would result in an increase in stromal CO_2 concentrations. von Caemmerer et al (2007) have soundly argued that there is a need

for a significant level of resistance to the diffusion of CO_2 out of the chloroplast for this simple system to function efficiently. Edwards et al (2004) referred to the “liquid-phase diffusive resistance to CO_2 ” that is created by the large vacuole in *Bienertia* and *Suaeda aralocaspica*. A lack of any significant diffusive resistance between rice chloroplasts and the surrounding cytosol may be a major impediment to the efficient function of a single-celled C_4 pathway in rice plants.

B. The Two-Cell Model

An argument has been made that distinct tissue compartments are required for the efficient operation of C_4 photosynthesis; indeed the large majority of C_4 plants, and all of the highly productive species, have a version of Kranz anatomy (Sage, 2002). The leaves of most C_3 plants have a bundle sheath layer surrounding the vascular tissue, but in nearly all cases, these cells are much smaller than Kranz bundle sheath cells and have few, if any, organelles (Sage, 2001). To introduce an efficient C_4 photosynthetic pathway into rice based on a two cell model, it would be necessary to inflate the bundle sheath cells and increase their organelle number. How this would be accomplished is not known, since the genes controlling bundle sheath characteristics are uncertain.

Chloroplasts are present in the bundle sheath cells of rice leaves however they are significantly less abundant compared to the bundle sheath cells of C_4 plants (Sheehy et al., 2007b). Furthermore, there is considerable variation in bundle sheath cell chloroplast density not only between different wild rice species but also within rice cultivars (Sheehy et al., 2007b) with some bundle sheath cells possessing chloroplasts while others do not. Interestingly rice bundle sheath chloroplasts have been shown to accumulate large amounts of starch in the late stages of leaf development indicating that they are capable of accumulating and storing starch (Miyake and Maeda, 1976).

These reports provide hope that there may be sufficient natural variation to accelerate a C_4 rice program by providing the variation for breeders to work with, and geneticists to dissect in a gene discovery program. However, it is clear that such variation is rare, and will require labour intensive screening to identify additional promising genotypes.

C. How Many Changes Are Required in the Two-Cell Model?

C₄ photosynthesis is a complex trait that has arisen from the modification of many genes that originally had a functional role in C₃ species. The number of genes modified is not known, but probably numbers in the thousands. The specific modifications can be summarized, and a list of these gives a sense of the challenges required to engineer C₄ photosynthesis into C₃ plants. Specific changes that would be required include the following:

- Differentiation of photosynthetic tissue into specialized mesophyll and bundle sheath cells
- Changes in the number, position and ultrastructure of bundle sheath chloroplasts
- Expression of carbonic anhydrase and PEP carboxylase in the cytosol of mesophyll cells
- Restriction of expression of Rubisco to the bundle sheath cells
- Decrease in expression of carbonic anhydrase in the bundle sheath cells
- Expression of PPDK in the chloroplasts of mesophyll cells
- Expression of a 4C acid decarboxylating enzyme in the bundle sheath cells
- Profusion of plasmodesmata linking the two cell types and proliferation of inter tissue transport systems
- Development of a CO₂ diffusion barrier between the two cell types
- Expression of individual regulatory systems of individual enzymes where present

All of the enzymes implicated in all three C₄ photosynthetic pathways are present in some form in C₃ plants. C₄ photosynthesis did not require the evolution of new enzymes, but the modification of expression, kinetics and regulatory properties of existing enzymes to create the C₄ pathway, and to coordinate the PCR cycle with the C₄ cycle. C₄ photosynthesis is, in essence, assembled from a pre-existing C₃ toolkit with a bit of tinkering here and there to get the pieces to fit together in a new configuration. The challenge for C₄ engineers is to identify the controlling genes and replicate C₄ evolution in a greatly accelerated manner. While the genetic pieces that can be co-opted for C₄ photosynthesis may be present in C₃ genomes, identifying all of them in a reasonable time frame

may be impossible. For this reason, attempts to engineer C₄ plants have exploited existing genes in C₄ species by transforming them into C₃ species.

VII. Early Attempts at Transferring C₄-Traits into C₃ Plants

A list of studies in which plants have been transformed in an attempt to increase photosynthetic rates is provided in Table 1. In most cases the introduced genes have been isolated from C₄ plants, although there are reports that bacterial isoforms that lack amino acid residues that are involved in the regulation of activity (e.g., bacterial PEPC) have also been introduced into plants. Most of the studies attempted to increase the expression of enzymes central to C₄ photosynthesis, especially PEPC, PPDK, NADP-ME and PEPCK. However, not all attempts to increase photosynthetic activity have involved C₄ enzymes and results of these studies are discussed below.

Early attempts to express enzymes of the C₄ photosynthetic pathway in C₃ plants involved either introducing cDNA or the gene for PEPC from various sources into tobacco (Hudspeth et al., 1992; Kogami et al., 1994), potato (Gehlen et al., 1996), and rice (Ku et al., 1999, Fukayama et al., 2001). The gene for PPDK has also been introduced into Arabidopsis (Ishimaru et al., 1997). Although the transgenic plants exhibited altered photosynthetic characteristics, there was no increase in the photosynthetic rates of transgenic plants from these studies. Rice plants transformed with a maize gene for C₄-specific PEPC, and which expressed PEPC in their leaves at a very high level, were less sensitive to inhibition of photosynthesis by increased O₂ (Ku et al., 1999). Claims by Ku et al. (1999) that photosynthetic rates were increased in transgenic rice plants exhibiting elevated levels of PEPC activity have not been independently confirmed (Fukayama et al., 2001). Ku et al. (1999) failed to demonstrate changes in CO₂ compensation points, which is an important criterion for demonstrating CO₂ concentration around Rubisco. Transgenic potato, transformed with *Corynebacterium glutamicum* PEPC had a lowered CO₂ compensation point (Häusler et al., 1999). Transgenic potato expressing both *C. glutamicum* PEPC in the cytosol and *Flaveria pringlei* (a C₃ species) NADP-malic

Table 1. Summary of attempts to increase photosynthetic rates in C3 plants.

Plant species	Attempted changes	Effect on plants	Effect on CO ₂ compensation point	Reference
Rice	PEPC (maize)	No effect	Not reported	Ku et al., 1999; Agarie et al., 2000 Jiao et al., 2002
Rice	PEPCK (<i>Urochloa panicoides</i>)	No effect	Unaffected	Suzuki et al., 2000
Rice	PEPC and PEPCK	C ₄ pathway detected Swollen thylakoid membranes and low chlorophyll levels	Not reported	Suzuki et al., 2006
Rice	PPDK	No effect	Not determined	Fukayama et al., 2001
Rice	NADP-ME	Photoinhibition and photo-damage detected	Not determined	Takeuchi et al., 2000 Tsuchida et al., 2001
Rice	Cyanobacterial <i>ic1B</i>	Higher photosynthetic rates. Increased plant productivity	Lower CO ₂ compensation points	M.S.B. Ku, unpublished results
Rice	Maize PEPCK and PPDK, rice NADP-ME and sorghum NADP- MDH	Stunting of rice Day/night expression of proteins	Not significantly altered	Taniguchi et al., 2008
Potato	PEPC (bacterial)	No effect	Unchanged	Gehlen et al., 1996
Potato and tobacco	PEPC (Corynebacterium) NADP- ME (<i>Flaveria pringlei</i>)	No effect	Decreased	Häusler et al., 2001
Tobacco	PEPC (maize)	No effect on CO ₂ assimilation	CO ₂ compensation point unaffected	Hudspeth et al., 1992; Kogami et al., 1994
Tobacco	NADP-ME (<i>Flaveria pringlei</i>)	No effect on photosynthetic per- formance	No reported	Lipka et al., 1999
Tobacco	<i>E. coli</i> genes in trehalose metabo- lism	Increased photosynthetic capacity; relative growth rates unaltered	Not determined	Pellny et al., 2004

enzyme in the chloroplasts had a reduced requirement for electrons for CO₂ assimilation in high light and high temperature (Lipka et al., 1999). Initial experiments in which a cDNA encoding PEPC of *Urochloa panicoides* (a C₄ monocot) was expressed in rice plants under the control of the maize promoter for PEPC or PPDK resulted in high chloroplastic PEPC activity (Suzuki et al., 2006). ¹⁴C-labelling experiments showed that expressing PEPC in the chloroplast resulted in a C₄-like carbon flow in which PEPC-catalyzed CO₂ released in chloroplasts was rapidly incorporated into photosynthetic intermediates (Suzuki et al., 2000). Transgenic rice simultaneously expressing both maize PEPC and *U. panicoides* PEPC exhibited a C₄-like carbon flow but was accompanied by a 10% decrease in the photosynthetic rates (Suzuki et al., 2006).

To date all attempts to construct C₄ rice plants have not addressed the fact that enzymes involved in photosynthesis are controlled by specific regulatory mechanisms. Both PEPC and PPDK are regulated by very different phosphorylation/dephosphorylation mechanisms. In sunlight, PEPC is phosphorylated, rendering the enzyme less sensitive to inhibition by malate and aspartate and more sensitive to stimulation by glycine (see review by Izui et al., 2004). Expression of PEPC in rice leaves without the co-expression of the PEPC kinase and PEPC phosphatase may produce transgenic rice plants containing elevated levels of PEPC that cannot catalyze PEP carboxylation at the full potential due to incomplete activation. Similarly, PPDK is regulated by a phosphorylation/dephosphorylation mechanism in response to adenylate energy charge. Under low adenylate energy charge at low light, PPDK is phosphorylated and inactive. The enzyme is activated under high adenylate energy charge when the enzyme is dephosphorylated (Chastain and Chollet, 2003). Expressing PPDK in the chloroplasts of rice leaves without the concomitant expression of PPDK regulatory protein may lead to unregulated hydrolysis of ATP. The same may be true for PEP carboxylase.

Mitchell and Sheehy (2007) liken the introduction of C₄ cycle enzymes into rice plants to the addition of a super charger to a car engine. Given this analogy, one could argue that unless the mechanisms that regulate the introduced enzymes (which are analogous to regulating

the supply of fuel to an engine) are also present in rice in an attempt to increase photosynthetic rates, the redox state and/or the adenylate charge of chloroplasts may become imbalanced with deleterious results to plant growth. The stunting of plants expressing transgenic NADP-ME (Tsuchida et al., 2001) appears to support this warning. Similarly, Taniguchi et al. (2008) overproduced PEPC and PPDK from maize, NADP-ME from rice and NADP-MDH from sorghum in rice. They reported that both the introduced PPDK and MDH were only active during the day while PEPC and ME were active during both the day and night. PEPC-over-producing rice plants were stunted, and they suggested this stunting might be remedied by decreasing the level of PEPC protein. Transgenic rice studies have revealed that higher levels of gene expression are achieved when rice plants are transformed with constructs containing gene sequences as opposed to cDNA sequences indicating that the intron sequences may be important in regulating levels of expression (Fukayama et al., 2001; Matsuoka et al., 2000).

In summary, to date, there has been limited success in increasing the photosynthetic rates of C₃ plants following the introduction of genes encoding enzymes of the C₄ photosynthetic pathway. Transgenic studies have also demonstrated that there is a diverse array of mechanisms that regulate the expression of genes encoding for C₄ enzymes in monocots and dicots alike (Suzuki et al., 2000, 2006; Suzuki and Burnell, 2003; Takeuchi et al., 2000, Taniguchi et al., 2008; Taylor et al., 1997). A key challenge for C₄ engineers in the future will be to identify the regulatory controls and develop the means to simultaneously introduce them into the target species with the genes encoding the enzymes they regulate.

In hindsight a blueprint for another attempt at introducing a C₄ photosynthetic pathway into rice is provided in Fig. 3. The major differences between Figs. 1 and 3 include the introduction of oxaloacetate and PEP transporters into the chloroplast membrane, replacement of PPDK by a bacterial PEP synthetase (PEPS, see below) and a mechanism to decrease expression of endogenous chloroplastic carbonic anhydrase. The operation of the pathway may also be assisted by the introduction of regulatory proteins (for PEPC and PEPS).

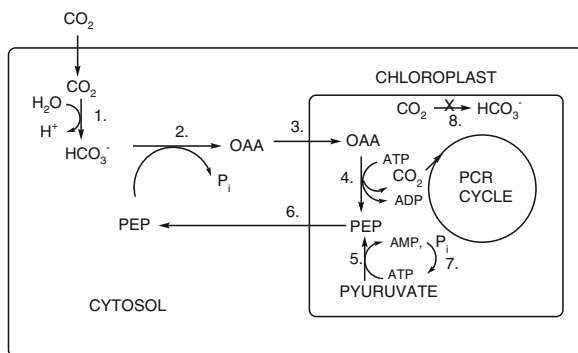


Fig. 3. Updated single celled C₄ photosynthesis. This diagram summarizes the modifications that could be introduced into rice to increase the photosynthetic rates. 1 – express carbonic anhydrase activity in the cytosol to ensure maximum rates of conversion of CO₂ to bicarbonate; 2 – express PEP carboxylase in the cytosol to maximize rates of OAA synthesis; 3 – express an effective oxaloacetate transporter in the chloroplast membrane to maximize OAA transport rates into the chloroplast; 4 – express a PEPCK in the chloroplast stroma to catalyze the release of CO₂ from OAA; 5 – express a PEP synthetase in the chloroplast stroma to ensure PEP synthesis is non-limiting; 6 – express a PEP transporter in the chloroplast membrane to ensure PEP export from the chloroplast; 7 – express an adenylate kinase in the chloroplast stroma to recycle AMP produced by PEP synthetase; 8 – down regulate the expression of endogenous chloroplastic carbonic anhydrase. There may also be a need to introduce enzymes that are involved in the regulation of PEP carboxylase and PEP synthetase.

VIII. Alternate Approaches to Improving Photosynthetic Rates

The introduction of a C₄ photosynthetic pathway into rice as a means of increasing photosynthetic rates is designed to decrease the endogenous rates of photorespiration. It may be possible to decrease endogenous rates of photorespiration by alternate means and these will be discussed in the following section.

A. Recycling Photorespiratory Products

An alternate biochemical mechanism to increase photosynthetic rates by decreasing the rate of photorespiration in C₃ plants in a single cell model has recently been reported (Kebeish et al., 2007). This has been achieved by introducing the glycolate catabolic pathway from *Escherichia coli* into the chloroplasts of *Arabidopsis thaliana*. Bacterial genes encoding glycolate dehydrogenase, glyoxylate carboligase and tartronic

semialdehyde reductase were introduced into *A. thaliana* to recycle glycolate formed during photorespiration to glycerate in the chloroplast. This minimizes the flux of photorespiratory intermediates through peroxisomes and mitochondria, and releases the photorespired CO₂ in the vicinity of Rubisco in the chloroplast. Transgenic plants grew faster and produced greater biomass compared to control plants (almost 70% more shoot tissue and 230% increase in root tissue on a dry weight basis. On a leaf area basis, transgenic plants produced more than 20% more sugar (glucose + fructose + sucrose) compared to wild type plants (Kebeish et al., 2007). So rather than attempting to increase photosynthetic rates by concentrating CO₂ around Rubisco, Kebeish et al (2007) engineered plants to recycle the glycolate within the chloroplasts via a pathway that was energetically more efficient than the generally recognized photorespiratory pathway. This variant of the photorespiratory pathway eliminated the release of ammonia, conserved energy by producing reducing equivalents and eliminated the need to convert pyruvate to PEP (which requires ATP). Unfortunately this engineered pathway has its limitations as the recycling of glycolate involves the loss of a carbon atom during the synthesis of one glycerate from two molecules of glycolate and this requires ATP to reform PGA. Because the CO₂ is released in the chloroplast and not another compartment, there is greater chance it would be refixed by Rubisco before escaping from the leaf. Hence chloroplast CO₂ levels are enhanced, but not to levels greater than found in the intercellular air spaces.

B. Introduction of an Alternate Carbon Concentrating Mechanism

The cyanobacteria, a group of photosynthetic aquatic bacteria, concentrate CO₂ around Rubisco. Bicarbonate is first pumped into the cell using an ATPase-linked cotransporter. CO₂ from the bicarbonate is then released within a compartment (the carboxysome) where Rubisco is localized using carboxysomal CA (Price et al., 1992). Four inorganic carbon transporters have been identified in cyanobacteria and reported to be involved in carbon concentrating mechanisms; two NADPH-coupled CO₂ transporters and two Na⁺-dependent bicarbonate transporters. The bicarbonate transporter, BicA, is

highly represented in marine cyanobacteria (Price et al., 2004) while SbtA is less widely distributed (Shibata et al., 2002). Both BicA and SbtA require about 1 mM Na⁺ for half maximal activity and the leaf cytosol possesses between 1 and 3 mM Na⁺ (Karley et al., 2000), indicating transformed BicA and SbtA may produce functional proteins in higher plants cells. Both BicA and SbtA are ideal candidates for expression in plants as they are both single subunit transporters and therefore easier to express than the CO₂ transporters which are multi-subunit proteins. This may not be necessary however, as higher plants may possess an inwardly directed Na⁺ gradient that would be capable of driving a Na⁺-dependent transport of bicarbonate into the chloroplasts. A proteomic analysis of the chloroplast envelope of *Arabidopsis* revealed the presence of several Na⁺-coupled transporters and Na⁺/H⁺ antiporters that are homologous to cyanobacterial forms. The challenge will be to upregulate the expression of these transport proteins and assemble them in a functional configuration with Rubisco and CA. Very recently Ku et al. (2007) reported enhanced photosynthetic capacity and greater growth and grain yield in rice plants possessing a constitutively expressed high affinity bicarbonate transporter gene, *ictB*, from a cyanobacterial species. They reported that transformed rice plants exhibited elevated photosynthetic rates (10–30% higher), higher carboxylation rates (15–20% higher) and lower photosynthetic CO₂ compensation points.

Currently, no terrestrial plant is known to naturally express a bicarbonate-based CO₂ concentrating mechanism, although they have been reported in aquatic plants (Raven et al., 1992). This implies there is an inherent barrier preventing the successful function of a bicarbonate/CO₂ concentrating system in land plants, but at present, it is not known. There is no theoretical reason why the concentration of inorganic carbon in the stroma could not be significantly increased if an active form of a bicarbonate transporter could be successfully inserted into the chloroplast membrane. If the insertion of an efficient carbon concentrating mechanism into the chloroplast membrane of rice plants were possible as indicated by the results of Ku et al. (2007), it would be interesting to test whether Rubisco levels could be manipulated downwards to reduce nitrogen requirements

and increase nitrogen use efficiency in the transformed plants. This may be relevant to any plant species in which an efficient carbon concentrating mechanism was introduced.

IX. Hurdles to Engineering C₄ Crops

The existence of plants exhibiting C₃, C₃–C₄ intermediate, C₄-like and C₄ photosynthesis in a single genus (e.g. *Flaveria*) indicates that the evolution of the C₄ photosynthesis phenotype is a long process (Edwards and Ku, 1987). The conversion from a C₃ to a C₄ photosynthetic plant involves a large number of modifications including genetic, structural, and anatomical and biochemical changes. Clearly, in trying to design and construct a C₄ rice plant, we do not have the luxury of introducing and studying the effect of changes to single characteristics in a model organism. Therefore the greatest challenge to constructing a C₄ rice plant lies in the fact that we do not possess a suitable genetic model like *Arabidopsis* that we could mutate or transform to study the effect of various genetic modifications. In addition we still lack even a basic understanding of the regulatory processes underpinning gene expression of many complex traits (e.g. development of bundle sheath cells).

In addition, there are still some serious gaps in our understanding of basic biochemistry in C₄ plants and I provide two specific examples of where we lack a basic understanding of the biochemistry of C₄ plants.

In relation to our understanding of photosynthesis and, specifically C₄ photosynthesis, one of the greatest gaps in our knowledge as plant biochemists is an understanding of the role and importance of carbonic anhydrase in plants. Although CA was first isolated from chloroplasts in 1938 (Neish, 1939), was first cloned in spinach in 1990 (Burnell et al., 1990; Fawcett et al., 1990) and subsequently cloned in a range of C₄ species (see Burnell, 2000), the roles of carbonic anhydrase isozymes are not completely understood. In recent studies three CA isozymes have been identified in a variety of C₄ species including maize, sugarcane and sorghum, all of which are NADP-ME type C₄ plants and in the dicot, *Flaveria bidentis*. To date the inter- and intracellular localization of monocot C₄ CA has defied

characterization. However, the location and suggested functions of the three CA isozymes in *F. bidentis* have recently been reported; the most abundant isozyme is involved in photosynthesis, another is involved in anaplerotic reactions and the third is involved in lipid metabolism (Tetu et al., 2007). Many C_3 plants have two CA isozymes with a major isozyme localized within the chloroplast stroma and is assumed to play a role in maximizing the rate of diffusion of inorganic carbon into the chloroplast. There has been little or no consideration given to altering the endogenous levels and location of CA in transgenic C_3 plants and the effect of high CA activity in the mesophyll chloroplasts and low levels in the mesophyll cytosol on an introduced C_4 pathway are unknown.

Most studies to improve photosynthetic rates have involved the use of the NADP-ME C_4 pathway and this has required the concomitant expression of PPDK to recycle the pyruvate released during malate decarboxylation, to PEP, the substrate for the initial carboxylation reaction. However, PPDK may not represent the most efficient PEP recycling enzyme. In C_4 plants the conversion of pyruvate to PEP is facilitated by the concerted activity of three enzymes, PPDK, adenylate kinase and inorganic pyrophosphatase; both adenylate kinase and inorganic pyrophosphatase catalyze the removal of inhibitory end-products of the PPDK-catalyzed reaction to ensure the conversion of pyruvate to PEP at rates that can support C_4 photosynthesis. In addition PPDK activity is tightly regulated by the surrounding adenylate energy charge (Edwards et al., 1985). The recent cloning of the maize PPDK regulatory protein (PDRP) led to the identification of PDRP homologs in many bacterial species (Burnell and Chastain, 2006). In some bacterial species the PDRP homolog (DUF299) catalyzes the regulation of PEP synthetase (PEPS), an enzyme that catalyzes the conversion of pyruvate to PEP at a significantly greater rate compared to plant PPDK. Furthermore, the bacterial PEPS is less cold sensitive compared to PPDK and, since it does not synthesize PP_i , does not require pyrophosphatase activity to remove an inhibitory end product. Therefore there may be biochemical and physiological advantages to introducing bacterial PEPS in place of plant PPDK in future attempts to introduce C_4 photosynthesis into C_3 plants. Recent experiments have shown that the endogenous

plant PDRP is not capable of catalysing either the inactivation or the activation of *E. coli* PEP synthetase (Burnell, 2010). Further studies have demonstrated that *E. coli* DUF299 is incapable of catalysing either the inactivation or the activation of plant PPDK indicating that transformation of plants with *E. coli* PEP synthetase and DUF299 would not directly interfere with the activity of endogenous PPDK.

X. Assessment of C_4 -ness

There have been a number of claims relating to increasing photosynthetic rates in plants following transformation with genes encoding enzymes associated with C_4 photosynthesis (Ku et al., 2007; Jiao, 2007); none of these claims have been independently assessed. At the 2006 meeting at Los Baños, Philippines there was some discussion regarding independent testing of plants claimed to be C_4 . The following recommendations were made.

1. Any claims regarding the construction of a C_4 rice plant should be tested in an independent laboratory or tested by a qualified independent researcher in the labs of the discovering scientist.
2. The CO_2 compensation points should be determined.
3. Labeling experiments should be conducted to confirm the biochemical pathway of the transgenic plants.

XI. Conclusions

The existence of plants that perform single-cell C_4 photosynthesis is evidence that Kranz anatomy is not a prerequisite for C_4 photosynthesis. However, biochemical and immunological examination of the location of enzymes associated with C_4 photosynthesis in plants that conduct single-cell C_4 photosynthesis provides overwhelming evidence that there is spatial separation of the C_3 and the C_4 components of the photosynthetic mechanism. This separation is probably related to the liquid-phase diffusive resistance to CO_2 that is required for the localized concentration of CO_2 in close proximity to Rubisco. Therefore it is highly likely that the diffusive resistance of CO_2 from the site of CO_2 enrichment will be critical to the successful conversion of C_3 plants to C_4 plants and

this probably represents the most difficult aspect of converting a C₃ plant into a C₄ plant unless a two-cell mechanism is adopted.

Earlier in the chapter I raised a number of questions relating to the conversion of rice to a C₄ plant; however it is probably wise to ask a final question – should we try to convert rice to a C₄ plant? In answering this question I draw the readers' attention to a recent letter to *Nature Biotechnology* that questioned the cloning of a transgenic pig rich in omega-3 fatty acids (Feister, 2006); justification for the project was provided with the statement “because we can”. In the case of converting C₃ plants to C₄ plants, and more importantly developing a C₄ rice plant we certainly have a significant way to go before we can make the claim that we should do it because we can. Clearly, there are strong moral and economic grounds to develop the ability to engineer C₄ photosynthesis into major food crops. Improving agricultural productivity has historically had huge benefits in terms of promoting food security, social stability, personal happiness and economic development (Dawe, 2007). Engineering C₄ photosynthesis into C₃ plants, however, is a far greater genetic modification of existing species than anything yet attempted by humanity. For plant biology, it would be the equivalent of the Manhattan project to build the atomic bomb, requiring great effort by many labs, large research outlays, and strong coordinating leadership. As well, a C₄ rice project will engender great controversy and raise many alarms from ecologists and social scientists. In addition to the scientific challenges that will require unprecedented cooperation to answer the question of *can we* engineer C₄ rice, there will have to be the parallel efforts to address the ethical, ecological and legal dimensions of the C₄ engineering challenge in order to answer *should we*. If the problem is restricted to the scientific basis alone, leaving the larger dimensions unaddressed, the probability that society would terminate the project would be high. Alternatively, if the science achieved the goal of C₄ rice and the larger dimensions were not addressed, the C₄ engineers could release the products upon an unprepared world. As with other great engineering feats, the sudden arrival of a new technology has radical consequences, for better or worse. Because C₄ rice may be the last, best means for dramatically increasing plant productivity at least for food production, it would be a shame if the

benefits of the end result could not be realized, or were misappropriated, because we failed to also address whether we should while addressing whether we can engineer C₄ rice.

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Patents Related to C_4 Rice

- Arai M, Suzuki S, Murai N, Yamada S, Ohta S and Burnell J (2003) Rice plants transformed to provide a PCK-type C_4 cycle and methods of making. United States Patent 6,610,913
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Chapter 19

C₄ Species as Energy Crops

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Summary	379
I. Introduction	380
II. What Are the Qualities of an 'Ideal' Energy Crop?	381
A. Light Use Efficiency	381
B. Water Use Efficiency	383
C. Nitrogen Use Efficiency	383
III. C ₄ Species as Energy Crops in Cool-Temperate Climates	383
IV. Examples of C ₄ Species as Biofuel Feedstock	385
V. Prospects for Energy Crop Improvement	388
VI. The Environmental Debate and Bioenergy Crops	389
VII. Economic and Energetic Costs and Benefits	391
VIII. Conclusions and Perspectives	392
References	392

Summary

The cultivation and utilisation of energy crops has the potential to provide, in the coming decades, part of the solution to the twin issues of substituting for fossil fuels and protection from damaging climate change by reducing carbon emissions. The ideal energy crop should have sustained capacity to capture and convert solar energy into harvestable biomass with maximal efficiency and with minimal inputs and environmental impacts. C₄ plants, and in particular rhizomatous perennial grasses (PRGs), have many of the characteristics of the 'ideal' energy crop. Herbaceous perennial species require far fewer energy and capital inputs than annual crops and they also sequester more carbon in the soil. C₄ photosynthesis also allows greater efficiencies in the conversion of solar energy to biomass energy, and of nitrogen and water use. Currently the most important feedstocks for biofuels are maize in the USA and sugarcane in Brazil, both C₄ species. In temperate climatic regions, where there is the greatest current demand for renewable energy, few naturally occurring species have C₄ photosynthesis. However, there are some notable exceptions, such as *Miscanthus* and switchgrass (*Panicum virgatum*), which show significant cold tolerance and are currently being developed as energy crops. The unusual features of the C₄ pathway in these species which appear to confer cold tolerance are reviewed. The recent drive to exploit the energy production and carbon emission mitigation potentials of C₄ energy crops has been controversial because of the anticipated competition for use of land for food or fuel. Despite this, the yield benefits provided by C₄ photosynthesis suggest that these species will make a significant contribution to bioenergy production over the near- and longer-terms.

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I. Introduction

Now that the world is arguably approaching the time of 'peak oil', when the maximum rate of global petroleum extraction is reached and followed by a terminal decline, human survival will depend on a transition from non-renewable carbon resources to renewable bioresources (U.S. DOE, 2006, Royal Society, 2008). It was the energy crisis of the 1970s that first stimulated the modern interest in the synthesis of fuels and materials from bioresources but the interest soon waned as oil prices declined. More recently the recognition of a rapidly increasing demand for energy, particularly in developing countries, coupled with the realisation that there are finite supplies of fossil fuel resources has led to a renewed interest in identifying and utilising renewable bioresources (Koonin, 2006; Smith et al., 2007). Concurrently, the concern about global warming has also stimulated interest in using biomass for energy because it is theoretically close to 'carbon neutral'. This means that these crops produce energy while only releasing carbon to the atmosphere that has recently been captured by plants, rather than emitting carbon that has been locked away from the atmosphere in geological deposits (Sims et al., 2006). While energy security has been the main driving force for recent interest in biofuels, in the longer term it is likely to be the mitigation of climate change.

Energy crops can take many different forms and can be utilised in a variety of ways from simple combustion (Tillman, 2000; Styles and Jones, 2008) to complex bioconversion processes (Ragauskas et al., 2006), although there is a strong case that it is in the context of transportation fuels (volatile liquid fuels) that energy crops have the biggest opportunity to make an impact (Somerville, 2007; Gomez et al., 2008; Heaton et al., 2008b). Although the use of plant biomass as a source

of energy was central to the early development of civilization the fact that the per capita energy demand has increased exponentially as societies have developed means that most nations are now largely dependent on fossil fuels. It is estimated that bioenergy currently contributes 12.9% (IEA 2007) of the global primary energy use but this is largely in the developing world where wood and dung are used in rural areas for heating and cooking. Energy crops, at present, contribute relatively little to the overall energy supply from biomass. However, this is set to grow substantially in the next few decades in order to meet recent national and international commitments such as the European Union 2008 Directive on Renewable Energy that specifies a 20% share of energy from renewable sources by 2020 in all member states, and a 10% share of renewable energy specifically in the transport sector (Gibbs et al., 2008).

In order to meet these demands it is inevitable that biomass for energy will compete for land with food production and consequently it will be essential to maximise biomass production per unit of land area (Heaton et al., 2008a). It will also be essential to produce energy biomass with minimum resource inputs, in other words the 'resource use efficiency' of production must be maximised. At present, most of the so-called 'first generation' feedstocks for liquid fuel production are produced from two crops that have been used for food; these are sugarcane (*Saccharum officinarum*) and maize (corn) (*Zea mays*), both of which are C_4 species. The two main producers of liquid biofuels today are Brazil (20 billion litres per year) and the United States (24 billion litres per year) who produce bioethanol from, respectively, the fermentation of sugars extracted from sugarcane or derived from the hydrolysis of starch in maize.

It is anticipated that the next (second) generation of feedstock for ethanol production will utilize, in addition to sugars and starch, the cellulose and hemicelluloses from perennial grasses, wood chips and agricultural residues (Milliken et al., 2007). However this depends on the development of processes which can extract fermentable sugars from the cellulose and hemicelluloses (Hamelinck et al., 2005; Himmel et al., 2007) or produce other products such as dimethyl furan for liquid fuels (Roman-Leshkov et al., 2007; Somerville, 2007).

Abbreviations: LCA – Life cycle analysis; LIHD, – Low input high diversity; NADP ME – Nicotinamide adenine dinucleotide-malic enzyme; NEB – Net energy balance; NUE – Nitrogen use efficiency; PEP-CK – Phosphoenolpyruvate carboxykinase; PNUE – Photosynthetic nitrogen use efficiency; PPDK – Pyruvate orthophosphate dikinase; PRG – Perennial rhizomatous grasses; RUE – Radiation use efficiency; SOC – Soil organic carbon; WUE – Water use efficiency;

The grand challenge for biomass production is to develop the second generation energy crops which have a suite of desirable physical and chemical traits to aid bioethanol production while maximising biomass yields (Vermerris, 2008). Achieving this will depend on identifying the fundamental constraints on productivity and addressing these constraints with modern genomic tools. It is now widely recognised that perennial rhizomatous grasses (PRGs) such as sugarcane, switchgrass (*Panicum virgatum*), reed canary grass (*Phalaris arundinacea*), *Arundo donax* and *Miscanthus* spp. are a plant life form which is particularly well-suited to maximising outputs in terms of biomass yield while minimising inputs in terms of resources, and it is likely that these will become dedicated bioenergy crops in the relatively near future (Venendaal et al., 1997; Lewandowski et al., 2003b; El Bassam, 2008). The most productive of the PRGs have C₄ photosynthesis and it will be these that are the main topic of this chapter. Currently, the potential second generation bioenergy crops are largely undomesticated and have not been subject to centuries of improvement as have our major food crops. Breeding of appropriate species and genotypes to suit specific climates and soil conditions will be required. Currently, we are in the preliminary stages of breeding programmes for the leading candidate crops and are unlikely to see significant productivity gains in the immediate future (Vermerris, 2008).

II. What Are the Qualities of an 'Ideal' Energy Crop?

The three distinct goals associated with development of biofuel feedstocks are: maximising the total amount of biomass produced per hectare per year, maintaining sustainability while minimising inputs, and maximising the amount of fuel that can be produced per unit of biomass. The precise values of these parameters will depend on the energy crop and the growing conditions. Fundamentally, bioenergy crop production should emphasize an optimal balance between input costs and yield, rather than simply maximizing yield. Research directed towards these goals will likely require the development of systems-level

predictive models that will integrate knowledge of molecular and genetic controls with physiological understanding and field crop management. The foundation of this approach will be to use the high-throughput tools of genomics, metabolomics and phenomics to rapidly develop the understanding needed to create novel, second generation bioenergy crops. It will be important for bioengineers using high-throughput techniques to work closely with agronomists and breeders, however, if the novel genotypes are to be optimized for the multitude of field conditions around the world.

Plants can be viewed as a set of structures and mechanisms for 'capturing' resources from the environment (Press et al., 1999). An ideal energy crop should have a sustained capacity to capture and convert the available solar energy into harvestable biomass with maximal efficiency and with minimal inputs and environmental impacts; in other words a high resource use efficiency. These characteristics are largely a consequence of the photosynthetic pathway and maximising the efficiency of light, nutrient and water use (Long, 1999; Long and Beale 2001; Heaton et al., 2004a). C₄ photosynthesis is the most efficient form of photosynthesis in warm to hot terrestrial environments where high growth rates of crops can be supported by the warm conditions. The superior efficiency of C₄ photosynthesis is typically expressed in terms of use of the major resources that plant require, that is, light, water and nutrients use efficiency.

A. Light Use Efficiency

The ultimate limit on biomass yield is determined by the amount of available light, its efficiency of interception by the plants, and the efficiency with which intercepted light is converted into biomass (Heaton et al., 2008b). C₄ plants substantially reduce the energetically wasteful process of photorespiration, but at the cost of more energy being required for each molecule of CO₂ that is assimilated. As a consequence, the maximum efficiencies with which plants convert light energy, using the existing pathways of energy transduction into stored carbohydrate, are 6.0% and 4.6% for C₄ and C₃ plants, respectively (Zhu et al., 2008; Heaton et al., 2008b) (Fig. 1).

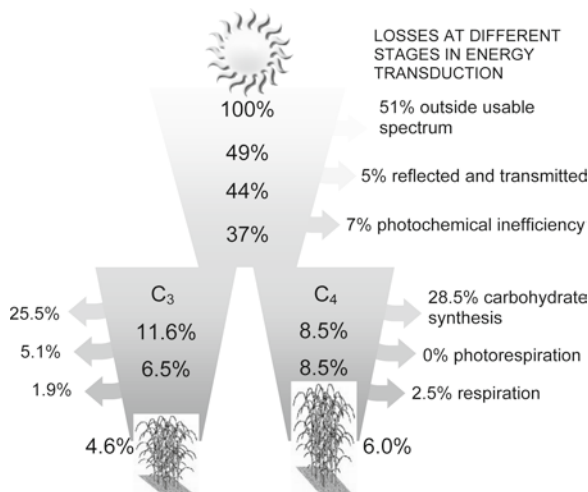


Fig. 1. A theoretical analysis of the maximum efficiency of conversion of incident solar energy into biomass energy (Adapted from Heaton et al., 2008b).

The potential yield of an energy crop can be estimated using an equation based on the principles developed by Monteith (1977):

$$W_h = S_i \cdot \epsilon_i \cdot \epsilon_c \cdot \eta / k$$

where W_h is the dry matter at final harvest (g m^{-2}), S_i is the incident solar radiation (MJ m^{-2}), ϵ_i is the efficiency with which the radiation is intercepted by the crop (dimensionless), ϵ_c is the efficiency with which the intercepted radiation is converted to biomass energy (dimensionless), η is the amount partitioned into the harvested components (dimensionless) and k is the energy content of the biomass (MJ g^{-1}). The quotient of dry matter yield to accumulated intercepted radiation is often referred to as the radiation use efficiency (RUE, g MJ^{-1}) (Kiniry et al., 1999; Vargas et al., 2002; Jorgensen et al., 2003). While S_i is dependent on the location and k varies little between species (Beale and Long 1995) the final dry matter produced at harvest depends primarily on ϵ_i and ϵ_c . Interception efficiency (ϵ_i) depends on the duration, size and architecture of the canopy. A crop that can maintain a closed canopy of viable leaves throughout the year, or at least through the period of maximum insolation, will have the highest efficiency of interception (Monteith, 1977; Kiniry et al., 1999; U.S. DOE, 2006). In temperate regions the main factor determining this will be the ability to develop leaves rapidly at

the start of the growing season (Clifton-Brown and Jones, 1997; Farrell et al., 2006). Monteith (1977) was the first to show that for healthy crops, ϵ_c varies relatively little within each photosynthetic group (C_3 or C_4) so that the potential dry matter productivity of a biomass crop, at a given site, will be determined primarily by the ability to form and maintain a closed canopy and by the photosynthetic type.

By contrast to the situation within a photosynthetic pathway, ϵ_c differs significantly between photosynthetic pathways, and these differences explain, in part, the superior performance of C_4 plants in warmer environments. Long et al. (2006) and Zhu et al. (2008) calculated a theoretical maximum ϵ_c of 0.051 in C_3 plants and 0.060 in C_4 plants at a leaf temperature of 25°C while the maximum measured ϵ_c for C_3 crops over a growing season is around 0.024, and 0.034 for C_4 crops (Monteith, 1977). The highest short-term efficiencies are 0.035 for C_3 plants and 0.043 for C_4 plants, about 70% of the theoretical maxima (Piedade et al., 1991; Beale and Long, 1995). The difference in ϵ_c between C_3 and C_4 plants increases with temperature because of the increase in photorespiration as a proportion of photosynthesis so that the advantage is most pronounced in the tropics (Long et al., 2006). As a result the highest recorded plant productivities are found in a C_4 perennial grass, *Echinochloa polystachia*, growing in flooded conditions in central Amazon at 100 t (dry matter) $\text{ha}^{-1} \text{year}^{-1}$ (Piedade et al., 1991; Long, 1999; Morison et al., 2000) and a C_4 perennial giant sedge, *Cyperus papyrus*, in east African swamps at 51.5 t (dry matter) $\text{ha}^{-1} \text{year}^{-1}$ (Muthuri et al., 1989; Jones and Muthuri, 1997). However, Long (1999) and Long et al. (2006) using models that combine leaf photosynthesis and canopy radiation distribution show that, while the advantage of C_4 photosynthesis diminishes with temperature, there is still a C_4 advantage for canopy photosynthesis even as low as 5°C. Consequently, even in temperate climates some advantage is gained from C_4 photosynthesis and this is supported by the observation that the highest dry matter production in NW Europe has been measured for the cold-adapted C_4 perennial grass *Miscanthus × giganteus* that has produced as much as 29 t (dry matter) $\text{ha}^{-1} \text{year}^{-1}$ and has a measured ϵ_c of 0.039 (Beale and Long, 1995; Beale et al., 1999).

B. Water Use Efficiency

To maximise productivity during the growing season, adequate supplies of water are required to maintain optimal rates of photosynthesis and to maintain green leaf area to maximise the efficiency of light interception. At the leaf level, C₄ species have, in theory, a higher water use efficiency than C₃ species (Long, 1999), which is largely explained by the fact that C₄ leaves have typically a 30–40% lower leaf conductance while maintaining a higher photosynthetic rate than C₃ leaves. These differences appear to be maintained at the whole plant level when WUE is expressed as a ratio of dry weight gained per unit of water transpired (Downes, 1969; Begg and Turner, 1976; Long, 1999) and it is now well established that the WUE of C₄ species is generally twice that of C₃ species, although at lower temperatures this difference is much smaller due to the reduced humidity gradient which drives transpiration (Downes, 1969). However, when Beale et al. (1999) determined the WUE of *Miscanthus × giganteus* and another C₄ perennial, *Spartina cynosuroides*, in the temperate climate of the UK, they found that when normalized by the daily maximum vapour pressure deficit the values for both were comparable with typical values for C₄ crops in a range of environments. Functionally, the advantage of increased WUE is most important in conserving soil moisture and extending the period of maximum photosynthetic activity of the canopy. However there is some evidence that under severe drought an increase in leakage of CO₂ from the bundle sheath in C₄ plants could lower their WUE and reduce their tolerance of drought (Buchmann et al., 1996).

C. Nitrogen Use Efficiency

Two terms are used to describe productivity per unit of nitrogen resource. While photosynthetic nitrogen use efficiency (PNUE) is the net rate of leaf CO₂ uptake in full sunlight per unit leaf nitrogen content (Sage et al., 1987), nitrogen use efficiency (NUE) is the ratio of increase in plant biomass to increase in plant nitrogen over the growing season (Hirel et al., 2007). In energy crops the latter approximates to the ratio of biomass to nitrogen at the end of the growing season. In terms of field crops, NUE is determined at three levels in perennial species. First, NUE is enhanced

by increasing the amount of biomass produced per unit of nitrogen invested into the photosynthetic apparatus. Second, NUE can be enhanced by increasing the fraction of soil nutrients that are assimilated by the plant (Lewandowski and Schmidt, 2006). Third, in perennial species, NUE is enhanced by increasing the fraction of nitrogen translocated out of the leaf canopy and stems during senescence; the translocated N can then be stored in the rhizomes for use in the following year (Beale and Long, 1997). Efficient recovery of N during senescence increases the efficiency of internal recycling of nutrients. The combined effect of these properties is to both minimise the quantities of nitrogen that need to be applied as fertiliser and the amount lost to drainage water (Christian and Riche, 1998; U.S. DOE, 2006).

Because C₄ species concentrate CO₂ at the site of Rubisco, the theoretical requirement for nitrogen in photosynthesis is less than in C₃ species (Long, 1999; Ghannoum et al., Chapter 8, this volume). At the estimated concentration of CO₂ at Rubisco in C₄ plants, Long (1991, 1999) has shown that a C₄ leaf would require, at 30°C, between 13.4% and 19.8% of the Rubisco in a C₃ leaf to achieve the same rate of light saturated photosynthesis. The benefit of a lower requirement for Rubisco in C₄ leaves is, however, partially offset by the N requirement for the enzymes of C₄ metabolic cycle, primarily PEP carboxylase and PPDK (Sage et al., 1987). At the whole plant level the difference in leaf nitrogen concentration between C₃ and C₄ plants combined with the higher leaf photosynthetic rate of C₄ species results in a PNUE that is approximately twice as high in C₄ compared to C₃ plants (Brown, 1978). In perennial crops this leads to a more than doubling of NUE in C₄ compared to C₃ plants which is maintained under well fertilised and unfertilised conditions (Long, 1999; Beale and Long, 1997; Lewandowski and Schmidt, 2006).

III. C₄ Species as Energy Crops in Cool-Temperate Climates

The cool-temperate climatic zone of Eurasia and North America represents a vast area that could be potentially cultivated to meet a future demand for productive C₄ bioenergy crops. C₄ species currently, however, are not common in the flora

of the cool-temperate climate zones, indicating there may be significant yield restrictions in these regions (Teeri and Stowe, 1976; Collins and Jones, 1985). Physiological explanations for the relative rarity of C_4 plants in cold climates generally argue that there is a fundamental restriction within the C_4 pathway that prevents photosynthetic performance at low temperature relative to what is possible in C_3 plants. These physiological restrictions include lower quantum yield, a low Rubisco capacity at low temperatures, lability of C_4 cycle enzymes such as PPDK, and greater likelihood of high light stress that in turn increases photoprotection costs or photoinhibition (Long, 1999; Sage et al., 1999; Pittermann and Sage, 2000; Sage and Kubien, 2007). Adopting a different approach, Edwards and Still (2008) have recently placed another interpretation on the significance of the physiological restrictions inherent in the C_4 pathway. They have shown that the restriction of C_4 grasses to warmer areas is due largely to phylogenetic constraints that reflect their evolutionary origins in warm climates. Instead of inherent physiological limits associated with the C_4 pathway, the strong positive correlation between temperature and C_4 grass abundance could simply arise because C_4 grasses are adapted to warmer habitats and are absent from cooler regions due to a general cold-intolerance, as would be the case with C_3 species from warm environments. There are nevertheless, irrespective of which of these two explanations is correct, a few dozen C_4 species that are tolerant of low temperatures (Long et al., 1975; Jones et al., 1981). These include native grasses and dicots found in high latitude and high elevation environments, and some species that grow in cool maritime environments and during late-winter and spring (Sage et al., 1999). Among these species is foxtail millet (*Setaria italica*), a proposed genetic model species whose genome is currently being sequenced (Doust et al., 2009). In addition, varieties of the C_4 crop *Zea mays* have been successfully selected for improved cold tolerance although both the initial establishment of the crop and subsequent canopy development is still frequently limited by cool spring temperatures (Meidema, 1982). Low temperatures can influence leaf photosynthesis both by reducing the efficiency of existing leaves and by affecting the development of new leaves which, as a consequence, have reduced efficiency

at maturity (Nie et al., 1992). Typically, when leaves of C_4 plants are exposed to bright sunlight at temperatures below 15°C photoinhibition of photosystem II occurs (Long, 1983), resulting in a reduction of maximum quantum efficiency. If, on the other hand, leaves develop at low temperatures there are reduced levels of numerous thylakoid proteins and stromal enzymes which leads to a reduction in the light saturated rates of CO_2 uptake (Nie et al., 1995; Wang et al., 2008).

One C_4 species which appears to be uniquely cold tolerant is *Miscanthus* $\times$ *giganteus* (Beale et al., 1996; Naidu et al., 2003; Naidu and Long, 2004; Farage et al., 2006). Unlike other C_4 species with the same NADP-ME pathway, such as sugarcane, *M* $\times$ *giganteus* is capable of developing photosynthetically competent leaves under chilling temperatures below 10°C (Earnshaw et al., 1990). Also, unlike other low temperature tolerant C_4 species it is able to achieve high efficiencies of light energy conversion and accumulate large amounts of biomass at low temperatures by maintaining physiologically active leaves at temperatures 6°C below the minimum for *Z. mays* (Beale and Long, 1995; Naidu et al., 2003). Beale et al. (1996) concluded that *Miscanthus* $\times$ *giganteus*, in contrast to all other C_4 species that they had examined, is able to realise the high photosynthetic potential of C_4 plants when grown under temperate conditions in the field in southern England. Even the cool-temperate C_4 grass *Spartina anglica* and the cold-tolerant annual grass *Echinochloa crus-gali* are susceptible to chilling damage, showing significant reductions in leaf photosynthesis after exposure to chilling temperatures (10–15°C) (Dunn et al., 1987; Potvin, 1987). *M.* $\times$ *giganteus* is an exception in that when it is exposed to chilling temperatures there is a large increase in the zeaxanthin content of its leaves, which is maintained overnight in the dark and is associated with a large increase in the non-photochemical quenching of excitation energy (Farage et al., 2006). This mechanism appears to underlie the remarkable capacity of this grass to grow in cool climates and greatly outyield other C_4 species at these temperatures (Heaton et al., 2004b). Naidu and Long (2004) in a comparative study using *M* $\times$ *giganteus* and *Zea mays* grown at low temperatures (14/11°C, day/night), have shown that the ability of *M* $\times$ *giganteus* to maintain high rates of photosynthe-

sis at low temperatures is due to different properties of Rubisco and/or pyruvate orthophosphate dikinase (PPDK), reduced susceptibility to photoinhibition, and the ability to maintain high levels of leaf absorbance and photosynthetic protein during growth at low temperatures.

It is predicted that C₄ species in temperate environments will benefit from global warming (IPCC 2007). Brown et al. (2000) modelled switchgrass yields in the Great Plains and showed an increase by as much as 50% for 3.0–8.0°C warming. However, in the tropics maize and sorghum yields will probably decrease in response to warming, with an average of 8% yield-loss for each degree Celsius rise in temperature (Lobell and Field 2007). The direct effects of elevated CO₂ will be small because C₄ photosynthesis is typically CO₂ saturated at present atmospheric concentrations (Ainsworth and Long 2005).

IV. Examples of C₄ Species as Biofuel Feedstock

Currently, maize and sugarcane are the two most widely exploited examples of C₄ species used as sources of first generation biofuels, providing starch and sugar respectively (U.S. DOE, 2006). Although grain starch from maize is currently the predominant source of biofuel in the United States, corn stover, the vegetative residue remaining after the grain is harvested represents approximately 50% of the above-ground dry matter and could also be used as a lignocellulosic feedstock for ethanol production (de Leon and Coors, 2008). It has been estimated that approximately 256 Mt year⁻¹ of corn stover will be available in the US by 2030 (Graham et al., 2007), which could provide 20% of the biomass needed to replace 30% of the current transportation fuel use. Future exploitation of maize as a biofuel depends on the selection of varieties with increased biomass production and bioconversion efficiency. The essential requirement for breeders is to either change the architecture of the maize plant to transform a primarily grain-producing plant to a biomass producing plant or to increase total biomass while maintaining grain yield potential. The advantage of the latter is that it would retain a high-value feed product while increasing the yield of lignocellulosic material for bioethanol

production (de Leon and Coors, 2008). However, there is still a requirement to alter the cell wall composition to remove the recalcitrance to the hydrolytic enzymes required for the conversion of the polysaccharide fraction into simple sugars for fermentation. Unfortunately, attempts so far to lower the lignin content to increase the digestibility of maize stovers has lead to lower yields (Dhugg, 2007).

Sugarcane is a subtropical C₄ species which has, in both Australia and Hawaii, achieved biomass yields that are about half the theoretical maximum (Moore et al., 1997). Muchow et al. (1994) recorded maximum biomass production of 72 t ha⁻¹ but the world average is considerably lower at about 17 t ha⁻¹ (Tew and Cobill, 2008). Energy output/input ratios between 1.8 and 4.0 have been reported for a number of countries (Mrini et al., 2001; Yuan et al., 2008). Additionally, the association of sugarcane with an endophytic diazotrophic bacteria results in enhanced N supply to the plant through biological nitrogen fixation of atmospheric N₂ (Andrews et al., 2003; Samson et al., 2005). In Brazil, the historical use of low N fertilisation rates may have selected for genotypes with a high proportion of fixed N.

Other first generation sub-tropical and tropical C₄ biofuel feedstocks are *Sorghum bicolor* (sorghum) (Saballos, 2008; Yuan et al., 2008), *Penisetum purpureum* (Napier grass) and *Erianthus* spp. (Samson et al., 2005). Sorghum can be cultivated for three processing streams: grain starch, similar to corn starch for the production of ethanol, high-sugar stem juice that can be used directly for fermentation, and dry bagasse left after juice extraction that can be used for lignocellulosic feedstock for fermentation (Saballos, 2008; Yuan et al., 2008). In addition to this versatility of utilisation, sorghum is a stress tolerant species that can be grown on poor quality land with low inputs (Saballos, 2008). Because industrialised nations with large biofuel targets such as the United States and European Union may not have the land needed to meet their growing demand for current first generation agricultural biofuels there is an incentive for land-rich tropical counties to help meet these rising targets (Gibbs et al., 2008). Consequently there is an increasing opportunity for high yielding C₄ crops to be grown for biofuel feedstock in the tropics (Samson et al., 2005; Koh and Ghazoul, 2008). Napier grass has, for

instance, been shown to achieve annual yields in excess of 55 t ha⁻¹ and because of the provision of N through biological nitrogen fixation of atmospheric N₂ it appears that very little N fertilizer may be necessary to maintain yields of approximately 30 t ha⁻¹ year⁻¹ (Samson et al., 2005).

In cool temperate regions, the sterile triploid of *Miscanthus* called *M. × giganteus* Greef et Deu ex Hodkinson et Renvoize (Hodkinson et al., 1997) has attracted the most attention in Europe (Venendaal et al., 1997; Lewandowski et al., 2000, 2003b). Taxonomically, *Miscanthus* is classified with several other species of high economic value such as maize, sorghum and sugarcane, in the predominantly tropical grass tribe Andropogoneae (Hodkinson et al., 2002a, b, c; Clifton-Brown et al., 2008). Within the Andropogoneae all species have C₄ photosynthesis of the NADP-ME type. *Miscanthus sensu lato* (*s.l.*; in a broad sense) contains approximately 14–20 species (Clayton and Renvoize 1986; Hodkinson et al., 1997) but its genetic limits have been re-evaluated using molecular phylogenetics (Hodkinson et al., 2002c) and has been reduced to approximately 11 species, all with a basic chromosome number of 19. Three *Miscanthus* species have been identified as having the highest potential for biomass production (Jones and Walsh, 2001); these are *M. × giganteus*, *M. sacchariflorus* and *M. sinensis*. *M. × giganteus* is a naturally occurring sterile hybrid so that all plantings are with the same clone. *M. × giganteus* has been wrongly called *M. sinensis* ‘Giganteus’, *M. giganteus*, *M. ogiformis* (Honda) and *M. sacchariflorus* var. *brevibarbis* (Honda). Several varieties and horticultural cultivars of *M. sacchariflorus* and *M. sinensis* have been described and they can hybridise and form a species complex with *M. × giganteus* (Hodkinson et al., 2002b; Clifton-Brown et al., 2008). This complex is considered to be the primary gene pool of *Miscanthus* available for plant breeding. The *Miscanthus* genus is native to eastern or south-eastern Asia and presumably originated in the broad area. Its natural geographic range extends from north eastern Siberia, 50°N in the temperate zone to Polynesia 22°S, in the tropical zone, and westwards to central India. It is therefore found in a wide range of climatic zones. The range of altitudinal zones are from sea level tropics where *M. floridus* is found to altitudes up to 3,100 m on dry moun-

tain slopes in Guizhou, Sichuan and Yunnan in China where *M. paniculatus* occurs (Chen and Renvoize, 2006; Clifton-Brown et al., 2008).

In Europe, *Miscanthus × giganteus* has been developed as an energy crop with productivity trials going back to the 1970s. Beginning in 1992, *Miscanthus* was trialed in 16 locations throughout ten European Union countries as part of the EU *Miscanthus* Productivity Network (Jones and Walsh, 2001). Results of these and additional trials indicate harvestable *Miscanthus* yields range from 10 to 40 t ha⁻¹ year⁻¹ throughout Europe (Lewandowski et al., 2000, Clifton-Brown et al. 2001, Price et al., 2004; Christian et al., 2008). Based on these European studies a model of *Miscanthus* productivity (MISCANMOD) was developed by Clifton-Brown et al. (2000, 2004) and Stampfl et al. (2007) to predict yields throughout Europe (Fig. 2). When this model was used to explore the likely productivity of *Miscanthus* in Illinois in the Midwestern US, projections of peak annual biomass prior to senescence ranged from 27 to 44 t ha⁻¹ (Heaton et al., 2004b). A meta-analysis of the effects of management factors on *M. × giganteus* growth and biomass production

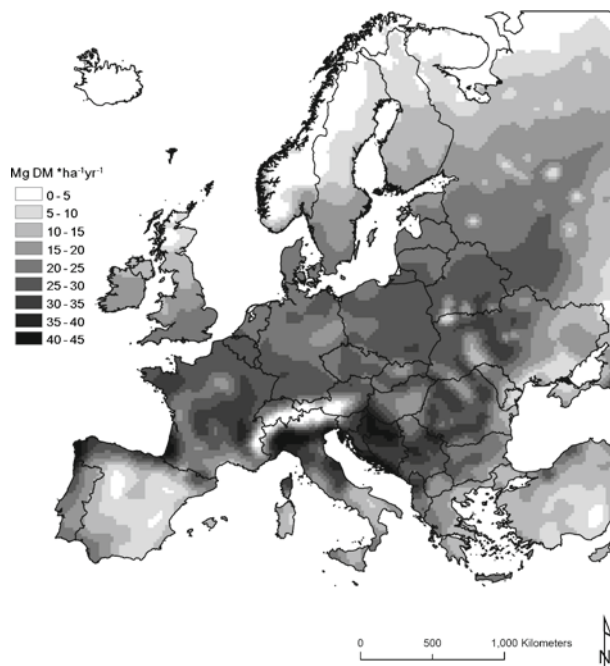


Fig. 2. Predicted current harvestable yields of *Miscanthus* in Europe using the crop growth model, MISCANMOD (Adapted from Stampfl et al., 2007).

in Europe (Miguez et al., 2008), has shown that one of the simplest models for predicting potential biomass production based on the thermal units accumulated during the growing season provides a remarkably good fit to the observed data. The data indicate that once the normal agronomic practices such as weed control and water availability are in place, temperature accounts for most of the variation in growth patterns.

In the United States the favoured C₄ species is switchgrass (*Panicum virgatum* L.), a large perennial grass native to the North American prairie that has been historically used as forage (Lemus et al., 2002). It was chosen by the US Department of Energy in 1991 as a model energy crop and in productivity trials of different varieties in a range of locations yielded an average of 13.4 t ha⁻¹, ranging from 9.9 to 23 t ha⁻¹ (McLaughlin and Kszos 2005). A quantitative review of annual production values from peer-reviewed articles describing trials of both switchgrass and *Miscanthus* in the United States and Europe found that *Miscanthus* produced an average peak annual biomass of 22 t ha⁻¹ (97 observations) while switchgrass produced 10 t ha⁻¹ (77 observations) (Heaton et al., 2004b). Parrish and Fike (2005) have suggested, following a thorough review of the biology and agronomy of switchgrass for biofuels, that it should be feasible to develop well-adapted cultivars that can sustainably produce more than 15 t ha⁻¹ year⁻¹ biomass at site in the United States that receive more than 700 mm annual rainfall.

Switchgrass was a widespread component of the tall grass prairie and occurred in non-forested areas throughout the eastern two thirds of the United States before the Europeans arrived (Bransby et al., 1998; Huang et al., 2003; Parrish and Fike, 2005). Its original use was as a forage and it is only recently, in the last 20 years, that it has been adopted as a biofuel (Parrish and Fike, 2005). The species' open pollination pattern and self-incompatibility mechanisms results in each plant in a population of switchgrass possessing a unique, heterozygous genotype. Morphologically, switchgrass is a rather coarse grass that grows from 0.5 to 3.0 m tall, with rooting depths of up to 3 m. The rhizomes show a good deal of variability which influences the spread of the stems to form a more bunched or open plant. There are two 'forms', the 'upland' and 'lowland' forms

which are associated with more hydric mid- to northern latitudes and drier lower latitudes, respectively. The result has been the development of a high level of genetic variability resulting from site-specific conditions and unique genotypes, which interact to produce a wide range of phenotypes (Casler, 2005). The possession of broad adaptation to environmental conditions, both climatic and edaphic, was a key factor in identifying switchgrass as a potential herbaceous energy crops for use across North America (U.S. DOE, 2006).

Although switchgrass is a C₄ species it is unusual in that it uses the nicotinamide adenine dinucleotide-malic enzyme (NAD-ME) photosynthetic pathway while it has a leaf structure more commonly seen in grasses using the phosphoenolpyruvate carboxykinase (PEP-CK) pathway (Prendergast et al., 1987). The *Panicum* genus contains species that operate with C₃, C₄, and C₃-C₄ intermediate photosynthetic pathways (Raghavendra and Das 1978; Smith and Brown 1973). Switchgrass single-leaf photosynthetic rates are generally lower than the warm-season grasses (Kiniry et al., 1999). However, the canopy radiation use efficiency (RUE) is high (Kiniry et al., 1999) due to a high leaf area index (LAI) and a low light extinction coefficient (k). Values of RUE between 2 and 4 g MJ⁻¹ of photosynthetically active radiation have been reported (Kiniry et al., 1999; Madakadze et al., 1998). There have also been reports that photosynthetic rates in switchgrass are higher at higher ploidies as a result of greater activity of the photosynthetic enzymes ribulose-1,5-bisphosphate carboxylase (Rubisco) and phosphoenolpyruvate carboxylase (Warner et al., 1987; Warner and Edwards 1993). However, Wulfschleger et al. (1996) found that, although early in the growing season tetraploid lowland cultivars had higher photosynthetic rates than octaploid upland cultivars, this was reversed later in the growing season. It was suggested that this may be associated with the better drought tolerance of the upland cultivars. Certainly, upland switchgrasses are considered to be more drought tolerant than lowland cultivars (Parrish and Fike, 2005). There are also reported differences among cultivars in WUE at the whole plant level (biomass produced per mass of water transpired) (Byrd and May, 2000). Kiniry et al. (2005) have successfully used the ALMANAC growth simulation

model to model mean biomass yields and year to year variability at sites in North America.

Most currently available commercial cultivars of switchgrass have been selected for their forage qualities as breeders have only recently begun to select for traits that may be exploited in energy cropping (Parrish and Fike, 2005). Breeders are selecting for lines which combine broad adaptation to the environment with greater biomass productivity. Increases in biomass are most frequently linked to phenological and morphological traits (van Esbroeck et al., 2003). The phenological ideotype of a high yielding biomass grass is one that triggers spring growth soon after the danger of freezing injury is passed and then prolongs its vegetative activity late into the growing season but also allows time for good seed set and complete senescence before the first killing freeze in autumn.

V. Prospects for Energy Crop Improvement

The candidate C₄ energy crops so far identified are largely undomesticated and have not undergone the centuries of improvement that characterise our current major food crops (Koonin, 2006; Vermerris, 2008). The requirement will be to select appropriate species and genotypes which are adapted to local soil and climatic conditions. Selection criteria need to be based on the triple goals of maximising productivity, minimising inputs and maximising utilisation for energy production. Some of the traits of particular interest in breeding programmes are drought tolerance (Clifton-Brown et al., 2002), frost tolerance (Clifton-Brown and Lewandowski, 2000; Jorgensen and Schwarz, 2000), maintenance of growth at low temperature (Farrell et al., 2006), chemical composition (Lewandowski et al., 2003a), resistance to pests and diseases (Clifton-Brown et al., 2008), altering plant architectural features such as dwarf structure and erect leaves (Yuan et al., 2008) and differences in photosynthetic capacity (Carver and Hocking, 2001). Karp and Shield, (2008) have identified the following three main challenges to achieving yield improvement. First, there should be a reduction in the thermal threshold for growth of the canopy leaves which extends the growing season. Second,

above ground biomass should be increased without depleting below ground biomass so much that there are insufficient reserves available for next years' growth. Third, above ground biomass should be increased without restricting growth due to excess water depletion and developing water stress.

Traditional plant breeding, selection and hybridisation techniques are slow and for some PRGs there is a limited availability of germ-plasm. *Miscanthus* × *giganteus*, for example, is a sterile triploid, which is normally propagated from rhizome pieces. There has, however, been some long-term conventional breeding of switchgrass, which has produced large yield gains. In the future new biotechnological routes may produce even greater improvements. Genetically modified (GM) energy crop species may be more acceptable to the public than are GM food crops, particularly in Europe (Koh and Ghazoul, 2008), but there are still concerns about the environmental impact of such plants including gene flow from non-native to native plant relatives. Consequently non-GM biotechnologies may be more attractive. Initially it is likely that the use of molecular biology will focus on the use of molecular markers that can be used in the rapid screening of germ-plasm within the breeding population. However, linking these molecular markers to complex traits such as yield is difficult because yield is controlled by many genes. With the advent of cost-effective and rapid sequencing technologies, there will be a rapid expansion of knowledge of genes and their expression profiles in potential biofuel crops including maize, sorghum and switchgrass (Kebrom and Brutnell, 2007). This knowledge should accelerate breeding strategies aimed to maximise biomass yield and quality. The availability of large amounts of sequence information facilitates identification of DNA polymorphisms (Rubin, 2008). Having complete genome sequences also makes possible the identification of genes located near polymorphisms on chromosomes. This knowledge has practical applications in 'marker assisted breeding' where a DNA polymorphism, closely linked to a gene coding for a trait of interest, such as drought resistance, is used to track the trait among progeny of sexual crosses between plant lines. If a large number of polymorphisms are available, the amount of time required to breed an improved plant cultivar is greatly reduced (U.S. DOE, 2006).

VI. The Environmental Debate and Bioenergy Crops

With the recent emphasis on bioenergy as a means to simultaneously reduce both dependence on fossil fuels and emissions of greenhouse gases, there is rising concern that bioenergy crops will divert resources from food production, as well as promoting global environmental degradation from increased pollution and accelerated land use change (Field et al., 2007; Righelato and Spacklen, 2007). Biofuel production competes for fertile land with food production, increases pollution from fertilisers and pesticides and threatens biodiversity when natural lands are converted to biofuel production. Escalating demands for both food and energy have raised issues about the potential for biofuels to be sustainable, abundant and environmentally beneficial energy sources (Tillman et al., 2006; Hill et al., 2006). Scientific and technological assessments of the performance of the different forms of bioenergy are urgently needed to quantify the potential benefits of growing bioenergy crops along with their accompanying dangers and limitations (Rowe et al., 2007; Schmer et al., 2008). A widely adopted approach is to use Life Cycle Analyses (LCAs) but unfortunately, many bioenergy LCAs have failed to account for GHG emissions from associated land use changes, largely because they are difficult to quantify (Searchinger et al., 2008). When this has been done using a worldwide agricultural model to estimate CO₂ emissions from land use change associated with the conversion of forest and grassland to new cropland to replace the grain directed to biofuels, it has been demonstrated that corn-based ethanol in the USA, instead of producing a 20% saving as suggested without accounting for this, nearly doubles GHG emissions over 30 years and actually increases GHG emissions for 167 years (Searchinger et al., 2008). Furthermore, this work has also shown that if biofuels are produced from switchgrass grown on U.S. corn lands, then replacing the corn would, through the triggering of emissions from land-use change, result in increase emissions of 50% over a 30 year period (Searchinger et al., 2008). On a global scale, Fargione et al. (2008) have shown that for a range of case studies where native rainforests, savannas, peatlands, and grasslands are converted to produce food-based biofuels in Brazil, South-

east Asia and the USA this would create a 'bio-fuel carbon debt' by releasing 17–140 times more CO₂ than the annual GHG reductions these biofuels provide by displacing fossil fuels. On the other hand, biomass grown on abandoned agricultural land in the tropics planted with perennials have little carbon debt and offer a short return GHG credit (Fargione et al., 2008; Gibbs et al., 2008).

Recently, Tillman et al. (2006) proposed that biofuels derived from low-input high-diversity (LIHD) mixtures of native tall-grassland perennials in North America can provide more usable energy, greater greenhouse gas reductions, and less agrichemical pollution per hectare than can either corn grain ethanol or soybean biodiesel. In North America, the tall grassland biome tends to be dominated by C₄ species, such as big bluestem (*Andropogon gerardii*), little bluestem (*Schizachyrium scoparium*) and switchgrass. Furthermore LIHD biofuels are carbon negative because net ecosystem carbon sequestration exceeds fossil carbon dioxide release during production, and they can be produced on agriculturally degraded land, which is not used for food production. These systems are extremely efficient in their use of nutrients (Dubeux et al., 2007) and retain their habitat diversity because they are only minimally disturbed. Also, in these nitrogen limited systems, plant productivity is enhanced by complementarity between legume and C₄ grass species. Complementarity occurs because nitrogen fixation by legumes facilitates growth of C₄ grasses, which have a high NUE, and because legume nitrogen supply and its use by C₄ grasses are differentiated in time (Formara and Tilman, 2008). Steppe, pampas and savannah ecosystems have similar growth patterns as the tall grass biome, and could also be suitable sources of bioenergy based on LIHD systems. In all of these ecosystems, C₄ grasses and sedges are significant contributors in terms of biomass, productivity and cover (Sage et al., 1999). The main disadvantage of the LIHD system is the relatively low yield of ~4 t ha⁻¹, which means that large areas need to be harvested to make a significant impact on the energy supply with the negative consequences that there are high costs of harvesting and transport between the field and the processing station. It has recently been suggested that a compromise may be pure stands of very productive perennial species which still have many of the benefits of

the prairie life form characteristic of the LIHD mixtures (Heaton et al., 2008a). Indeed, Schmer et al. (2008) have shown that, compared with low input prairies, switchgrass grown and managed as a biomass crop can produce significantly greater biomass per hectare and is therefore a more feasible system for providing sufficient supplies of biomass to meet energy demands.

In addition to carbon sequestered in living biomass, the growth of energy crops also leads to changes in soil organic carbon (SOC) and the accumulation of relatively large quantities of root-stock below ground. The below-ground carbon pool is a continuum from living root biomass and freshly senesced leaf and root material through to more recalcitrant soil humic fractions. The slower rates of turnover of the humic fractions means that the greatest long-term benefits arise from sequestration in these recalcitrant soil carbon pools (Jones and Donnelly, 2004; Lemus and Lal, 2005). Estimates of the net sequestration in SOC by *Miscanthus* have been made at a number of locations in Europe (Kahle et al., 2001; Foe-reid et al., 2004). Of the four sites investigated in one study, two showed an increase on SOC compared to adjacent grassland areas while in the other two there was no significant change (Kahle et al., 2001). In this particular case, the sites that did show an increase were on sandy soils compared with silty clay in the other two sites, suggesting that soil texture is an important factor in regulating carbon sequestration. In other studies, Schneckenger and Kuzyakor (2007) in Germany showed that carbon accumulation under *Miscanthus* was similar to that under perennial grasses and 1.6–1.8 times higher than with maize grown under similar climatic conditions while in Denmark, Hansen et al. (2004) recorded carbon sequestration rates that ranged from 0.78 to 1.12 t C ha⁻¹ year⁻¹ and in Ireland, Clifton-Brown et al. (2007) measured a rate of 0.6 t C ha⁻¹ year⁻¹ over a 15 year period from crop establishment on land converted from grassland. The measurement made by Hansen et al. (2004) and Clifton-Brown et al. (2007) used the ¹³C signal to detect C₄ plant sequestration to the soil and therefore did not take into account carbon losses from the old C₃ vegetation. The general consensus is that the net change on conversion from arable land to *Miscanthus* will result in substantial carbon sequestration while the conversion of grassland may

lead to a net loss of carbon, at least in the short term (King et al., 2004). However, Yazaki et al. (2004) demonstrated that long-established *Miscanthus sinensis* dominated grasslands in Japan were, over a 2 year period of study a small carbon source, and they suggested that for many of these grasslands the carbon budget is near to equilibrium. The LIHD mixtures described by Tillman et al. (2006) have also been shown to increase carbon accumulation on agriculturally degraded soils. Formara and Tilman (2008) showed that the high-diversity mixtures of perennial grassland plant species stored 500% more soil carbon than did monocultures of the same species and that the presence of C₄ grasses and legumes increased soil carbon accumulation by 193% and 522% respectively over a 12 year period. Ma et al. (2000) have demonstrated that the establishment of switchgrass resulted in more soil carbon than an adjacent fallow soil but suggested that several years of switchgrass culture will be required to realise a soil carbon sequestration benefit. Frank et al. (2004) have, however, argued that switchgrass has potential for storing a significant quantities of SOC in the northern Great Plains of the USA.

In a recent study by Wynn and Bird (2007) the rates of decomposition of C₄ and C₃ derived plant material in soils have been shown to differ significantly. They found that the active pool of SOC derived from C₄ plants collected over a major environmental gradient across Australia decomposed twice as fast as the C₃ derived SOC pool. Wynn and Bird (2007) suggest that the primary mechanism explaining the selective preservation of C₃-derived biomass in the SOC pool is a difference in the quality of organic matter due to differences in lignin content and/or mean particle size. Clearly, these observations have significant implications for the long term sequestration of carbon in C₄-derived SOC and could potentially reduce the contribution of C₄ biomass crops to total carbon mitigation.

A major environmental concern, related to the large scale deployment of energy crops, is the impact on biodiversity (Murray et al., 2003; Hill et al., 2006; Rowe et al., 2007; Groom et al., 2008; Koh and Ghazoul, 2008). There is particular concern that monocultures of C₄ grasses grown on large areas of formerly diverse marginal land will reduce biodiversity dramatically. To date, there have been few studies of the potential effects of

perennial grasses like *Miscanthus* on biodiversity, but those that have been carried out suggest that in the years immediately after establishment there is an increase in invertebrate diversity, but that as *Miscanthus* plantations mature there may be a decline in diversity (Rowe et al., 2007; Semere and Slater, 2007). However, long-term trials need to be carried out to verify this (Haughton et al., 2009). Of course, a clear benefit of the LIHD mixtures of native grassland perennials as a source of biomass is that this system conserves existing biodiversity (Hill et al., 2006; Tillman et al., 2006).

A further environmental concern highlighted by Raghu et al. (2006) is that many of the ecological traits identified as being advantageous for energy crop production (Heaton et al., 2004a), and listed in Table 1, are the same that contribute to the invasiveness of many species. Raghu et al. (2006) point out that, globally there has been little success in eradicating or even controlling most invasive grasses and that introduced biofuel sources need a rigorous agronomic and ecological risk analysis to ensure that they do not add to the already major global problems with invasive species.

Finally, another environmental controversy has arisen recently in relation to the apparently large emissions of N₂O arising from nitrogenous fertilizer applications to energy crops. In the case of rapeseed bioenergy feedstock it has been

demonstrated that emissions of a powerful GHG like N₂O can outweigh the carbon benefits (Crutzen et al., 2007). However, the very low requirements for nitrogenous fertilizer in PRGs, and in particular C₄s, probably mean that this is far less of a concern for these sources of bioenergy (Jorgensen et al., 1997).

VII. Economic and Energetic Costs and Benefits

For biofuels to be viable as alternatives to fossil fuels they should not only have superior environmental benefits but they must be economically competitive, be producible in sufficient quantities to make a meaningful impact on energy demands and provide a net energy gain over energy sources used to produce them. Determining whether alternative fuels provide benefits over the fossil fuels they displace requires detailed accounting of both direct and indirect inputs and outputs for the whole life cycle. This requires information on farm yields, commodity and fuel prices, farm energy and agricultural inputs, production plant efficiencies, GHG emissions and other environmental effects discussed above (Farrell et al., 2006; Somerville, 2007; Field et al., 2007).

The Net Energy Balance (NEB) is a measure of the input energy to grow crops and convert them to biofuels relative to the biofuel energy

Table 1. Comparisons of attributes of types of energy crops with C₄ photosynthesis.

Attributes	Annuals (e.g. <i>Zea mays</i>)	Perennial rhizomatous (e.g. <i>Miscanthus</i>)	Low intensity High diversity (e.g. North American prairie)
C ₄ photosynthesis	✓	✓	Both C ₄ and C ₃
Long canopy duration		✓	✓
Recycles nutrients to roots		✓	✓
High output/input energy ratios		✓	✓
Non-invasive	NA	✓	✓
Winter standing		✓	
High water use efficiency	✓	✓	✓
Few pests and diseases		✓	
Uses existing farm equipment	✓	✓	✓

NA: not applicable

content upon combustion (Schmer et al., 2008). The NEB of a biofuel is determined by subtracting the value of all fossil energy inputs used in producing the biofuel from the energy value of the biofuel and its co-products. The NEB is positive when the biofuel energy content exceeds fossil fuel energy inputs. The NEB ratio is calculated by dividing the sum of the energy outputs by that of the inputs. Using this type of analysis, Hill et al. (2006) have shown that corn grain ethanol, a food-based biofuel, provides only a 25% net gain in energy (NEB ratio = 1.25) and a 12% reduction in GHGs while at the same time there are environmental and human health impacts associated with increases in air pollutants, nitrate, nitrite and pesticides. Non-food, C_4 feedstocks have been shown to have substantial environmental, energetic and economic advantages over food-based biofuels with NEB ratios as high as 50 estimated for switchgrass and *Miscanthus* (Lewandowski and Schmidt, 2006; Clifton-Brown et al., 2008; Schmer et al., 2008). In general, low-input biofuels have the potential to provide much higher NEBs as well as much lower environmental impacts per net energy gain than food-based biofuels. The long term prospects for second-generation biofuels for cellulosic ethanol is that they will have the potential to be grown on agriculturally marginal lands with minimal fertilizer, pesticide and fossil energy inputs and therefore provide fuel supplies with greater environmental benefits than either fossil fuel or current food-based biofuels.

VIII. Conclusions and Perspectives

Energy crops have been both lauded as a solution to our problems over declining reserves of fossil fuels and the consequence of their use on CO_2 emissions and denigrated because of their negative impact on food prices and the environment (Scharlemann and Laurance, 2008). More recently the negative aspects have been emphasised by researchers who have identified a number of issues which have rung alarm bells. These include the food or fuel debate and a number of environmental concerns. It is now clear that, if biofuels are to have a future in the medium to long term it is most likely to require the development

of second and third generation technologies that are capable of producing lignocellulosic based energy products such as bioethanol. At the heart of this will be more productive agricultural systems and the introduction of high-yielding, dedicated bioenergy crops which maximise cellulose production. In order to meet the production goals envisioned for future bioenergy feedstocks, the C_4 photosynthetic mechanism will have to play a major role. Although it is clear that biofuels are not a panacea it is essential that as interest and investment in energy crops increases there needs to be an active and continuing discussion on strategies for balancing the pros and cons of biomass energy production and utilisation. This will recognise the role that bioenergy can play in combating climate change and improving the security of energy supply, as well as providing a range of improved ecosystem services such as soil carbon sequestration, reduced soil erosion, reduced water pollution risks and increased biodiversity.

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Γ values, 65, 70, 72, 76

A

Abaxial, 66, 76
Abildgaardia, 325
A. hygrophila, 325
 Abildgaardieae, 323, 325
 Absciscic acid, 116, 242–243
 Acclimation, 132, 143, 185–187
 antisense reductions, 182
 and quantum yield, 177–179
 Acetylation, 245
 A/Ci response, 176
Actinobacillus succinogenes, 290–291
 Activator, 266
 Adenosine 5'-phosphosulfate reductase (APR), 115–119, 122
 Adenylate energy charge (AEC), 307, 371, 374
 Adenylate kinase, 302
 ADP-glucose pyrophosphorylase, 289
 AEC. *See* Adenylate energy charge
 Aerial leaves, 74
 Ala, 87–88
Alinula, 325–326
 Alismataceae, 74
 Alismatales, 72, 75, 320–323
 Allosteric activators, 262
 Allosteric effectors, 266
 Allosteric inhibitors, 261, 262
Alloteropsis, 332
Aloe arborescens, 284
Alternanthera, 268
A. pungens, 258
A. sessilis, 266, 267
A. tenella, 267
 Alternative oxidase (AOX), 235
 Amaranth, 22, 222, 226, 228–230, 240
 chloroplasts, 240
Amaranthus edulis, 288
Amaranthus hypochondriacus, 267, 288, 290
Amaranthus tricolor, 241, 288
Amborella, 75
Amborella trichopoda, 69
 Amino transferase, 86, 87
 Ammonium, 110, 111, 113
 transport, 112
 transporters, 111

Anaerobiospirillum succiniciproducens, 290–291
 Anaplerotic reaction, 290
 Anatomical features, C_4 , 147–155
 Anatomy, 20
 Antiporters, 206–207
 Antisense, 289
 AOX. *See* Alternative oxidase
 Apical meristem, 226
 APR. *See* Adenosine 5'-phosphosulfate reductase
 APS reductase, 114
Aptenia cordifolia, 284
 Aquaporins, 71
 Aquatic, 19, 20
Arabidopsis, 83, 85–95, 115, 118, 122, 150, 153, 310–313, 366
A. thaliana, 258, 264, 283, 372
 Araceae, 231
 Arctic, 168, 175
Argentia mutant, 243
Aristida longifolia, 326
 Aristidoideae, 326
Arundinella hirta, 151
Arundo donax, 381
Ascaris suum, 286
Ascolepis, 325–326
 Ascorbate, 117, 118
 Asparagine, 112
 Aspartate/Asp, 70, 260, 262
 Atmospheric CO_2 , 167
 ATP sulfurylase (ATPS), 115–117, 122
Atriplex, 64
Atriplex rosea, 230

B

Bacterial-type PEPCs, 259
 Basal meristem, 229, 230
 Basic leucine zipper proteins, 271
 Basipetal, 228, 233
 Basipetal sink-to-source transition, 229
 Bayesian analyses, 328
 BicA, 372–373
 Bicarbonate, 260
Bienertia, 367
B. cycloptera, 367
B. sinuspersici, 267
 Big bluestem (*Andropogon gerardii*), 389
 Biochemical models, 175

Biodiversity, 390–391
 Bioethanol, 24
 Biofuel, 18
 feedstock, 385–388
 Bioinformatic analysis, 310–313
 Biomass, 24
 Biophysics, 4, 5
 Blue light, 230
 Boreal zone, 168
 Bose Institute, 5
 Bose, Sir Jagadish Chandra, 3–10
 Boundary layer, 174
Bouteloua, 327
Brassica campestris, 293
Brassica juncea, 118
Brassica napus, 122, 258, 293
Brassica oleracea, 293
 Bromeliaceae, 320
 BSCs. *See* Bundle sheath cells
Bulbostylis, 325
 Bundle sheath cells (BSCs), 64, 74–75, 263,
 269, 277–295
 C₄ gene expression, 221–249
Bundle sheath defective 1 (bsd1), 245

C

¹³C heavy isotope, 15
¹⁴C-pulse/¹²C-chase, 68
 $\delta^{13}\text{C}$ values, 67–68
 C₂ cycle, 83–95
 C₃-C₄, 98, 117, 232
 intermediacy, 20, 98, 100, 110, 113, 116
 intermediate artificial, 249
 C₃/C₄ intermediate, 72
 C₃-like, 228, 229, 230, 241
 C₃-pathway, 7–8
 C₃ plants/species, 283, 284, 285, 289, 292–293
 C₃-to-C₄ transition, 226, 228, 229, 230,
 232, 237, 241
 C₄ acids, 277
 C₄ cycle, 176
 chilling sensitivity, 180
 CO₂ and light effects on, 167
 CO₂ effect on A/T response, 176
 genes, 66
 C₄ determinants, 266
 C₄ evolution, 269
 C₄ grasslands, 340, 347–353
 C₄ PEPC, 264, 266
 C₄ photosynthesis model, 305

C₄-plants, 278–280, 284–286, 288–292,
 294, 295
 C₄ type, 226, 230, 231, 241, 242
 CA1, 239
 CA2, 239
 CA3, 239, 244
Cabomba caroliniana, 14
Callitriche cophocarpa, 76
 Calvin cycle, 83, 100, 286
 CAM. *See* Crassulacean acid metabolism
 CAM-like, 76
 cAMP-dependent protein kinase, 294
 CaMV promoter, 246, 247, 283, 289
 Carbon assimilation, 241–242
 Carbon concentrating mechanism, 372–373
 Carbonic anhydrase (CA), 66, 70, 139, 364,
 367–369
 Carbon isotope discrimination, 129, 130,
 139–141
 Carbon isotopes, 340
 Carbon sequestration, 390
 Carboxysomes, 69
 Catalase (CAT), 87
 Catalytic mechanism, 302
 Catalytic turnover rate (k_{cat}), 136, 138,
 143
 CCM. *See* CO₂ Concentrating mechanism
 cDNA library, 284
 Cell position, 242
 Cellulose, 141
 Centothecoideae, 326
Centropodia/Merxmullera, 327
Ceratophyllum demersum, 76
 Challenges, 249
 Character reconstruction, 328
 Chemical composition, 141
 Chenopodiaceae, 39
 Chimeras, 282
Chlamydomonas reinhardtii, 258
ChlMe1, 232, 233, 244
ChlMe2, 232, 233, 244
 Chloridoideae, 23, 326, 327
Chloris, 327
 Chlorophyll, 134, 135
 Chlorophyll A/B binding proteins
 (*Cab* genes), 239
 Chloroplasts, 85, 235, 239–241
 ATP synthase, 309
 decarboxylase, 69
 inner envelope membrane, 200
 leader sequences, 312

outer envelope membrane, 200
 structure
 differentiation, 31
 grana deficient, 31
 grana development, 31
 Chromatin modifications, 245, 269
 Cis-acting, 243, 249
 sequences, 244, 246
 Cis-regulatory elements, 269, 270
Cleome gynandra, 264, 366
Clostridium symbiosum, 302
 CO₂ compensation point, 82, 96, 98
 CO₂ concentrating mechanism, 7, 64–66, 130, 133, 136, 139, 141, 143, 291
 CO₂ conductance, 71
 CO₂-depletion/CO₂ limiting, 65, 66
Codium fragile, 72
 CO₂ flux mechanism (CFM), 76
 Cold climates, 162
 Cold lability, 181–182
 Cold-stability, 304
 Cold tolerance, 173
 CO₂ leakage from bundle sheath, 185
 Competition, 166
 deactivation, 180–181
 Competitive inhibitor, 307
 Computational models, 151
 Concentration gradient, 203
 Conductance, 71
 Conductivity balance, 6
 CO₂ permeability, 133
 CO₂ response (A/c_i) curves, 96
 Corn, 380, 385, 389
Corynebacterium glutamicum, 290–291, 369, 371
 CO₂ transport, 211
 Cotyledons, 229, 236
Courtoisina, 325–326
Crassula argentea, 288
 Crassulacean acid metabolism (CAM), 8, 74, 75, 281
 Crescograph, 6
 Cretaceous, 75
Crosslandia, 325
 Crystal structures, 280
Cucumis sativus, 293
Cucurbita pep, 285
 Cyanobacteria, 75, 97, 258
 Cyperaceae, 30, 37–39, 151, 320, 323–326
 chlorocyperoid: NADP-ME, 37–38
 C₄ types, 37–39

 eleocharoid: NAD-ME, 39
 fimbristylloid: NADP-ME
 and NAD-ME, 37
 rhynchosporoid: NADP-ME, 39
 Cypereae, 325–326
Cyperus, 325–326
Cyperus papyrus, 382
 Cysteine, 112, 114–118, 120–122
CytMe, 232–234
 Cytochrome *f*, 135
 Cytokinins, 112, 116
 Cytoplasmic form, 237
 Cytoplasmic streaming, 203

D

Danthoniopsis, 326
 Dark-grown, 246
 Day respiration, 94
 Decarboxylating enzyme, 69
 Default state, 240
 Density of leaf venation, 151–152
 Developmental gradient, 226, 229
 Developmental processes, 241
 Di-and tricarboxylate carrier
 (DTC), 210
 Diatoms, 71–72, 75, 291
 Dicarboxylate carrier (DIC), 210
 Dicarboxylate translocator (DiT), 206
 3,3-dichloro-2-(dihydroxyphosphinoylmethyl)
 propenoate, 67
 Dicot C₄ PEPC, 266
 Dicotyledons, 39–45
 Diethyloxalacetate, 67
 Differential display, 70
 Diffusion barrier, 153
 Diffusion resistance, 71, 76
Digitaria sanguinalis, 113
 Dimer-of-dimers structure, 263
 Dioecious, 65, 73
 Diurnal regulation, 112
 Diurnal rhythm, 115
 DNA methylation, 245
 DNA/protein interaction, 272
 Dof1, 245
 Dof2, 245
 Dof-domain, 245
 Dof transcription factors, 245
 DR (distal element), 243, 244
 Drought, 140, 142
 tolerance, 387, 388

E

Echinochloa crusgalli, 363, 384
Echinochloa polystachia, 382
Egeria, 69, 72, 73, 75
 E. densa, 64, 65, 284, 322–323
Egregia, 367
 Electron transport, 175, 183
Eleocharis, 74, 154, 325
 E. acicularis, 75
 E. baldwinii, 74, 75, 325
 E. nuttalli, 76
 E. vivipara, 74, 75
Eleocharis vivipara, 231, 325
Eleusine coracana, 113, 288
 Elevation limits, 168, 174
Elodea, 72, 73
 E. canadensis, 64, 65, 76, 325
 Energetics, 46–48
 minimum energy requirements, 45–48
 in vivo requirements
 NAD-malic enzyme type, 30, 46, 48
 NADP-malic enzyme type, 30, 46–48
 PEP-carboxykinase type, 30, 36–37, 48
 Energy crops, 379–392
 Engineering C_4 , 249, 361–375
 photosynthesis, 214
 Enhancer/promoter, 243–245
 Enzymes, 269
 Eocene, 75
 Epidermis, 285
Eragrostis, 327
Eriachne, 326
Erianthus spp., 385
Escherichia coli (*E. coli*), 259, 370,
 372, 374
 Ethanol, 18
 production, 385
 Etiolated, 229, 230, 232, 241, 248
 Evolution, 263, 268
 transport proteins, 202
 Evolutionary constraint, 180

F

Facultative C_4 , 65, 76, 322–323
 Fat-storing seeds, 292
FbRbcS1, 246
 Feedback inhibitors, 260
 Fens, 175
Fimbristylis, 325
 F. variegata, 325

Fire-climate feedback, 351–352
 Fire hypothesis, 351
 First generation biofuels, 385
 Flanking region, 244, 245
Flaveria, 21, 22, 98, 151, 238, 240, 264,
 269, 366, 373
 F. australasica, 117
 F. bidentis, 237, 247, 262, 270, 281–283, 304,
 373–374
 F. brownii, 242, 264, 267, 268, 270, 304
 F. cronquistii, 264, 270
 F. palmeri, 270
 F. pringlei, 237, 243–244, 264, 267, 268, 270,
 283, 369, 370
 F. pubescens, 267, 268, 270
 F. ramosissima, 242, 264
 F. robusta, 264
 F. sonorensis, 264
 F. trinervia, 117, 230, 237, 243, 244, 245, 258,
 262, 266–270, 285, 292, 294
 F. vaginata, 270
 Florida, 64
 Floristic analysis, 168
 Fossil record, 342–343
 Foxtail millet (*Setaria italica*), 384
 Frost tolerance, 388
 Fructose diphosphate, 14
 Fructose phosphate, 14
 Futile cycle, 295

G

G2. *See* Golden2
 Gas exchange measurements, 96–97
 GDC. *See* Glycine decarboxylase
 Gel mobility shift, 248
 Gene duplication, 264
 Gene expression, 22, 221–249, 269
 Gene networks, 154
 Geologic evidence, 340–344
 Geologic history, 339–354
 GFP. *See* Green fluorescent protein
 GHG emissions, 389
 Glc-6-phosphate, 68
 G2-like (*Glk*) genes, 245
 Gln synthetase (GS), 82, 93
 Global warming, 380
 Glopnet, 129, 133, 134
 Glu, 87, 88
 α -glucan phosphorylases, 235
 Gluconeogenesis, 290

Glucose-6-phosphate, 260, 262, 266, 268
 β -glucuronidase (GUS), 244
 Glu:glyoxylate aminotransferase (GGT), 86, 87
 Glumes, 238
 Glu synthase, 82
 Glutamate, 112, 117
 dehydrogenase, 111, 113
 synthase, 21, 111
 Glutamate synthase (GOGAT), 112, 113
 Glutamine, 111, 112
 Glutamine synthetase (GS), 21, 111–114, 120
 Glutathione (GSH), 115–122
 Glutathione reductase (GR), 118, 119
 Gly, 87–88
 Glycerate, 91–93
 Glycerate 3-kinase (GLYK), 92
 Glycerate 3-phosphate (3PGA), 82, 92–93
 Glycine, 117
 decarboxylation, 111
 Glycine decarboxylase (GDC), 110, 116, 121, 234
 Glycine dehydrogenase complex (GDC), 20
Glycine max, 259, 285
 Glycolate, 85–87
 dehydrogenase, 372
 Glycolate oxidase (GOX), 86, 87, 92
 Glycolate 2-phosphate (2PG), 82
 Glycolate 2-phosphate phosphatase (PGLP), 82
 Gly decarboxylase (GDC), 86, 88
 Glyoxylate, 86–88
 carboligase, 372
 Gly shuttle, 100
Golden2 (G2), 245
Gomphrena
 G. globosa, 120
Gomphrena globosa, 113
 GR. *See* Glutathione reductase
 Grana, 280
 Grape berries, 285
 Grass, 264
 Grass C₄ PEPC, 266
 Grass-grazer co-evolution hypothesis, 350–351
 Grazing, 352–353
 Green fluorescent protein (GFP), 247, 312
 GS. *See* Glutamine synthetase
 g_s. *See* Stomatal conductance
 GUS. *See* β -glucuronidase
 Gymnosperm, 75

H

Haloxylon persicum, 20, 283
 Hartt, C.E., 13–16
 Hawaiian Sugar Planters' Association, 14
 HCO₃-use, 76
 Heat shock protein (Hsp70), 284
 Hexokinase, 14, 15
Hilaria, 327
 Histone acetylation, 245
 H₂O₂, 86–87
Homo, 18
 H-protein, 89–90
 Husk, 238, 242
hvca1, 70
hvm1, 69
 HVPEPC, 66, 68
Hydrilla, 3–10, 19, 65, 68–75, 367
 H. verticillata, 8, 64, 286, 322–323
 Hydrocharitaceae, 65, 72
 3-hydroxy-3-methyl glutaryl coenzyme A reductase, 294
 Hydroxypyruvate, 84
 reductase, 83
 Hypersensitive response, 92

I

ictB, 373
 Illumination, 229, 230, 233, 241
 Immunogold labelling, 68
 Immunolocalization, 226, 238
 Inhibitor, 266
In situ hybridization, 222, 238
 Intercalary meristem, 229
 Intercellular transport, 203
 International Rice Research Institute (IRRI), 18, 24
 Interveinal distance, 149, 151–152
 Intracellular pH, balance of, 285
 Invasiveness, 391
 IRRI. *See* International Rice Research Institute
Isoetes howellii, 74
 Isotope discrimination, 140

J

Jasmonate, 116

K

K_c. *See* Michaelis–Menten constant for CO₂ (K_c)
 k_{cat}, 164, 281

K_m , 283, 285, 291
 K_m (CO_2), 69
 K_m (malate), 69
 Kranz, 29–56, 64, 133
 Kranz anatomy, 30, 100, 148, 293–294
 C_4 types
 angustifolioid: NAD-ME, 45
 aristidoid: NADP-ME, 35
 arundinelloid: NADP-ME, 34–35
 atriplicoid: NAD-ME & NADP-ME, 40–41
 chlorocyperoid: NADP-ME, 37–38
 classical NAD-ME type (Poaceae), 32–33
 classical NADP-ME type (Poaceae), 32–33
 classical PEP-CK type (Poaceae), 34
 eleocharoid: NAD-ME, 39
 eriachneoid: NADP-ME, 35–36
 fimbristylloid: NADP-ME and NAD-ME, 37
 glossocardioid: NAD-ME & NADP-ME, 43–45
 isostigmoid: unknown, 45
 kochioid: NAD-ME & NADP-ME, 41
 Kranz-Tecticornoid: NAD-ME, 43
 neurachneoid: NADP-ME and PEP-CK, 36
 portulacelloid: NADP-ME, 43
 rhynchosporoid: NADP-ME, 39
 salsinoid: NAD-ME, 42
 salsoloid: NAD-ME & NADP-ME, 41–42
 schoberioid: NAD-ME, 42–43
 simplicifolioid: NADP-ME, 45
 stipagrostoid: NADP-ME, 35
 trioid: NAD-ME, 36
 C_4 without Kranz (*see* Single cell C_4)
 mestome sheath (MS), 35
 Suberin lamella, 31
Kyllinga, 325–326
Kyllingiella, 325–326

L

Lagarosiphon, 72, 73
 L. major, 65
 Latitude, 168
 Leaf area index (LAI), 387
 Leaf development, 150
 C_4 leaves, 147–155
 Leaf developmental gradient, 230
 Leaf dry mass per area (LMA), 130, 132–134, 137, 138
 Leaf primordium/primordia, 226, 229, 230, 237
 Leakiness, 130, 140, 141
Leptochloa, 327

Life cycle analyses (LCAs), 389
 Light, 233, 241, 242, 246
 activation, 68, 72, 305
 harvesting, 175
 intensity, 166
 limitations in photosynthesis, 179, 181, 183, 187
 low temperature studies, 183
 use efficiency, 381–382
 Light/dark regulation, 305–310
 Light-grown, 232
 Lineage, 226, 242
Lipocarpha, 325–326
 Little bluestem (*Schizachyrium scoparium*), 389
 LMA. *See* Leaf dry mass per area
 Low-input high-diversity (LIHD) mixtures, 389–391
 L-protein, 90–91
Lycopersicon esculentum, 294

M

Macroalga, 75
 Macrofossils, 343
 Magnetic crescograph, 6
 Magnetic radiometer, 6
 Maize, 18, 24, 69, 70, 116–122, 151, 152, 154, 222, 230, 233–235, 240, 241, 244–246, 260, 262, 269, 281, 303–307, 309–313, 380, 385
 Malate, 7, 14–15, 65, 70, 260, 262, 266, 268
 exchange at the chloroplast, 205–206
 import, 206–207
 import into chloroplast, 207–208
 inhibition, 68
 oxidation, 280
 Malate dehydrogenase, 14, 67, 289
Mallotus
 M. paniculatus, 386
 Marker assisted breeding, 388
 MCs. *See* Mesophyll cells
 Mehler reaction, 95
Mel1, 232
 MEM1, 271
 MEM-1. *See* Mesophyll expression module 1
 β -Mercaptopicolinic acid, 72
Mesembryanthemum crystallinum, 284
 Mesophyll, 65, 74–75, 263, 269
 Mesophyll cells (MCs), 278
 C_4 gene expression, 221–249

Mesophyll expression module 1 (MEM-1), 244, 270
 Metabolic, 239, 241
 enzymes, 231
 Metabolite effectors, 310
 Metabolite transport, 200, 201, 203, 208, 210, 214
 Methionine, 114
 Michaelis–Menten, 68
 Michaelis–Menten constant for CO₂ (K_c), 136
 Michaelis–Menten kinetic, 263
 Micrairoideae, 326
 Microarray analyses, 234
 Microelectrode, 66
 Microfossils, 343–344
Mimosa pudica, 4
 Miocene, 23, 75, 340, 342–354
Miscanthus, 18
 M. floridus, 386
 M. ogiformis, 386
 M. sacchariflorus, 386
 M. sacchariflorus var. *brevibaris*, 386
 M. sinensis, 386
Miscanthus × *giganteus*, 305, 382, 384, 386
Miscanthus spp., 381
 Mitochondria, 86
 inner membrane, 200
 outer membrane, 200
 Mitochondrial, 240
 enzymes, 240
 genomes, 240
 Molecular markers, 388
 Monocots
 C₄ origin, 319–334
 Monoecious, 65, 73
Moricandia, 98, 100
 MP-specificity elements, 244
 mRNA processing, 226
 mRNA-protein interaction, 247–248
 mRNA stability, 223, 239, 246
Muhlenbergia, 327
 Multi-lineage, 234
 Multiple levels of regulation, 223, 243, 249
 Mutagenesis, 281
 Mycorrhiza, 110
Myriophyllum
 M. brasiliense, 76
 M. heterophyllum, 76
 M. spicatum, 69, 76

N

NAD-malic enzyme (NAD-ME), 277
 subtype, 363
 NAD-malic enzyme biochemistry
 Kranz type, 37–45
 NAD-ME, 223, 231, 232
 NAD-MEL, 231–232
 NAD-MES, 231
 NADPH, burst of, 284, 285
 NADPH-MDH, 305
 NADP-malic enzyme (NADP-ME), 21, 231, 234, 239, 242, 277, 304
 compartmentation of reactions, 203–209
 species, 65
 subtype, 363
 transport, 203–209
 NADP-malic enzyme/NAD-ME, 64, 69, 74, 75
 NADP-malic enzyme type biochemistry
 Kranz type, 34–45
 Single-cell C₄: terrestrial, 34–45
 N assimilation, 121
 N budget, 129, 134–136
Nelmesia, 325
Nemum, 325
 Neo-functionalization, 264
Neostapfia colusana, 74
 Net energy balance (NEB), 391–392
Neurachne munroi, 332
Nicotiana tabacum, 286
Nitella, 76
 Nitrate, 110–112, 120–122
 assimilation, 93
 reductase, 111, 294
 reduction, 114
 transport, 112
 transporters, 110, 111
 Nitrite, 112, 113, 121
 Nitrite reductase (NR), 111, 120
 Nitrogen, 15, 110–114, 243
 assimilation, 21
 fixation, 385–386, 389
 metabolism, C₄, 109–122
 Nitrogen use efficiency (NUE), 21, 66, 129–143, 383
 Nodules, 110, 112
 Non-leaf tissues, 249
 Non-photosynthetic ancestors, 223, 237
 Non-photosynthetic PEPC, 263, 264, 266
 NR. *See* Nitrite reductase
 Nuclear, 222, 223, 232, 233, 240, 243, 245

NUE. *See* Nitrogen use efficiency

N uptake, 120

N use efficiency, 113, 120

Nutrient use efficiency, 122

O

2-Oxoglutarate, 111

O₂ inhibition, 65, 70

Oligocene, 17, 343, 345–346, 349–351

Oligomeric states, 282

One-carbon metabolism, 94–95

Orchidaceae, 320

Orcuttia

O. californica, 63, 74

O. viscida, 63, 68, 74

Organelle, 222, 240, 243

Origin, 345–347

C₄ photosynthesis, 319–334

Orthologs, 240

Oryza sativa, 258, 270, 284, 289, 290

Oscillating recorder, 6

Overcycling, 201

Oxaloacetate (OAA), 67, 70, 260

transporter, 205

transport into chloroplast, 205–206

Oxidative stress, 83, 116–119

Oxycaryum, 325–326

P

p⁴⁷, 248

Paleoclimate hypotheses, 349, 352

Panicoideae, 23, 327–332

Panicoideae/Arundinoideae/Chloridoideae/

Centothecoideae/Micraioideae/

Aristidoideae/Danthonioideae

(PACCMAD), 326

Panicum, 100, 152

P. dichotomiflorum, 288

P. maximum, 113, 291, 294

P. miliaceum, 269

P. virgatum (switchgrass), 18, 24

PAT. *See* Polar auxin transport

Patatin promoter, 289

Pennisetum purpureum (Napier grass),
385

PEP. *See* Phosphoenolpyruvate

PEPC. *See* Phosphoenolpyruvate carboxylase

PEP-carboxykinase type biochemistry

Kranz type, 36

PEP carboxylase (PEPC), *see*

Phosphoenolpyruvate carboxylase

PEPC inhibitor, 67

PEPCK, PEPC-kinase. *See* Phosphoenolpyruvate
carboxykinase 261, 294, 371

PEPC kinetics, 68

PEPC phosphatase, 371

PEP synthetase (PEPS), 374

Perennial grasses, 380

Perennial rhizomatous grasses (PRGs), 381

Permo-Carboniferous, 346

Peroxisome, 86–87, 92

Pheidochloa, 326

pH gradient, 207

Phosphoenolpyruvate (PEP), 17, 64, 67, 68, 260
regeneration, 176, 305

Phosphoenolpyruvate carboxykinase (PEPCK), 72,
75, 277

subtype, 363

Phosphoenolpyruvate carboxylase (PEPC), 22, 64,
65, 67–68, 72, 75, 176, 222, 223, 235–238,
242, 258, 261, 263, 266, 278, 294, 302,
304, 310, 341, 364, 371–372

Phosphoenolpyruvate phosphate translocator
(PPT), 205

2-Phosphoglycolate, 85–86

Phosphorus, 15

Photoassimilate, 241

Photoinhibition, 70, 83, 87, 175, 183

Photophosphorylation, 305

Photorespiration, 20, 65, 76, 81–103, 177, 208,
362–363

energetics in C₄, 48

Photosynthesis Recorder, 6

Photosynthetic, 241–242

form, 244

function, 241, 244

Photosynthetic leaf water use efficiency (W), 138,
139, 141

Photosynthetic nitrogen use efficiency (PNUE),
130–134, 136, 383

Photosystem I (PSI), 239, 240

Photosystem II (PSII), 21, 70, 134, 135, 239,
240, 280

Phylogenetic history, 165

Phytochrome, 230

PIN proteins, 150

Plant movements, 4

Plasmodesmata, 21–22, 148, 153–154,
203, 364

Plasticity, 154

Pleurostachys, 323
 Pliocene, 340, 342, 343, 345–349, 351, 353
 PNUE. *See* Photosynthetic nitrogen use efficiency
 Poaceae, 31–37, 63, 74, 320, 326–333
 aristoideae, 32, 34
 chloridoideae, 32, 34
 C₄ types
 aristidoid: NADP-ME, 32, 35
 arundinelloid: NADP-ME, 34–35
 classical NAD-ME type, 32
 classical NADP-ME type, 32
 classical PEP-CK type, 34
 eriachneoid: NADP-ME, 35–36
 neurachneoid: NADP-ME and PEP-CK, 36
 stipagrostoid: NADP-ME, 35
 trioid: NAD-ME, 36
 micrairoideae, 32, 34
 panicoideae, 32, 34
 Polar auxin transport (PAT), 150
 Polysomes, 246, 248
 Positive selection, 266
 Postembryonic cotyledons, 229
 Post-illumination burst, 82, 95–96
 Post-transcriptional, 223, 243, 245–247, 249
 Post-translational phosphorylation, 68
 Post-translational regulation, 301–313
 Potassium, 14
Ppc, 245
PpcA, 237
PpcA1, 237, 238, 243, 244
PpcA1-GUS, 237
PpcC, 237
PpcZm1, 238
PpcZm2, 238
 PPK. *See* Pyruvate orthophosphate dikinase
 PPK regulatory protein (PPK RP), 23, 305, 310–313
 P-protein, 89
 PR (proximal element), 243, 244
 Promoter, 269
 Promoter/enhancer, 243, 244
Proserpinaca palustris, 76
 Protein kinase/protein phosphatase, 302
 Protein phosphatase, 292
 Protein phosphatase 1, 313
 Protein phosphatase 2A, 313
 14–3–3 Proteins, 112
 γ-Proteobacteria, 258
 Proteomic, 234
 analysis, 213
 Provascular centers, 230

Pycnus, 325–326
 Pyridoxal-5-phosphate (PLP), 89
 Pyrophosphatase, 309
 Pyruvate, 67, 71
 dehydrogenase, 94
 export, 207
 import, 207–208
 kinase, 260
 sodium dependent transport, 207–208
 Pyruvate orthophosphate dikinase (PPDK), 63, 67, 70, 260, 289, 301–313, 369
 Pyruvate orthophosphate dikinase regulatory protein (PDRP), 374
 Pyruvate phosphate dikinase (PPDK), 23, 223, 235, 236
 Pyruvate Pi-dikinase (PPDK), 176, 181–182
 quantum yield of (Φ_{PSII}), 179–180
 response of photosynthesis, 163

Q

Quantitative elements, 244
 Quantitative traits, 155
 Quantum yield, 71, 141

R

Radiation use efficiency (RUE), 382
 Rainfall, 142
 Raman, Sir Chandrasekhara Venkata, 4
Ranunculus peltatus, 76
 Rate-limiting enzyme, 304–305
 Ratio of adenylates, 292
rbcL, 225, 240, 243, 246
RbcS, 225, 243, 246, 247
 Reactive oxygen species (ROS), 116
 Recombinant, 269
 enzyme, 311
 protein, 281
 proteins, 69
 Redox regulation, 235
 Reducing equivalent shuttle, 205, 208
 Redundancy, 243
 Reed canary grass (*Phalaris arundinacea*), 381
 3' region, 283
 5' region, 283
 Regulatory factors, 249
 Regulatory phosphorylation, 262
 Regulatory processes, 249
 Regulatory protein (RP), 305–313
 Regulatory sequences, 246, 249

Remirea, 325–326
 Renewable bioresources, 380
 Reporter gene, 243, 246
 Resonant recorder, 6
 Resource use efficiency (RUE), 381, 382
 Respiration, 14
 Reverse-transcription PCR, 284
 Reversible phosphorylation, 260, 291, 305–310
Rhynchospora, 323
 Ribulose biphosphate carboxylase-oxygenase, 63, 64
 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), 20–22, 64, 82, 84, 85, 96–99, 101, 175, 222, 225, 230, 231, 263, 278, 304, 340–341, 362–365, 369
 activase, 175, 235
 RuBP regeneration, 175–176
 type II, 363
 Rice, 24, 70, 72, 85, 91, 151
 C₄, 361–375
 cab promoter, 283
Ricinus communis, 259, 284
 RNA-antisense, 305
 RNA binding proteins, 246, 248
 ROS. *See* Reactive oxygen species
 Rossman fold, 282
 RP. *See* Regulatory protein
 RT-PCR, 232
 Run-on transcription, 246

S

Saccharum officinarum, 380
Sagittaria, 323
 S. eltonica, 267
 S. latifolia, 74
 S. linifolia, 267
 S. subulata, 63, 74
Sartidia, 326
 SbtA, 372–373
 Sedges, 74
 Senescence, 283
 Ser hydroxymethyltransferase (SHMT), 88, 91
 Serine, 114, 121
 Serine/Ser C₄-signature, 63, 68
 SHMT. *see* Ser hydroxymethyltransferase
 Sigmoid saturation kinetic, 263
 Signaling, 243

Single cell C₄, 19, 20, 222
 aquatic plants, 63–77
 Single cell C₄, terrestrial, 29–56
 Bienertia cycloptera, 49–53, 55
 Bienertia sinuspersici, 50, 51, 53
 Bienertia (common name), 49–55
 Bienertioid anatomy, 49–55
 biogeography, 49–50
 C₄ Type Carbon Isotope Composition, 51–52
 development, 53
 dimorphic chloroplasts, 52–54
 evolution, 55–56
 phylogeny, 49, 50
 physiological response, 52
 salinity, 175
 spatial compartmentation, 53
 Suaeda aralocaspica, 49, 51, 53
 Borszczowia (section/common name), 49–55
 Borszczowoid anatomy, 49–55
 Single-cell model, 367–368
 Sink-to-source transition, 228, 230, 232, 236, 241
 Site-directed mutagenesis, 68
 Sodium-dependent pyruvate transport, 207
 Soil organic carbon (SOC), 390
Solanum tuberosum, 289
Sorghum, 18, 119, 235, 239, 246, 269, 281, 385
 S. bicolor, 258, 259, 285, 286, 385
 S. sudanense, 113
Spartina, 327
 S. anglica, 292, 294, 385
 S. cynosuroides, 383
 Specificity factor, 68–69, 85, 97, 98
Sphaerocyperus, 325–326
Sporobolus, 327
 Stability, 248
Steinchisma hians, 332
Stipagrostis, 326
 Stomata/Stomates, 65, 185
 Stomatal conductance (g_s), 138–140, 142
 Stomatal guard cells, 290
 Stress, 242
 Stromal pH, 310
 Stromal redox state, 310
 Structural diversity, 31–45
Suaeda aralocaspica, 265, 267, 367, 368
 Suberin lamellae, 153–154

Submerged form/Submersed leaf, 74–75, 231
 Substrate K_m , 304
 Substrate saturation constants, 266
 Subunits, 235
 Suc-P synthase, 294
 Sucrose, 19–20
 phosphate, 14, 15
 Sugarcane, 13–16, 18–20, 24, 235, 281, 395
 ripening, 14
 Sulfate, 114, 121
 assimilation, 116–118, 120–122
 reduction, 115
 transporters, 114
 Sulfide, 114, 121
 Sulfite, 114, 121
 reductase, 114–116
 Sulfur
 assimilation, 21
 metabolism, C_4 , 109–122
 reduction, 21
 Sulfur use efficiency, 121, 122
 Switchgrass (*Panicum virgatum*), 381, 387
 Symplastic transport, 203
 System data, 155

T

Tartronic semialdehyde reductase, 372
 T-DNA-mutagenesis, 92, 117
 Tef, 18
 Temperature, 21, 136, 139, 142, 143
 C_4 photosynthesis, 161–188
 thresholds of occurrence, 169–170
 Terrestrial form, 231
 Tetrahydrofolate (THF), 82, 88
 Tetrameric PEPC holoenzyme, 263
Thalassiosira
 T. pseudonana, 72
 T. weissflogii, 63, 71–72, 75
 Thermogenesis, 231
 Thermotolerance, 83
Thermus thermophilus, 290–291
 Thiol-modifying reagents, 291
 Thioredoxin, 88
 Tobacco, 312
 Tomato, 285
 T-protein, 89, 90
 Trans-acting factors, 245
 Trans-acting regulatory, 245

Transcription, 93, 223, 239
 Transcriptional, 243, 249
 control, 263
 repressor, 270
 Transcriptomics, 213
 Transgenic C_4 plants, 223, 237, 245, 304
 Transgenic maize, 269
 Transgenic plants, 243
 Transgenic rice, 269, 371
 Transient expression, 247
 Transit peptide, 313
 Translation, 248
 Translational elongation, 246
 Translational initiation, 246
 Transpirograph, 6
 Transport processes, C_4 , 199–214
 Trans-regulatory factors, 269
 Tricarboxylic acid (TCA)
 cycle, 94, 285
 Triose-phosphate, 14
 Triose-phosphate phosphate translocator (TPT), 201, 204, 208
Tristachya, 326
Trypanosoma cruzi, 290–291
Tuctoria greenii, 74
 Tundra, 168
 VPD effect, 185
 Two cell model, 368

U

Udotea, 75
 U. flabellum, 63, 72
 Untranslated regions (UTRs), 246, 247
 Uptake, 110
Urochloa panicoides, 244, 288, 291, 370–371
 UV crosslinking, 248

V

Vallisneria spiralis, 323
 Variations, 231
 Vascular centers, 238, 242
 Vascular tissue, 285
 Vascular transport, 228
 Vegetative organs, 285
 Vein density, 152
 Vein ontogeny
 Arabidopsis, 150
 other species, 151

Venation, 148, 149

Vitis vinifera, 259

W

Water splitting complex, 239

Water stress, 75

Water use efficiency (WUE), 21, 64, 130,
138–143, 383

X

Xanthophyll cycle, 183

Y

Yeast one-hybrid, 245

Z

Zea mays, 267, 380, 384

Zinnia, 150

ZmChlMe1, 233

ZmChlMe2, 233

ZmGlk1, 245

Zostera marina, 323

Zoysia japonica, 270, 292, 294

Zoyzia, 327