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The Plant Cytoskeleton: a Key Tool for Agro-Biotechnology

Edited by
Yaroslav B. Blume
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The Plant Cytoskeleton: a Key Tool for Agro-Biotechnology

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PREFACE

Essential processes in biology such as cell and nuclear division, development, intracellular transport and physiological response, rely on the perception of environmental and intracellular signals and their transduction to subcellular targets. The mechanisms by which these signals are received by cells and transduced towards the proper targets by cytoskeletal components constitute one of the most important and rapidly developing areas in modern plant biology. In addition, fundamentally important responses of plants to biotic and abiotic factors also involve signalling to and through the cytoskeleton, which helps explain the current interest of biotechnology in this field of fundamental research. Manipulation of cytoskeletal components, the microtubules and microfilaments, had, until recently, not been a priority issue for plant biotechnology. However, given the fundamental role of the cytoskeleton during plant growth and development, the potential for biotechnological applications is immense.

The NATO Advanced Research Workshop, "**The Plant Cytoskeleton: Genomic and Bioinformatic Tools for Biotechnology and Agriculture**" was held in Yalta, Ukraine, from September 19 to 23, 2006 – which continued the tradition of the first two International Symposia "Plant Cytoskeleton: Molecular Keys for Biotechnology" (Yalta, Ukraine, 1998) and "The Plant Cytoskeleton: functional diversity and biotechnological implications" (Kiev, Ukraine, 2002). This meeting represented efforts by scientists specializing in the field of plant cytoskeleton research and its practical implications to focus on the most-recent aspects of cytoskeletal investigations, to present state-of-the-art research, and to discuss how this knowledge and understanding could help advance various biotechnological applications. This book was inspired by the topics raised and discussed at the last Plant Cytoskeleton Conference, and these have been further expanded and updated.

The cytoskeleton, both microtubules and microfilaments, is involved one way or another in most of the important cellular events such as mitotic and meiotic division, cytokinesis, molecule and organelle trafficking, cell wall deposition, gravity perception, cell motility, etc. For this reason, these components (especially microtubules) are very attractive targets for various

chemically or biologically active compounds (such as herbicides, fungicides, anticancer drugs) and physical factors (i.e. cold stress, gravity, irradiation, etc.). The cytoskeleton also participates in the interaction of plant cells with pathogens such as viruses, fungi and bacteria. With this in mind, it can be assumed that directed influences (including the use of genetic engineering) on the main structural components of the cytoskeleton could be powerful tools for the development of improved germplasm and new commercial products. Such an approach can be effective for producing herbicide-, virus- and fungus-resistant crops, for the development of highly efficient procedures for polyploid cell production, for enhancing the effectiveness of embryogenesis, for influencing cold-resistance of plants, for manipulating protoplast fusion, for increasing the recovery rates of various secondary metabolites from plant cell cultures, etc.

The scope and aim of this book is to introduce some cytoskeletal-mediated key processes that provide potential targets for biotechnological manipulation by genetic, agricultural, pharmacological or ecophysiological approaches. It provides an up-to-date and critical overview of many aspects of this exciting and rapidly expanding field.

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PART 1

CYTOSKELETON FUNDAMENTALS

PLANT GAMMA-TuSC-LIKE COMPONENTS: THEIR ROLE IN MICROTUBULE NUCLEATION

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Abstract. The molecular mechanisms of microtubule nucleation are still poorly understood in higher plant cells. We have isolated and characterized plant homologues of the γ-Tubulin Small Complex (γ-TuSC) components and studied their activity in microtubule nucleation. In tobacco BY-2 cells, γ-tubulin and Spc98p/GCP3 were colocalized by immunolabelling at the nuclear surface which was functionally demonstrated as a dispersed microtubule nucleating site. Spc98p/GCP3 is not associated with γ-tubulin along microtubule arrays. Transient expression of AtSPC98/AtGCP3:GFP targeted the fusion protein to three main cellular domains: cortical sites at the plasma membrane, the nuclear surface and intranuclear double dots in G2. These data suggest that higher plants possess multiple sites of microtubule nucleation and that the nucleation activity of γ-TuSC-like complexes is under specific cell cycle control.

Keywords: γ-Tubulin Small Complex (γ-TuSC), plant homologues, tobacco, BY-2, microtubule nucleation

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1. Introduction

The microtubular cytoskeleton is formed by the assembly of α-/β-tubulin heterodimers into polarized polymers. It fulfills either identical functions in animal and plant cells e.g. chromosome segregation, or specific mechanisms e.g. those leading to cytokinesis. Cytoskeleton activity is based on microtubule dynamics and reorganization during the cell cycle and developmental processes. For example, an intense microtubule assembly is revealed at the equatorial region of dividing plant cells, during centrifugal expansion of phragmoplast microtubules which drive cell-plate development toward mother cell walls. The microinjection of fluorescent tubulin[1] or specifically stainable tubulin[2] has shown an extremely rapid incorporation of exogenous tubulin within the polymers. Furthermore, taxol treatment which blocks microtubule disassembly,[3] as well as colchicin treatment which blocks microtubule assembly,[4] inhibit cell wall expansion, reinforcing the link between microtubule dynamics and function. Molecular motors are involved in the transit of various cargoes on microtubules e.g. Golgi derived vesicles on phragmoplast microtubules,[5] and microtubules themselves translocate within the cytoplasm,[6] leading to modifications in the cytoskeleton organization.

In most eukaryotes, the interphase microtubule network is, for a major part, organized in a radial fashion from the microtubule organizing centre, which in animal cells consists of a pair of centrioles – the centrosome – surrounded by an electron-dense pericentriolar material, and which corresponds in fungi and yeasts to the Spindle Pole Body (SPB), a specialized part of the nuclear envelope.

In plant cells, different microtubule arrays succeed one another during the cell cycle (Figure 1), and no structured organizing centre is present. Cortical microtubule arrays forming microtubule sheets beneath the plasma membrane (A), pre-prophase band microtubules with radiating ones emerging from the nuclear surface (B, C) and phragmoplast microtubules (F) are not found in other eukaryotes. The dynamic and continuous reorganization of each plant microtubule array raises the fundamental questions of how and where plant microtubules are assembled and how microtubule orientation is controlled.

Although this debate has been the centre of several reviews for the last few years[7–12] the recent identification of molecular mechanisms that control plant microtubule nucleation sheds a completely new light on this process.[2,13–17] In the present review, we will integrate the current knowledge on plant γ-TuRC components through the identification and/or functional characterization of proteins that control microtubule nucleation and spatial distribution. This concerns:

- The identification of plant homologues of γ-tubulin complexes (γ-TURCs) and both the molecular and functional characterization of their components.
- The intracellular distribution of these components and their use as markers for microtubule nucleation sites, and
- The analysis of mutants affected in microtubule assembly and distribution.

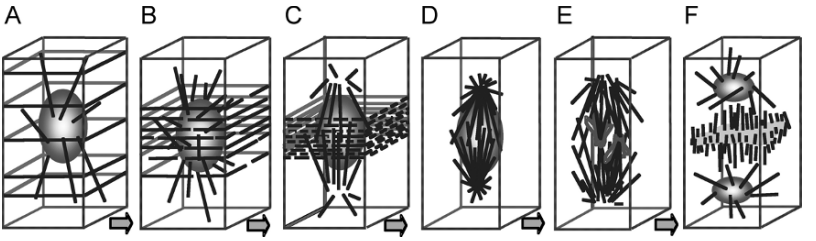

Figure 1. Reorganization of the plant cytoskeleton throughout the cell cycle.
Cortical microtubules (A) progressively shorten and rearrrange into the pre-prophase band (C). Simultaneously, the density of perinuclear microtubules increases in G2 (B). Spindle microtubules begin to surround the nucleus and focalize at poles (C, D). Then, after nuclear envelope breakdown, kinetochore microtubules and interpolar microtubules assemble, poles spread during prometaphase (E) and later re-focalize during anaphase. During telophase, the centrifugal expansion of phragmoplast microtubules leads to cell plate formation, and perinuclear microtubules nucleate around the new daughter nuclear surfaces (F).

2. Microtubule nucleation complexes

In vitro microtubule assembly occurs with expenditure of energy supplied by GTP and depends on the concentration of α-/β-tubulin dimers and adequate buffered medium. Critical GTP-tubulin concentration for autoassembly has been estimated at 18 ± 2 μM.[18] The hydrolysis of GTP is also associated with the formation of linear oligomers of tubulin, which are precursors in microtubule assembly.[19] However, γ-tubulin, a member of the tubulin superfamily has first been identified in *Aspergillus nidulans*,[20] and then found in all eukaryotes[21]; it is required for *in vivo* microtubule nucleation, when the α-/β-tubulin concentration is too low to promote autoassembly. *In vitro* translated monomeric γ-tubulin nucleates microtubules by lowering the size of the nucleus from seven to three tubulin subunits.[22] Nevertheless, *in vivo* microtubule nucleation depends not only on γ-tubulin but also on its associated proteins that structure the nucleating complexes. Cytoplasmic soluble complexes become active after their recruitment at specific sites and under cell cycle control.[23] This nucleation step precedes microtubule growth and shortening activities corresponding to dynamic instability.

2.1. THE GAMMA-TuSC: A MINIMAL NUCLEATING UNIT

γ-Tubulin Small Complexes or γ-TuSCs, with a sedimentation coefficient of 6S and a molecular mass of about 280 kDa have been identified in *Saccharomyces cerevisiae*[24,25] and *Drosophila*[26] so far. γ-TuSCs are considered as ubiquitous microtubule nucleators.[27] In *Saccharomyces cerevisiae*, two molecules of γ-tubulin associate with two Spindle pole components i.e. one molecule of Spc97p and one of Spc98p, forming a heterotetramer. Within the γ-TuSC, γ-tubulin interacts directly with Spc98p which itself interacts with Spc97p. The homologues of γ-tubulin, Spc97p and Spc98p have also been described in nucleating complexes of other cell types e.g. human cells in which they are called Gamma-tubulin Complex Proteins 1–3 (GCPs) respectively (Table 1).

In higher plants, γ-tubulin/GCP1, Spc97p/GCP2 and Spc98p/GCP3 homologues have been identified,[28] suggesting that γ-TuSCs may exist in plants. Moreover, these three proteins are part of a complex *in vivo* and interact with each other *in vitro*.[29] γ-Tubulin has been characterized in *Arabidopsis* in which two genes (TUG1 and TUG2) show a constitutive expression in all tissues,[30,31] but also in maize,[32] rice,[33] barley and tobacco.[34] In the latter case, the authors have shown that the γ-tubulin gene expression coincides with mitotic rather than post-mitotic cells. Low γ-tubulin RNA levels were found in elongating cells in which cortical microtubules reorganize, whereas α-tubulin RNA levels remain high, indicating a development-dependent regulation. A differential expression of two *Helianthus annuus* γ-tubulin isoforms has been characterize.[35] One isoform is expressed in all tissues whereas a smaller one is only detected in meristematic and dedifferentiated tissues where it is not bound to the nucleus. All these data suggest that γ-tubulin may be a multifunctional protein, playing a role in microtubule nucleation and dynamics. Comparisons between γ-tubulin genes show that they are conserved among higher plants and ferns,[36] with about 90% identity. Higher plant γ-tubulin sequences share 65–75% identity with those of vertebrates[37] and invertebrates.[38,39] With fungi, the sequence identity drops to about 60%.[40–42] The homologues of SPC97 and SPC98, identified in the *Arabidopsis* genome have been cloned from a genomic library.[13] At the protein level, *At*Spc98p/*At*GCP3 has been compared with partial sequences of rice and tobacco homologues as well as the other eukaryote counterparts.[2] As expected, amino-acid similarity is higher with mammalian Spc98p homologues than with others, suggesting that functional mechanisms have mainly been conserved through evolution.

TABLE 1. γ-Tubulin associates with various components characterized in *Aspergilus*, [46] *Xenopus*,[47] yeast,[23,24] *Drosophila*,[48] *Homo sapiens*,[49] mouse[49] and sheep.[50]

γ-TuSC					γ-TuRC					
S. cerevisiae	Drosophila		H. sapiens		Xenopus	Sheep	Mouse	A. nidulans	S. pombe	A. thaliana
Spc	Dgrip		GCP		Xgrip				Alp	Spc/GCP
						250				
		163	6	210	210	195	211		16	/6
		128	5	116	133	130	128			/5
98	91	91	3	97	109	105	101	105	6	98/3
97	84	84	2	97	110		100	95	4	97/2
		75s	4	66	75s	75	71	80	Gfh1p	75/4
		72			72					
Tub4p	γ-tubulin	γ-tubulin	1	γ-tubulin	γ-tubulin	γ-tubulin	γ-tubulin	γ-tubulin	Tug1p	γ-tubulin/1
					β-tubulin	β-tubulin				β-tubulin
					α-tubulin	α-tubulin				α-tubulin

2.2. THE GAMMA-TuRC: A STRUCTURED SEED CONTROLLING MICROTUBULE NUCLEATION

A large γ-Tubulin-Ring Complex (γ-TuRC), first identified in *Xenopus* egg extracts (Table 1), derives from the smaller complex by condensation and association with few other proteins.[43] Four proteins of respectively 72, 75, 133 and 210 kDa have been called *Xenopus* Gamma-tubulin ring proteins (*X*Grips). These additional components favour the formation of a ~25 nm wide core which coincides with the diameter of most microtubules.[44,45] This complex then seeds the microtubule growth from its minus end. Such γ-TuRC nucleation activity is 25-fold higher than that of isolated γ-TuSCs. γ-TuRCs show a sedimentation coefficient of 20–32S and molecular masses up to 2 MDa. Human γ-TuRC components have been called Gamma-tubulin Complex Proteins 1–6, in which GCP1 represents γ-tubulin. The so far identified non-tululin components of the γ-TuRC share structural motifs similar to GCP2 and GCP3, suggesting that they may belong to the same superfamily and arise from a common origin. Some of the complexes also associate with α- and β-tubulins.

In higher plants, a wide range of γ-tubulin containing complexes have been identified from various species in cytosoluble and membrane bound fractions. Large complexes (over 1,500 kDa) have been described in extracts of cultured maize cells and pollen[51] and of *Arabidopsis* and Fava bean.[52] Intermediate range complexes (400–900 kDa) and small complexes (280 kDa) were observed simultaneously; α- and β-tubulins were often associated to these complexes. These data suggest a sort of dynamic instability of γ-TuRC components or differential properties of γ-tubulin containing complexes which would not only be involved in microtubule nucleation.

3. Microtubule nucleation is a conserved process

Two models were proposed for microtubule nucleation by the γ-TuRC.[53,54] In one model, the γ-TuRC acts as a template in which 13 γ-tubulin subunits forming a ring interact with the bottom side of 13 α-tubulin subunits corresponding to the minus end of the nascent microtubule. In the second model, the γ-tubulin ring organizes perpendicularly and extends into a protofilament interacting laterally with α-/β-tubulin dimers to seed a bidimensional microtubule sheet which curves during its assembly into a microtubule. Nevertheless, γ-TuSC components are probably involved in nucleating microtubules and associated GCPs in folding the γ-TuRC, anchoring the nascent seed and modulating its activity. For example,

XGrip210 plays a role in both γ-TuRC assembly and the recruitment of γ-tubulin and XGrip109 at the centrosome.[55] Figure 2 schematizes a currently favoured model in which γ-TuSC components are closer to the microtubule end than the other γ-TuRC proteins which would form a microtubule cap.

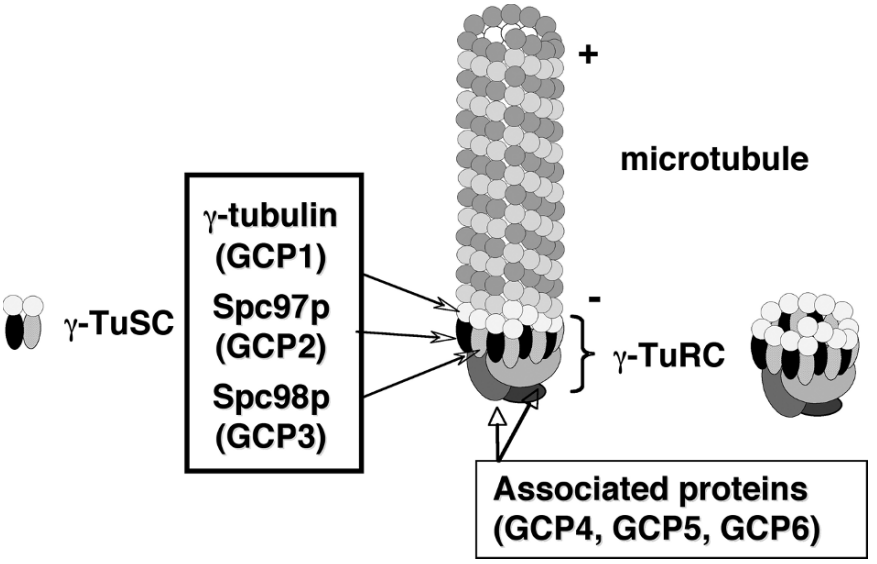

Figure 2. Microtubule nucleation by γ-tubulin containing complexes. Two molecules of γ-tubulin/GCP1, and one molecule of both Spc97p/GCP2 and Spc98p/GCP3 oligomerize in small complexes called γ-TuSCs. The addition of associated proteins (GCP4 to 6) to 6 or 7 γ-TuSCs structures a high molecular weight complex (γ-TuRC) which is able to nucleate microtubules.

3.1. PLANT EXTRACTS RESTORE THE NUCLEATING FUNCTION OF INACTIVATED CENTROSOMES

Tobacco BY-2 cytosol extract is able to induce a recovery of microtubule nucleating activity of urea inactivated mammalian centrosomes.[56] This indicates that plant complexes may complement depleted nucleating factors and activate the centrosome nucleating machinery.

However, the introduction of purified potential active centrosomes by microinjection in living *Haemanthus* cells did not induce ectopic microtubule nucleation around them, neither in interphase nor in mitosis (Figure 3). This indicates a specific regulation of nucleation sites *in vivo*.

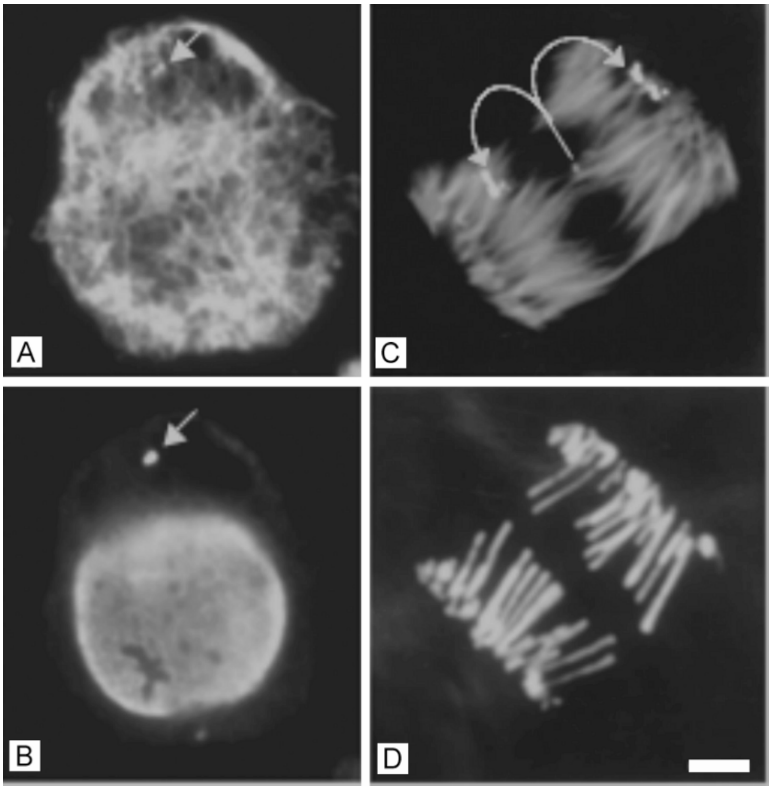

Figure 3. Microinjection of mammalian centrosomes into *Haemanthus katherinae* endosperm cells.
Interphase cell labelled with anti-α-tubulin (A) and mAb 6C6 antibodies (B), which reveal MTOC sites (the injected centrosomes and the plant nuclear surface). Injected centrosomes do not nucleate microtubules.
Anaphase cell fixed 40 min after injection in metaphase (C, D). The merged image of centrosome staining using mAb 6C6 and anti-α-tubulin antibodies (C) show that the injected centrosomes migrated in the cytoplasm (arrows) but did not inhibit mitosis progression. No microtubules were nucleated in their close vicinity. Moreover, they seem to be excluded from the centrosomal areas. (D) Corresponding DAPI staining of chromatids. Bar = 20 μm.

3.2. THE PLANT NUCLEAR SURFACE ACTS AS MICROTUBULE NUCLEATOR

The organization of perinuclear radiating microtubules in plant G2 cells has for a long time suggested that in acentrosomal cells, the whole nuclear surface acts as a microtubule organizer. Using monoclonal antibodies directed against centrosomal epitopes (mAb 6C6), the labelling of the plant

nuclear surface is an additional argument for this hypothesis[57] (Figure 3B). This has been functionally characterized by inducing microtubule nucleation at the surface of isolated nuclei, at a purified tubulin concentration below that necessary for spontaneous autoassembly (<15 μM).[58,59]

Furthermore, a pre-incubation of plant nuclei with specific anti-γ-tubulin or anti-Spc98p/GCP3 antibodies dramatically inhibits microtubule nucleation. Seventy percent to 96% inhibition has been obtained compared to non specific antibody pre-incubation, indicating that both γ-tubulin and Spc98p/GCP3 are essential for the nucleation process14. These data demonstrate the recruitment and activation of γ-tubulin and Spc98p/GCP3 containing complexes at the nuclear surface of interphase acentrosomal cells.

3.3. MEMBRANE-ASSOCIATED COMPLEXES SHOW A MICROTUBULE NUCLEATION ACTIVITY

γ-Tubulin has been shown to distribute in multiple maize cell sub-fractions whether soluble or membrane associated.[51]

Recently, various γ-tubulin containing complexes have been enriched in plant microsomal fractions from *Arabidopsis* and Fava bean.[52] High speed microsomal pellets contain 2 MDa complexes mainly, in which γ-tubulin associates with membranes. Nucleation assays have shown that these fractions induced microtubule assembly at a low tubulin concentration (0.5 mg/mL), suggesting that plant γ-TuRCs may anchor to membranes.

4. Tracking microtubule nucleation sites

4.1. CENTROSOMES AND SPBS

γ-TuRCs have been identified in the amorphous material that surrounds the centrioles, or in association with the outer and inner plates of the SPB, i.e. at sites where microtubules are initiated and generally remain anchored. These structured microtubule organizing centres[60] play a vital role, not only in nucleating microtubules, but also in determining their polarity. γ-Tubulin complexes are recruited in the pericentriolar material or anchored in the SPB by Spc110p,[25] where they nucleate cytoplasmic and/or intranuclear microtubules.

4.2. MICROTUBULE NUCLEATION IN ANIMAL CELLS POSSESSING INACTIVE CENTROSOMES

Various specialized animal cells, like epithelial cells and neurons contain arrays of non-centrosomal microtubules.[61] Microtubule release from the centrosome, breakage or severing with stabilization from non-centrosomal sites and/or nucleation at particular sites are some ways in which non-centrosomal microtubules can arise. Once formed, microtubules that are not tethered to the centrosome rearrange within the cytoskeleton through self-organization or capture. Minus ends may be stabilized, either by microtubule-associated proteins or by an end-capping complex. γ-Tubulin may participate in such a mechanism as suggested by its location in the mitotic spindle.[62]

The laser destruction of centrosomes in mammalian cells which enter in mitosis still leads to bipolar spindle formation, indicating that an alternative mechanism allows microtubules to assemble a functional bipolar spindle independently of centrosomes.[63]

Moreover, meiotic spindles in many oocytes, such as mouse,[64,65] *Xenopus*,[66] *Drosophila*[67] or *Caenorhabditis*[68] do not possess centrioles. And human myoblast differentiation into myotubes leads to a lack in active centrosomal structures.[69] The periphery of each nucleus in myotubes becomes labelled with centrosomal antibodies suggesting a major reorganization of microtubule-nucleating material soon after myoblast fusion. Regrowth experiments after Nocodazole treatment established that microtubules were growing from the periphery of the nuclei, mimicking what happens in higher plant cells.[69]

In spermatocytes of *Drosophila* asterless (*asl*) mutants, centrosomes do not nucleate microtubules and γ-tubulin is dispersed in small cytoplasmic aggregates without microtubule nucleating activity.[70] In an acentriolar *Drosophila* cell line, γ-tubulin still concentrates at spindle poles.[71] In such systems, microtubule nucleation may either occur from dispersed γ-TuRCs, as in plant cells, or through an alternative process in which chromatin and molecular motors may promote and organize the cytoskeleton.[72,73] This is also emphasized by the inhibition of spindle assembly in the absence of chromosomes.[74,75]

4.3. ARE KINETOCHORES NECESSARY FOR SPINDLE ASSEMBLY?

Centrosome-nucleated aster microtubules exhibit rapid growing and shrinking phases at mitosis onset. When they hit kinetochores, some microtubules may be captured and stabilized,[76] forming kinetochore fibres, a specialized population of spindle fibres. In the absence of centrosomes, spindle assembles according to a different pathway, first through the

nucleation of microtubules around chromatin, then by rearranging in a bipolar structure. In *Melanoplus differentialis*, the partial ablation of chromosomes leads to the reduction of spindle microtubules, proportionally to the chromatin mass removed, not to the number of kinetochores lost,[77] suggesting that chromatin possesses the ability to promote spindle assembly.

Furthermore, secondary spermatocytes devoid of chromosomes in *Drosophila fusolo* and *solofuso* mutants form large asters which give rise to 'functional' spindles and cell cleavage.[78]

Moreover, an artificial *in vitro* system has been established, in which the plasmid DNA containing no centromeric sequences is linked to magnetic beads and forms artificial chromosomes devoid of kinetochores.[79] Their incubation in *Xenopus* mitotic extracts triggers the formation of a bipolar spindle, indicating that neither centrosomes nor kinetochores are essential for its assembly.

All these results suggest that *in vivo*, the origin and activity of spindle fibres are balanced by both chromosomes and centrosomes, each one on its own being able to supplement the second in particular conditions.

Nevertheless, microtubule nucleation may be due to the activation of γ-TuRCs and regulated by various factors including motor and non motor MAPs.

4.4. ORGANELLE AND MEMBRANOUS NUCLEATING SITES

In fission yeast, a so-called equatorial microtubule organizing centre is involved in cytokinesis progression.[80] It contains γ-tubulin and Spc97p and organizes cytoplasmic microtubules after septation. γ-Tubulin, also present in the midbody of animal cells, is located at microtubule minus ends. Selective depletion of midbody γ-tubulin impedes cytokinesis.[81] Purified Golgi membranes bind α-, β- and γ-tubulin, and possess intrinsic microtubule nucleating properties.[82] The presence of centrosomal epitopes at the surface of Golgi vesicles[16] migrating towards the equator during cell plate formation suggests that membranous associated γ-TuRCs may be selectively switched on.

4.5. PLANT MICROTUBULE NUCLEATION SITES

For plant microtubule assembly, finding a selective marker is essential to identify functional nucleation sites in the absence of structured organizing organelles. Only few plant homologues of centrosomal proteins has been located so far, either using immunocytochemistry on fixed cells or by expressing GFP fusions.

4.5.1. *Interphase cells*

The immunolabelling of various proteins concentrated within the centrosome was used to check plant microtubule organizing centres. Mab 6C6 labelled the nuclear envelope[57]; Mab2E10 labelled equatorial vesicles during telophase[2]; γ-tubulin antibodies directed against various peptides showed microtubule arrays[30,83] or branch start points of cortical microtubules,[84] polar regions[85] and/or chromatin areas.[86] Spc98p/GCP3 antibodies did not label microtubules whereas the nuclear envelope and the plasma membranes became highlighted.[14]

By expressing GCP-GFP fusions, multiple sites were detected.[29] The transient expression of GFP-*Zm*TUG1 and *Zm*TUG1-GFP constructs in tobacco BY-2 cells revealed a fibrillary fluorescence overlapping endogenous microtubules (Figure 4A, C). No ectopic nucleation was induced by γ-tubulin overloading. The stable expression of maize TUG1 in tobacco did not reveal dramatic phenotypes. Plants showed fewer and larger cells and did not show fertility alterations. At the cellular level, GFP-TUG1 organized in linear arrays either formed by γ-tubulin polymers or surrounding microtubules which then became more difficult to label using anti α- or β-tubulin specific antibodies (Figure 4B, D).

The transient expression of GCP3-GFP (Figure 4E) did not colocalize with microtubule arrays, suggesting that they may be a better tool for the detection of nucleation sites. Three subcellular domains were identified: the nuclear envelope, the plasma membrane and double dots within the G2 nucleus.[2] These data reinforce the idea that cytosolic γ-TuRCs are recruited at dispersed subcellular sites which become activated under cell cycle controls as proposed in the comparative model (Figure 5).

A bipartite NLS sequence which is only conserved in angiosperms was characterized in *Arabidopsis* GCP3. This sequence was fused with GFP and chalcone synthase to produce a fusion protein with a molecular mass exceeding the nuclear pore exclusion limit. The expressed protein was specifically triggered within the nucleus. Nevertheless, as endogenous GCP3 was only partially addressed within the nucleus, with a peak in G2 phase,[2] the regulation of such an import remains to be investigated.

4.5.2. *Spindle microtubule assembly and cell plate development*

The presence of both γ-tubulin[86,2] and GCP3[2] within the nucleus, at prekinetochores, suggests that nucleating complexes may be associated to chromosomes, activated after nuclear envelope breakdown and be responsible for mitotic spindle assembly in acentrosomal cells. It will be of high interest to check the behaviour of both components during mitosis.

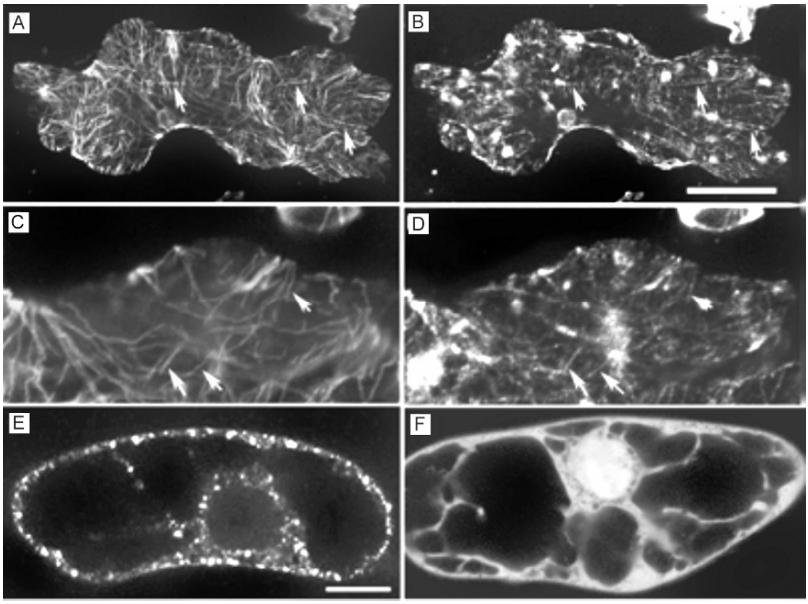

Figure 4. *In vivo* expression of ZmTUG1-GFP and AtGCP3-GFP in interphase tobacco cells. (A–D) Limb cell constitutively expressing γ-tubulin-flagM2 proteins. (A, C) Immunolabelling of α-tubulin revealed by Alexa 568 (B, D) Immunolabelling of transgenic γ-tubulin using anti-flagM2 antibodies. γ-Tubulin colocalizes with microtubule arrays (arrows). (E) In a tobacco BY-2 cell, transiently expressed AtGCP3 localizes at the nuclear surface and at the plasmalemma, suggesting that dispersed nucleating sites may be activated depending on cell cycle signalling. (F) Control expression of GFP which diffuses in the cytoplasm and the nucleus. Bar = 10 μm.

Cytokinesis is achieved by the centrifugal development of phragmoplast microtubules whose density suddenly increases during anaphase-telophase transition. The origin of these microtubular arrays is not completely demonstrated. Part of them derive from interzonal anaphase microtubules and others may nucleate from the daughter perinuclear regions and/or from still unidentified equatorial nucleation sites. The progressive centrifugal assembly of microtubules allows the polarized migration of Golgi derived vesicles towards the equator. The presence of specific kinesin related proteins (KRPs), i.e. AtPAKRP1 and AtPAKRP2 at microtubule plus ends and on vesicles respectively, suggests their role in microtubule rearrangement and vesicle transport.[5]

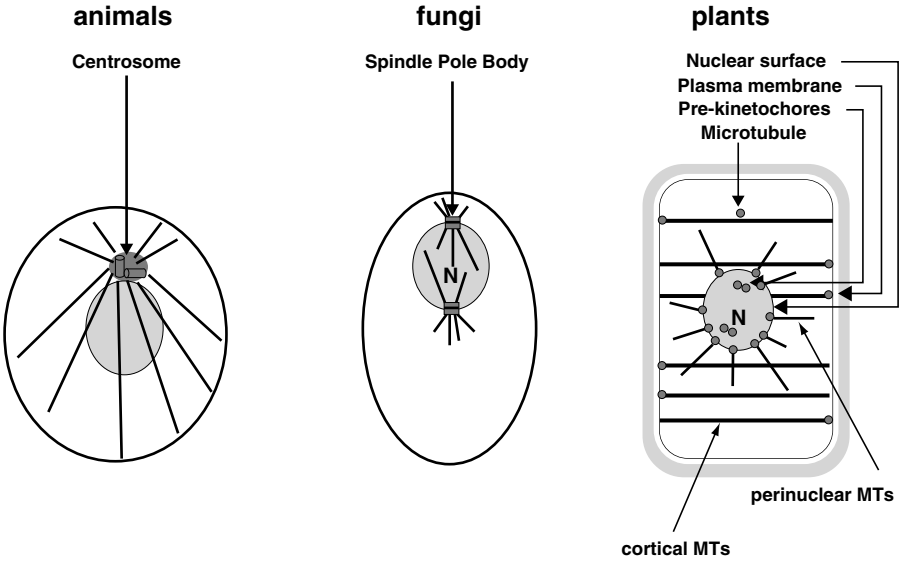

Figure 5. Microtubule nucleation sites in eukaryotes.
In higher plant cells, microtubule nucleation occurs at dispersed sites whereas structured organelles regulate the microtubule cytoskeleton in most animal and fungi cells all over the cell cycle.

5. Multiple functions of γ-TuSC components

Defects in γ-tubulin or SPC97/GCP2 and SPC98/GCP3 gene expression which did not abolish microtubule nucleation led to new cytoskeletal organization.

5.1. GAMMA-TUBULIN MUTANTS

Isolated centrosomes depleted of γ-TuRCs lose their ability to nucleate microtubules, but may be reactivated through the addition of purified γ-TuRCs.[87] γ-tubulin depletion or antibody inhibition also prevents microtubule nucleation from centrosomes in cultured mammalian cells and in *Xenopus* egg extracts.[88,89] Furthermore, the RNAi depletion of γ-tubulin in *Caenorhabditis elegans* showed defects in spindle organization and function,[90] and the inactivation of γ-tubulin genes in *Paramecium* inhibited basal body duplication.[91] Mutants, null for the γ-tubulin gene of *Aspergillus* failed in assembling spindles.[92] Nevertheless, γ-Tubulin mutants of *S. cerevisiae*, *S. pombe* and *Drosophila* impaired but did not completely block the assembly of mitotic spindles.[39,93–96] In *Arabidopsis* cells, γ-tubulin knock-out mutants are lethal at the gametophytic stage,[97] confirming its

essential role in microtubule nucleation and organization of all microtubular arrays.

5.2. SPC97/GCP2 AND SPC98/GCP3 MUTANTS

Temperature-sensitive *alp4* and *alp6* mutants in fission yeast show defects in Spc97p and Spc98p respectively. γ-Tubulin containing complexes still form in the cytoplasm but are not recruited at the SPB in G1 phase. Both mutants form monopolar spindles at restrictive temperature, suggesting that these proteins also play a role in SPB duplication.[98]

In *Drosophila*, mutants in the DGrip91 gene corresponding to the SPC98 homologue show abnormal spindles and abnormal γ-tubulin location,[99] suggesting a defect either in γ-TuRC assembly or recruitment.

6. Concluding remarks

The characterization of microtubule nucleating complexes from animals, fungi and plants reveals the conservation of at least γ-tubulin, Spc97p/GCP2 and Spc98p/GCP3 – the three γ-TuSC components – suggesting that similar mechanisms are involved in building a microtubule. Homologies between other components of animal and plant γ-TuRC components (GCP4 to 6) suggest similarities in anchoring γ-TuRCs to their recruitment sites, even if the location of these sites is specific to species. Recent convergent data demonstrate the importance of additional factors including importins and Ran GTPase in the regulation of microtubule nucleation.[100] It will be of particular interest to further characterize how they modulate spindle assembly in plant cells.

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GAMMA-TUBULINS AND THEIR FUNCTIONS IN PLANT CELLS

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Abstract. Higher plants represent an important group of eukaryotes where discrete sites for microtubule nucleation are absent in all mitotic and meiotic cells, and the concept of dispersed microtubule-nucleation sites has been generally accepted. Moreover, even in cells containing centrosomes or spindle pole bodies, microtubules can nucleate non-centrosomally but molecular mechanisms behind the process are so far poorly characterized. γ-Tubulin is a ubiquitous phylogenetically conserved member of tubulin superfamily, present within the cells in various types of microtubule-organizing centers and cytoplasmic protein complexes. Down regulation of γ-tubulin showed its essential role in non-centrosomal nucleation in plants. Plant γ-tubulin is localized with all microtubule arrays, and in nuclei. Protein complexes of γ-tubulin are present in soluble forms in cytoplasm and also with membranes. Association of γ-tubulin complexes with microtubules and dynamic membranes might ensure the flexibility of non-centrosomal microtubule nucleation. However the presence of various molecular forms of γ-tubulin suggests that, in addition to its nucleation role, additional roles for non- centrosomal γ-tubulin might exist.

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1. Introduction

Microtubules are highly dynamic cytoskeletal components that are essential for many cellular functions in eukaryotes such as intracellular organization, ordered vesicle transport and cell division. The basic building blocks of microtubules are heterodimers of globular α- and β-tubulin subunits. They are arranged in a head-to-tail fashion to form 13 protofilaments that constitute cylindrical microtubules with outer diameter around 25 nm. Microtubules are thus inherently polar, with α-tubulin at one end of the polymer, the (-) end, and β-tubulin at the other, the (+) end. Protofilaments are slightly offset from one another and form a "3-start" helix. This means that the helix spans three subunits of a protofilament before it completes one turn. There is a "seam" in the microtubule wall, where each helix makes a complete turn. While the protofilaments interact laterally with each other mainly through α-α and β-β contacts, at the seam an α-subunit interacts with a β-subunit.[1] Microtubule ends also differ in their assembly and disassembly characteristics. Tubulin subunits are added to the fast growing (+) ends of microtubules and are preferentially lost from the (-) ends. Microtubules can self-assemble *in vitro* from high concentrations of tubulin heterodimers in the presence of GTP and Mg^{2+}, but such microtubules are randomly organized. However, the concentration of αβ-tubulin heterodimers in cells is below the level needed for spontaneous nucleation *in vitro*. In spite of this, microtubules in cells are precisely organized. This is because microtubules are anchored in microtubule-organizing centers (MTOCs) and these organelles assist in microtubule nucleation.

2. Microtubule organizing centers

Though present in various cell types, MTOCs have different structural arrangements, but they have similar functions in initiating and maintaining the polar assembly of cellular microtubules. Centrosomes and basal bodies of cilia and flagella are the most frequently studied MTOCs in animal cells. The centrosomes are formed by an electron-dense fibrogranular pericentriolar matrix that surrounds two perpendicular short microtubule structures – the centrioles. Centrosomes are usually located in perinuclear regions, and microtubules emanate from centrosomal peripheral regions.[2] Microtubule polymerization is thus oriented, i.e. the (-) ends of microtubules are in MTOCs and the growing (+) ends are directed to the cell periphery. Microtubules can also arise from centrosomes without

centrioles. The structure of basal bodies resembles that of centrioles but, in contrast to centrosomes, microtubules grow directly from basal bodies.[3] In yeast, the MTOC is located in the nuclear membrane and is called the spindle pole body (SPB). Spindle pole bodies do not have centrioles, and in some aspects they present a highly abridged version of centrosomes. A three-layer electron-dense structure participates in the organization of Cytoplasmic, as well as nuclear microtubules. It is now becoming more obvious, that centrosomes, in addition to its fundamental role in microtubule nucleation, may also serve as an important structural context for coordinating cell cycle regulation.[4] Recently, studies identified coiled-coil proteins forming a framework to which microtubules and key regulators of centrosome behaviour are anchored have been described.[5] Consistent with recent data, a broader function and higher flexibility of centrosomes behaviour is suggested.

3. Non-centrosomal microtubule nucleation

Centrosomes in animal cells or spindle pole bodies in yeast are dominant organizers of microtubule nucleation and the formation of bipolar mitotic spindle. However, in most differentiated animal cells, as well as in fungi and higher plant cells, microtubules are arranged in noncentrosomal arrays. Several distinct mechanisms of formation of noncentrosomal microtubules depending on cell type are suggested: release from centrosome, catalysed nucleation at noncentrosomal sites or breakage of pre-existing microtubules.[6] In *Schizosaccharomyces pombe* microtubules can nucleate not only from spindle pole bodies but also from cytoplasmic interphase and equatorial microtubule organizing centers. In cells normally containing centrosomes, the spindle can be formed when centrosomes are experimentally removed.[7] In the oocytes of some species centrosomes are absent and microtubules are nucleated around the chromosomes.[8] In mitotic egg extracts, mitotic spindles can assemble around artificial chromosomes in the absence of centrosomes and kinetochores.[9] Current models of mitotic spindle formation without centrosomes are based on chromatin mediated microtubule organization.[10,11] To elucidate the principles of non-centrosomal microtubule nucleation in eukaryotes is currently one of the main tasks of molecular and cellular biology. Both somatic and meiotic higher plant cells are acentrosomal, and they therefore naturally may use the non-centrosomal nucleation pathway for microtubule organization and spindle assembly. Moreover, completed genome sequencing has shown that many proteins required for microtubule nucleation and organization are highly conserved. Plants thus provide a model system for studying the

microtubule nucleation from dispersed sites and the spindle formation in the absence of centrosomes.

4. γ-Tubulin in eukaryotic cells is present in multiple forms and its structure suggests multiple interactions

γ-Tubulin has a central role in microtubule nucleation by MTOCs. It was first discovered in the filamentous fungus *Aspergillus nidulans* as a product of the *mipA* gene during genetic screening for proteins that interact with β-tubulin.[12] Experimental depletion of γ-tubulin led to a depletion of microtubules and to growth arrest. Immunolocalization of γ-tubulin revealed an enrichment in MTOCs at the mitotic poles. On the basis of these observations it was proposed that γ-tubulin might play a role in microtubule nucleation.[13] Up to now γ-tubulin cDNAs and genes have been cloned and sequenced from a huge variety of organisms, and it seems certain that γ-tubulin is present in all eukaryotes. While the α- and β-tubulins are encoded by multiple families[14] only a limited number of γ-tubulin genes were found in the majority of organisms studied to date. For example, there are only two functional genes in *Arabidopsis thaliana*,[15] *Drosophila melanogaster*[16] and mammalian cells (TUBG1, TUBG2).[17,18] What might be the selective advantage of retaining two genes that code for very similar γ-tubulins is still unclear. One possibility is that their expression could be differentially regulated, and consequently the level of γ-tubulin expression would be increased in particular tissues or cells. For example, whereas TUBG1 in mice has been found ubiquitously expressed in all cell types, TUBG2 has been found mainly in brain, and its deletion did not affect viability of mice.[18] Alternatively, γ-tubulin may be so important that there is a selective advantage in functional redundancy.

γ-Tubulin forms differing in their electrophoretic mobility in one-dimensional electrophoresis were distinguished in *Physarum*,[19] *Drosophila*,[20] brain[21] and plants.[22] Multiple charge variants were detected by two-dimensional electrophoresis in brain,[21,23] chicken erythrocytes,[24] mammalian cell lines[25] as well as in protozoan parasites[26] and plants (2007 unpublished). These findings indicate that γ-tubulin, like the α- and β-tubulin counterparts, could be subject to posttranslational modifications. Phosphorylation of the mouse[27] and budding yeast γ-tubulin has been reported.[28] In *Saccharomyces cerevisiae*, γ-tubulin is phosphorylated in G1 phase of the cell cycle, and dephosphorylated in mitosis. A highly conserved tyrosine-455 residue near the carboxy-terminus is required for phosphorylation.[28] Deletion of this tyrosine-containing motif results in localization defects of microtubule plus-end-interacting proteins (+TIPS).[29] In *Drosophila* γ-tubulin is phosphorylated

by Wee1 kinase.[30] Also, human γ-tubulin is mono-ubiquitinated at lysines 48 and 344 by the ubiquitin-ligase complex BRCA1/BARD1.[31]

A comparison of γ-tubulin sequences with those of α- and β-tubulins revealed a similar secondary and tertiary structure, and implied that highly conserved regions among tubulins might be involved in the binding of GTP. In contrast to α- and β-tubulin subunits, which have on their C-terminal regions acidic amino acids residues that bind microtubule associated proteins (MAPs), γ-tubulin lacks a substantially acidic C-terminal end.[32] Little is known about the self-assembly of γ-tubulin and/or interaction of γ-tubulin with αβ-tubulin dimers and microtubules. As the high-resolution structural knowledge on αβ-dimer and tubulin-tubulin interactions in the microtubule wall is available, the sequence/structure analysis resulted in a structural model of possible interactions of γ-tubulin. γ-Tubulin is likely to contact the (-) end of α-tubulin and may associate laterally with α- or β-tubulin. γ-Tubulin should also be capable of self-assembling to form either a dimer or a protofilament-like oligomer.[33] Lateral association of γ-tubulin with the αβ-dimer has been suggested from a systematic search for αβ-tubulin binding sites on the γ-tubulin using the SPOT peptide technique.[34] The structure of human γ-tubulin has been determined at 2.7 Å resolution by crystallisation of γ-tubulin bound to non-hydrolysable GTP analogue and X-ray analysis.[35] Structural analysis revealed the potential of γ-tubulin to form lateral interactions in a curved conformation. This is a key feature of γ-tubulin in serving as a template for microtubule nucleation.[35]

γ-Tubulin has been for a long time believed to be a key functional component of MTOCs. Disruption of the γ-tubulin gene in *A. nidulans* caused a loss of mitotic spindle,[13] and microinjection of anti-γ-tubulin antibody into mammalian cells prevented the nucleation of new microtubules by the centrosome and spindle pole bodies and changed the morphogenesis of mitotic spindle.[36] Functional conservation of γ-tubulin was demonstrated in experiments in which human γ-tubulin was expressed in *S. pombe* with defective endogenous γ-tubulin.[37] In addition, *Xenopus* egg extract depleted of the γ-tubulin-containing protein complex lost much of its ability to support the microtubule asters originating from the centrosome.[38] On the other hand, overexpression of γ-tubulin in mammalian cells caused γ-tubulin accumulation in the cytoplasm, where it formed ectopic microtubule nucleation sites.[39] These and many other experiments provide convincing evidence that γ-tubulin is the key functional component of the MTOCs. Surprisingly, when γ-tubulin was depleted by RNAi in *Caenorhabditis elegans* embryos, centrosomal microtubular asters were formed.[40] However, another set of experiments with *C. elegans* embryos, which were severely compromised for γ-tubulin function, revealed that γ-tubulin was kinetically dominant centrosomal microtubule nucleator,

although unknown mechanisms could support a partial assembly of mitotic centrosomal asters.[41] γ-Tubulin has been found to interact with various proteins of pericentriolar material, such as pericentrin[42] and ninein.[43]

5. Subcellular distribution of γ-tubulin in acentrosomal higher plant cells

The intracellular location of γ-tubulin in spindle pole body (SPB) was first determined in *A. nidulans.* Immunofluorescence experiments on various cell lines of different tissue origin and derived from different species revealed that γ-tubulin in metazoan cells was located in centrosomes of interphase cells and concentrated in poles of mitotic spindles.[44]

γ-Tubulin was found along microtubules of mitotic spindles, yet was lacking on astral microtubules, and was also detectable in late telophase in midbodies.[45,46] Data from immunofluorescence examination were supported by *in vivo* analysis of GFP-γ-tubulin.[47] γ-Tubulin was further located in the core of centrioles.[48] The distribution of γ-tubulin in different stages of the cell cycle in mouse embryonal fibroblast cell line 3T3 is shown in Figure 1. γ-Tubulin is in basal bodies in multi-ciliated epithelial cells[49] as well as in ciliate[50] and flagellate protozoa.[51,52] The distribution of γ-tubulin in MTOC is independent of the presence of centrioles, as can be inferred from its detection in acentriolar centrosomes in fertilized oocytes[53] and in centrosomes of an acentriolar cell line of *D. melanogaster*.[54] It was also shown that centrosomal association of γ-tubulin remained also in the case when centrosomes were formed *de novo* in the absence of centrioles.[7] γ-Tubulin localization is very dynamic, and centrosome-bound γ-tubulin is in an exchange with a pool of soluble, cytoplasmic γ-tubulin that represents approximately 80% of the total cellular content of γ-tubulin.[25,47] A small fraction of γ-tubulin was also found in mammalian cells in nuclear speckles, interacting with DNA repair protein Rad51.[55]

In comparison with other eukaryotic cells, γ-tubulin in plants is more abundant and is localized in different cellular compartments. The successive replacement of microtubular arrays (cortical microtubules, pre-prophase band, mitotic spindle and phragmoplast) by a continuous assembly and disassembly of the cytoskeleton structures during the cell cycle progression is unique to higher plants. γ-Tubulin was preferentially immunolocalized on microtubules of all microtubular arrays.[56] A centrosomal plant spindle is predominantly formed by kinetochore microtubular fibres that are organized in the vicinity of chromatin and the kinetochore region of chromosomes after nuclear envelope breakdown. Cell cycle specific accumulation of γ-tubulin in premitotic nuclei, its location with kinetochores and later on in mitosis with kinetochore microtubules (Figure 1) suggested its role in

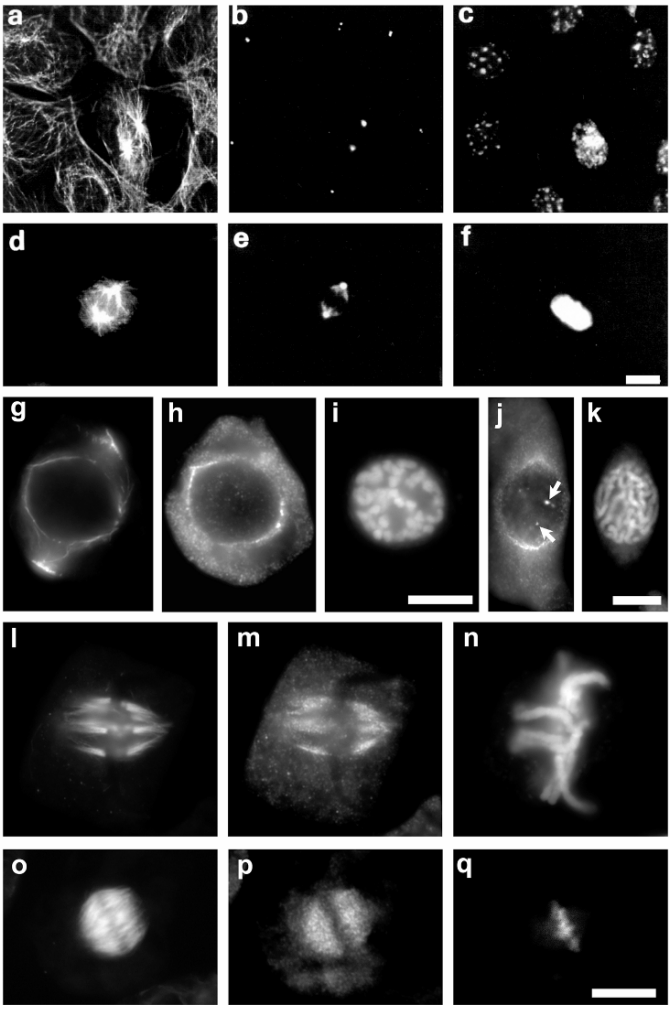

Figure 1. Localization of γ-tubulin in mammalian and plant cells. Distribution of γ-tubulin at interphase (a–c) and metaphase (d–f) in mammalian 3T3 cells, and subcellular localization of γ-tubulin in prophase (g–k) and metaphase (l–q) in plant cells. Triple-label staining of 3T3 cells with antibody against α-tubulin (a, d), antibody against γ–tubulin (b, e) and DNA-binding dye (c, f) in interphase (a–c), and in metaphase (d–f). Triple-label staining of root meristem cells of *Vicia faba* (g–n) and suspension cells of *Arabidopsis* (o–q) with antibody against α-tubulin (g, l, o), antibody against γ-tubulin (h, j, m, p) and DNA binding dye (i, k, n, q). (g) preprophase band of microtubules (h) γ-tubulin in the vicinity of nuclei is concentrated on spindle poles; (j) the same stage as in (g) but different focus level, nuclear γ-tubulin is located in nucleoplasm and concentrated in pre-kinetochore regions of the chromosomes, (l), metaphase spindle of *V. faba* – kinetochore microtubular fibres more focused to poles are decorated with γ-tubulin (m), γ-tubulin is located also with kinetochore microtubular fibres of *Arabidopsis* metaphase spindle with broad poles (o, p). Bars = 10 μm.

microtubule and spindle organization.[57,58] However γ-tubulin was found to be present in plant nuclei during the entire cell cycle; and another yet undefined roles for nuclear γ-tubulin might be expected. Immunolocalization studies with different antibodies reported a punctuated labeling for γ-tubulin in the vicinity of nuclei, on the nuclear envelope, in the area of cell plate formation, with cortical membranes and with organelle-like structures.[15,57–62]

6. γ-Tubulin complexes and their effect on microtubule nucleation

Although γ-tubulin is mainly localized on MTOCs or microtubule arrays, a large amount of γ-tubulin is in soluble form. Soluble γ-tubulin cytoplasmic complexes were first detected in embryonal extracts of *D. melanogaster*. In addition to γ-tubulin, they contain proteins DMAP190 and DMAP60.[63] Since then, γ-tubulin has been detected in the form of protein complexes of various sizes in all organisms investigated. Cytosolic forms of γ-tubulin were present in oocytes of *X. laevis* as 25S complexes, γ-somes.[64] Cytosolic γ-tubulin appears in two main complexes: the large γ-tubulin ring complex (γTuRC; ~2.2 MDa) and the γ-tubulin small complex (γ-TuSC; ~280 kDa). The γTuRCs can be dissociated from centrosomes and dispersed into γ-TuSCs, which are similar in size and composition to γ-tubulin complexes of *S. cerevisiae*.[65] The human γ-TuSC comprises two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins)[66] which are homologues of the *S. cerevisiae* proteins Spc97p and Spc98p associated with SPB.[67] Homologous proteins in *Drosophila*[68] and in *Xenopus*[69] were characterized. The γTuRC derives from 5 to 7 γ-TuSCs by condensation and association with proteins GCP4, GCP5, GCP6[70] and GCP-WD/NEDD1[71] in an, as yet, unknown stoichiometry. Electron microscopy revealed, in γTuRC isolated from *Xenopus* egg extracts or *Drosophila* extracts, an open-ring structure approximately 25 nm in diameter, and defined a subunit structure similar to a microtubule in cross section. The individual subunits visible within the ring walls have been interpreted as representing the γ-TuSC. Electron microscopic tomography indicates that associated proteins, not involved in γ-TuSC, form the cap of the ring structure.[72] Hundreds of γTuRC-like rings were found in pericentriolar material of *Drosophila* centrosomes. The existence of these rings correlated with the ability of centrosomes to nucleate microtubules.[73]

While in budding yeast only small complexes were identified, both small and large γ-tubulin complexes co-exist in fission yeast, *S. pombe*, thus resembling (in this respect) the cells of higher eukaryotes.[74] Homologues of all GCP proteins are present in plant genomes (2007 unpublished), and therefore interaction of γ-tubulin in the form of γ-TuSC or large complexes similar to γTuRC was expected. The analysis of plant γ-tubulin revealed

heterogeneous protein complexes of various sizes.[58,62] Heterogeneity of γ-tubulin complexes, differing in their molecular masses, is not unique to the acentrosomal plant cells. Similar large and intermediate size γ-tubulin protein complexes distributed within a broad range of molecular masses were reported in different organisms including neuronal cells[23] and *Aspergillus*.[75]

Two models were proposed to explain how the γTuRC nucleates microtubules, the so-called "template" and "protofilament" models. It is presumed by the "template model" that γTuRC contains laterally associated γ-tubulin subunits and nucleates by making longitudinal contacts with the terminal α-tubulin.[76] In the "protofilament" model, a ring of longitudinally stacked γ-tubulin subunits forms a short protofilament that nucleates microtubules via lateral contacts with αβ-dimers to seed a bidimensional microtubule lattice.[77] Although electron tomography and immunoelectron microscopy studies seem to confirm the template model, biochemical evidence[78] and X-ray crystallography data on γ-tubulin[35] rather supports a modified protofilament model. The γ-TuSC also nucleates microtubules, but with a much lower efficiency than the γTuRC, suggesting that the assembly into a larger complex enhances nucleating activity.[68] On the other hand, even monomeric γ-tubulin could nucleate microtubules. It was suggested that a single γ-tubulin formed a tight lateral bond with β-tubulin in an oligomer of three tubulin dimers.[78]

Coverslip nucleation assays in *A. thaliana* and *V. faba* revealed that only cytoplasmic fractions with large γ-tubulin complexes (>1 MDa) affected the nucleation activity strongly, while intermediate and small complexes were basically without effect or with only a small effect on microtubule nucleation.[62] In this respect, the large complexes resemble animal γTuRCs, which are the most potent soluble microtubule nucleators, while variable intermediate size plant complexes might represent other functional types of tubulin complexes. Despite the functional similarity, there are several significant differences between large γ-tubulin complexes in plants and between well defined animal γTuRC. First, in comparison with the γTuRC (Mw ≈ 2.2 MDa), large γ-tubulin complexes in plants (Mw > 1.5 MDa) are distributed in a broader range of molecular masses; second, in contrast to the reported salt sensitivity of γTuRC, large γ-tubulin complexes of *A. thaliana a*nd *V. faba* do not dissociate into smaller complexes in the presence of 500 mM NaCl; third, large γ-tubulin complexes in plants were found to be associated with membranes.[62]

7. Plant γ-tubulin complexes are associated with membranes

The nuclear membrane was believed to be the site of microtubule nucleation in plants, and numerous immunolocalization observation[15,58–60] support this hypothesis. The plant homologue of Spc98p, a protein

interacting with γ-tubulin in complexes in a broad range of eukaryotes, co-localizes with γ-tubulin on the nuclear envelope in tobacco. Later biochemical evidence of the presence of Spc98 homologue in plant γ-tubulin complexes was provided.[79,80] Association of large (>1 MDa) plant γ-tubulin complexes with membranes has been reported.[62] Although γ-tubulin itself does not have a consensus membrane-binding motif, it is possible that other proteins of the complex mediate its association with membranes. Our data indicate that αβ-tubulin dimers are associated with large membrane-bound γ-tubulin complexes. Interestingly, palmitoylation of the α-tubulin molecule was reported.[81] Moreover, an association of γ-tubulin with protein tyrosin kinases p53/p56^{lyn}, and Fyn whose significant fraction is located in membrane microdomains, has been reported previously.[82,83]

In acentriolar early mouse oocytes, γ-tubulin-positive membranous aggregates containing a variety of vesicular structures were suggested to be the centrosomal precursors of a unique ultrastructure.[84] It is possible that similar membrane vesicular structures containing γ-tubulin complexes exist in acentrosomal plant cells. It was reported that Golgi-derived vesicles acted as microtubule nucleating and organizing sites with γ-tubulin participating in the process.[85] Recently asymmetric CLASP-dependent nucleation of noncentrosomal microtubules at the *trans*-Golgi network was shown.[86] Golgi emanating microtubules may contribute to the asymmetric microtubule network in polarized cells. Ultrastructural studies showed that there are many examples among plants where membranes show intimate and specific association with acentrosomal mitotic apparatus.[87] Localization of *cis* and *trans*-Golgi membranes in the close vicinity of spindle poles containing γ-tubulin suggest that an interaction of γ-tubulin with these membrane compartments might exist in plants.[62] Microtubule nucleating and anchoring activities of Golgi apparatus that is associated with microtubules in pericentrosomal area throughout the cell division cycle may present, in general, a pathway of microtubule nucleation and organization.[88]

8. Association of plant γ-tubulin with αβ-tubulin dimers and microtubules

Apart from γTuRC and γTuSC, a portion of γ-tubulin is probably associated with the TCP-1 chaperonin complex, which is responsible for folding of α-, β- and γ-tubulins.[89] It was also found that γ-tubulin in HeLa cells under natural conditions existed as a homo- or a heterodimer.[90] Interestingly, like the αβ-dimers, γ-tubulin in porcine microtubule proteins has the ability to form oligomers under the conditions of native electrophoresis.[21] The co-precipitation of αβ-tubulin dimer and γ-tubulin confirmed independently an association of soluble γ-tubulin with tubulin dimers also in *A. thaliana* and

V. faba.[62] This might reflect the presence of tubulin dimers in γ-tubulin complexes; alternatively γ-tubulin could be present in cells in some other forms capable of interacting with αβ-tubulin dimers. Tubulin dimers were not found in the majority of analyzed animal γTuRC,[91] but variable amounts of tubulin dimers have been reported to co-precipitate with γ-tubulin in preparations from oocytes[76,92] or erythrocytes[24] for example. Structural models for self-assembly suggest that γ-tubulin should be capable of self-assembling into dimers or protofilament-like oligomers, as well as of interacting laterally with α- or β-tubulin.[33]

γ–Tubulin is bound to plant microtubules in living cells, as well as *in vitro*.[62] Dot-like γ-tubulin staining of microtubules along their entire length and its accumulation with microtubular bundles suggest a lateral association of γ-tubulin forms. It was shown that γ-tubulin peptides did not interfere with microtubule assembly *in vitro* and were associated with microtubules along the polymer length.[34] An association of γ-tubulin with marginal-band microtubules in chicken embryonic erythrocytes was also described.[24]

In plants highly dynamic cortical microtubules are arranged into regular arrays and rapidly adopt the directionality of anisotropic growth in response to hormones, environmental cues and differentiation. Recently a model was proposed for the self-organization of cortical microtubules. It was shown that intermicrotubule interactions among newly formed short microtubules are sufficient to facilitate organization of plant cortical microtubules into a parallel configuration.[93] Parallel cortical microtubules of differentiating Arabidopsis cells were found to be sensitive to the reduction of γ-tubulin level by RNAi; γ-tubulin rapidly disappeared from its locations on the plasma membrane and from cortical microtubules that became bundled and randomized.[94] Reduction of cortical microtubule bound γ-tubulin, which acts as a nucleator, might disturb the balanced rate of tubulin polymerization needed to maintain minimum cortical microtubules length that is a prerequisite for self organization and for guiding microtubules into parallel networks. Both γ-tubulin mediated nucleation and the severing of microtubules from existing nucleation sites are important to generate new microtubules and enable their self-organization into transverse arrays. Recently the nucleation of microtubules from pre-existing cortical microtubules was shown to require binding of cytoplasmic γ-tubulin to cortical microtubules in tobacco cells.[95] Linear microtubule arrays are not plant cell specific but they are formed in polarized animal cells such as neurones, epithelial cells or myotubes.[96] In *S. pombe*, nucleation from γ-tubulin complexes along the length of existing microtubules was shown to drive the formation of bipolar microtubule bundles.[97]

The γTuRCs not only nucleate microtubules, but they also have separate capping activities and may be important for modulating microtubule (-) end

dynamics.[98] It was shown that γTuRCs cap forming proteins GCP4,5,6 and GCP/Nedd1 may not be required for the localization of γ-tubulin to centrosomes, but they are required for its binding along the spindle microtubules and for its recruitment to the midbody.[99] Homologues of cap forming proteins exist in plant genomes and similar mechanisms might then optimize the generation and spacing of multiple bipolar MT bundles in fungi, animal cells and plants. However, more detailed molecular characterization of membrane and microtubule associated complexes is needed. Interestingly, several reports also provide evidence for a role of γ-tubulin, or γ-tubulin complexes in regulating microtubule dynamics at the plus ends.[100]

Accumulating data support the idea that γ-tubulin could participate in microtubule stabilization. γ-Tubulin has been found on stable kinetochore microtubules that are resistant to anti-microtubule drugs in plants[101] and in the cold-stable fraction of microtubules in animal cells.[23] Genetic data support the idea of γ-tubulin having, besides its nucleation role, also a role in microtubule organization. A γ-tubulin mutation in *S. pombe* permitted microtubule nucleation only at a restrictive temperature, altered the interphase microtubule arrays and caused an arrest of mitosis in anaphase A.[102] γ-Tubulin and the kinesin-like protein KLPA had overlapping roles in the establishment of spindle bipolarity.[103]

The presence of γ-tubulin in the kinetochore region of chromosomes, the massive association of γ-tubulin with kinetochore microtubular fibres in mitosis, and its presence in the vicinity of kinetochores in aberrant monopolar mitosis[57] imply a role of γ-tubulin in the organization of bipolar spindles (Figure 2). Reduced levels of centrosomal γ-tubulin by RNAi was found to lead to monopolar or multipolar spindle formation in *C. elegans*,[41] and to aberrant spindle formation in the vicinity of chromatin in *Drosophila*.[104] We found that severe depletion of γ-tubulin in *Arabidopsis* cells resulted in collapsed spindles with remnants of microtubules in the vicinity of chromatin. Moreover cytokinesis became frequently defective in RNAi cells showing misorientation of cell division and temporal uncoupling of the cytokinetic events.[95] Interestingly, a lack of coordination of mitotic events was also found in conditional γ-tubulin mutants in *Aspergillus*, and in *S. pombe*; mitotic microtubules were formed in spite of the impaired γ-tubulin function but cytokinesis became abnormal.[102,105] The position of the mitotic spindle provides positional clues for cell plate formation in *S. pombe*.[106] Dispersed γ-tubulin containing MTOCs are critical for correct anchorage of the cytokinetic actin ring and for proper coordination of mitosis with cytokinesis in *S. pombe*.[107] Our data suggest that a similar mechanism might also operate in plant cells with dispersed MTOCs. γ-Tubulin interconnects membrane organization with positional clues, such as centrosomes, during the cell cycle progression also in mammalian cells.[108]

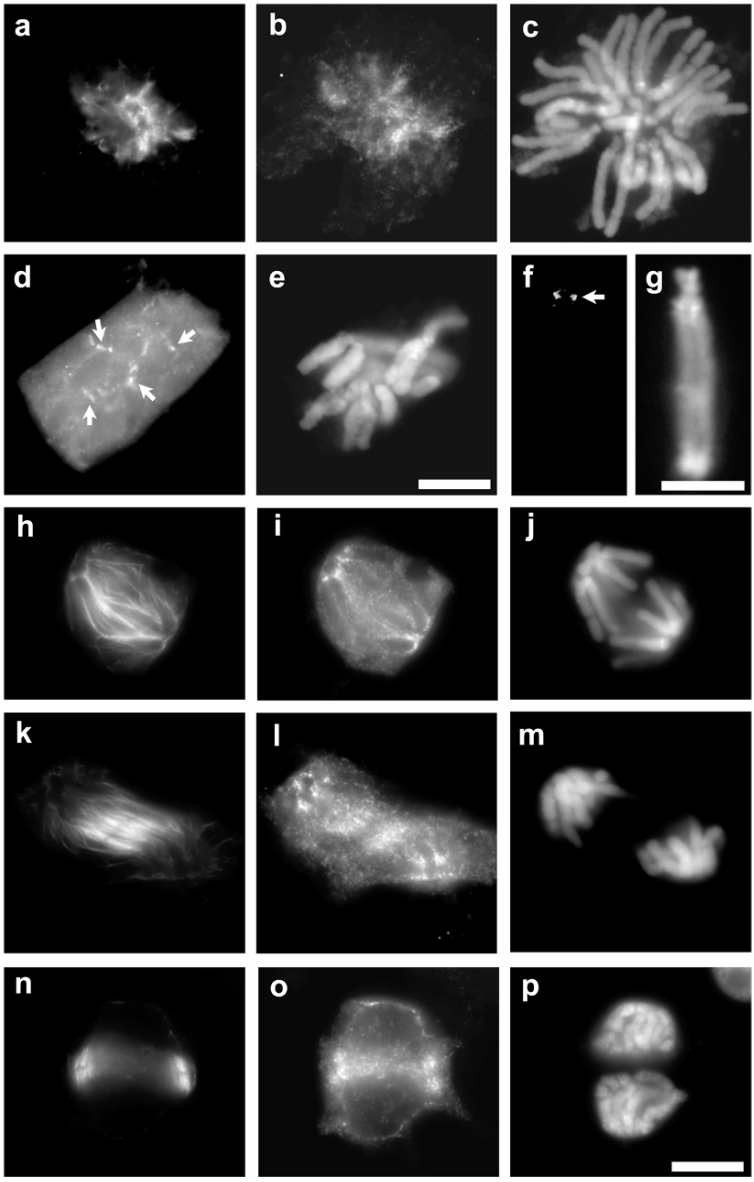

Figure 2. Subcellular localization of γ-tubulin during cell division of acentrosomal plant cells is microtubule dependent and regulated by cyclin dependent kinase signalling. Triple-label staining of root meristem cells of *V. faba* (a–p) with antibody against α-tubulin (a, h, k, n), antibody against γ-tubulin (b, d, f, i, l, o) and DNA binding dye (c, e, g, j, m, p).

Aberrant metaphase after treatment of *V. faba* with inhibitor of cyclin dependent kinase roscovitine: (a) remnants of short microtubular kinetochore fibers in the centre of metaphase circular chromosome configuration and long microtubules along the chromosome arms; (b) γ-tubulin labelling in the vinicity of kinetochores in the centre of metaphase circular chromosome configuration; Localization of γ-tubulin in cells treated with microtubule depolymerazing drug amiprophos methyl: (d) γ-tubulin labelling in the kinetochore regions of chromosome and in their vicinity where remnants of microtubules are present (f) isolated chromosome with γ-tubulin located in kinetochore region (arrow).

Polar localization of γ-tubulin during mitosis/cytokinesis: (h) anaphase spindle; (i) association of γ-tubulin with shortening kinetochore microtubular fibers in the vicinity of the spindle poles; (k) late anaphase/early telophase cell with early phragmoplast, (l) γ-tubulin labelling is present mainly in the area of the developing phragmoplast but a portion of protein remains at the poles, (n) late phragmoplast in telophase cell; (o) γ-tubulin labelling is present in the area of cell plate formation and in the vicinity of the reforming nuclear envelope.

Bars = 10 μm, bar in (g) = 5 μm.

As shown in Figure 2 γ-tubulin is dynamically relocated during mitosis and cytokinesis in a polar manner in acentrosomal plants. However regulatory mechanisms and impact on progression through cell division remain to be elucidated.

9. Conclusions

Experimental data obtained with different eukaryotic cells suggest that the distribution of γ-tubulin protein complexes among various species might underscore the specific need for an organism to regulate its microtubule dynamics and spindle functioning. The abundance of γ-tubulin in plant cells, the presence of γ-tubulin in protein complexes of various sizes, different molecular composition and subcellular location, reflect the specific needs of plant cells. In plants, all somatic and gametic cells are acentrosomal. Due to the presence of a rigid cell wall, plants develop specific microtubular arrays - cortical microtubules with preprophase band and phragmoplast that all reflect the requirement of plant cells for a precise control of division plane and polarity. In plants, microtubules are dominant cytoskeletal elements associated with plasma membranes, whereas in animal cells actin filaments are more abundant in the same location. Association of γ-tubulin with interphase and mitotic microtubules and with kinetochores suggests a key role for γ-tubulin in plant microtubule organization. A role for nuclear γ-tubulin, in addition to its non-centrosomal microtubule nucleation role, is less obvious. Membrane associated γ-tubulin complexes in plants could serve as a dynamic self-organizing system that provides flexible microtubule nucleation activity. More generally, an

association of γ-tubulin with membranes and microtubules and chromatin might ensure the nucleation of microtubule arrays not only in plants but also in other eukaryotic cell types with non-centrosomal microtubules. On the other hand, the membrane associated γ-tubulin complexes might participate in actions on microtubule plus ends, which are important for cell morphogenesis. After several plant genomes have been sequenced, one can expect that the current proteomics tools and available mutants will make it possible to elucidate the precise functions of various γ-tubulin forms and their protein interactions in nucleation and organization of microtubules or regulation of the other processes in plant cells.

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COILED-COIL- AND INTERMEDIATE FILAMENT-PROTEINS IN THE PLANT NUCLEOSKELETON

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Abstract. Plant cells have a nucleoskeleton formed by a peripheral lamina and an internal network of branched knobbed 15–25 nm filaments, with a periodic organization similar to that of intermediate filaments. We review here the features of NuMA and lamins, two main intermediate filament- type proteins of the vertebrate nucleoskeleton, and also some plant nucleoskeleton proteins sharing antigenic determinants with those of vertebrates. In addition, plants seem to have evolved unique intermediate filament-like nucleoskeleton proteins that are functional homologues of the vertebrate ones, such as NIFs, NMCP1, MFP1 and NMP1. The present challenge is to determine the specific subsets of proteins forming the lamina and the nucleoskeleton in plant systems.

Keywords: Intermediate filaments, lamins, NuMA, NIFs, NMCP1, MFP1, NMP1, nucleoskeleton, plants

Abbreviations: CaaX = isoprenylation box, cIF = cytoplasmic intermediate filament, IF = intermediate filament, INE = inner nuclear envelope, LAP = lamina-associated protein, LGN = homologue of mammalian Pins, MAR = matrix attachment region, NIF = nuclear intermediate filament, NOR= nucleolar organizing region, NPC = nuclear pore complex, NuMA = nuclear mitotic apparatus, NSK = nucleoskeleton

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1. The nucleoskeleton

The nucleus is organised in structural and functional domains. The nuclear envelope (NE) regulates molecular transport between nucleus and cytoplasm throughout nuclear pore complexes (NPCs), while the underlying lamina is involved in chromatin organisation and replication.[1–3] The interchromatin domains contain nucleoskeleton (NSK) filaments, interchromatin granules, splicing/transcription complexes and different sorts of nuclear bodies.[4] The nucleolus houses the rDNA genes, although nucleolar functions exceed those for rDNA expression. It is also essential for snRNP maturation, nuclear trafficking, and sequestration of proteins.[5] The genome is distributed in domains that occupy separate territories in the interphasic nucleus.[4,6] The fixed relative positions of genes in the nucleus define their spatial and temporal sequence of expression and replication.[7–9] Active genes replicate early, while the repetitive sequences of centromeres and telomeres, which in plants keep their anaphasic polar distribution,[7] replicate in late S phase.[9,10] Some gene clusters associate in nuclear domains, as do the tandem arrays of rDNA genes in the nucleolar organizers (NORs) from different chromosomes that often coalesce in a single nucleolar domain.[11]

Nuclear order also affects gene expression. The chromatin is organised in loop domains, attached to the NSK at their bases by specific DNA regions (matrix attachment regions or MARs), which have crucial functions in chromatin organisation, gene expression and transgene stability. Heterochromatin, a domain especially prominent in plants, forms large clusters to which temporarily silenced genes associate.[2,6,8] Nuclear functioning relies on a constant flow of proteins and RNAs between compartments. Their binding to specific nuclear domains prevents some of them from diffusing and from undergoing Brownian motion.[3] Interactions between specific addressing protein domains are essential for intranuclear topogenesis.[12]

Micromanipulation demonstrates that subnuclear structures and chromatin are interconnected. Isolation protocols that preserve the ultrastructure of the *in situ* NSK, suggest that the distinct nuclear domains remain in place after removal of soluble proteins and chromatin.[13]

Nuclear compartmentalization requires a NSK to establish its general organisation.[2] The discovery of proteins involved in the assembly of an expandable nucleoskeleton (EAST and skeletor), and the direct observation of their incorporation into the NSK in living cells,[14] makes it a readily observed structure and not an operational concept. NSK, nuclear matrix and nuclear scaffold are terms that refer to the intranuclear non-chromatin network that is observed in fixed cells, in extracted nuclear structures and also *in vivo*.[2,13,15–18] NSK is the term used to refer to such structure from here onwards.

2. Ultrastructural organization of the plant NSK

Resinless EM of cells after either electroelution of chromatin, or sequential extraction with detergent, DNase and 2M salts, revealed a branched NSK connected to the cytoskeleton. The NSK is made of 11 nm filaments with an axial 23 nm repeat similar to intermediate filaments (IFs).[13,15] The NSK is an RNA-stabilized intranuclear web[16] consisting of 3–5 nm lamin filaments to which NuMA islands are anchored.[17]

The NSK of plant cells forms a fibrogranular network that contains the interchromatin granules network. After sequential extraction to produce *in situ* NSKs, a branched and fibrous NSK network connected to the cytoskeleton, is visualized. It includes the nucleolar and interchromatin residual domains and the lamina.[18] The NSK obtained from isolated nuclei retains the original

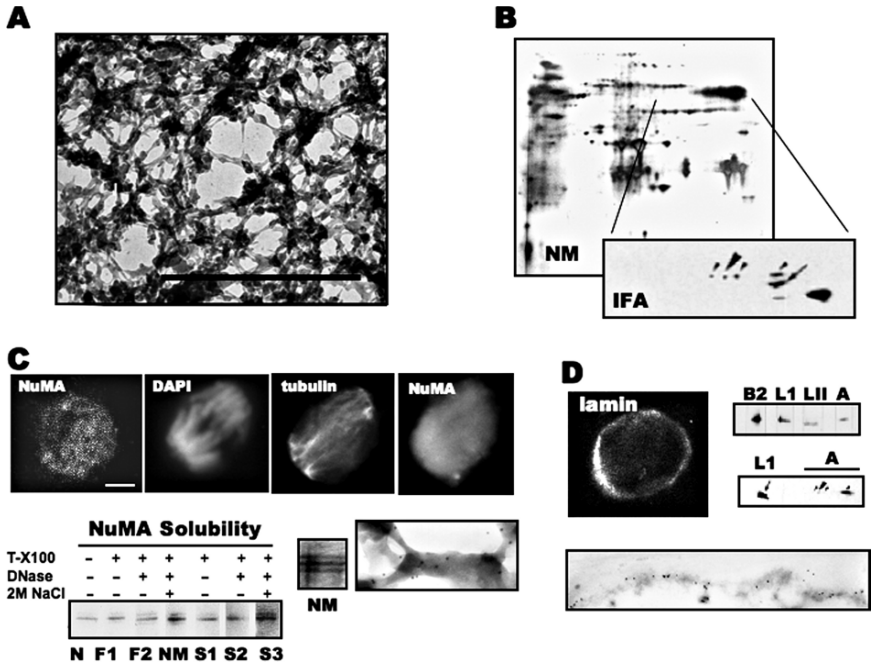

Figure 1. IF-type proteins of the plant NSK. (**A**) Ultrastructural organization of the NSK network displaying IF-like core filaments, either individual or with complex associations. (**B**) The universal anti-IF Mab IFA reveals that only a subset of the multiple silver stained NSK proteins corresponds to IFs. (**C**) Main features of the plant NuMA. (**$C_{1,4,6}$**) Distribution of NuMA in the interphasic nucleus, mitotic spindle and NSK filaments, respectively. (**$C_{2,3}$**) Distribution of chromosomes and MT in the same mitotic cell. (**C_5**) solubility of the NuMA isoforms during NSK extraction. (**D**) Lamin-like proteins. (**D_1**) Cross-reactivity with several Abs against vertebrate lamins in 1D and 2D western blots, respectively. (**$D_{2,3}$**) Distribution of lamin-like proteins in the lamina and internal foci. Bars in A and D_3 = 1 μm. Bars in $C_{1–4}$ and D_2 = 10 μm. Bar in C_6 = 0.1 μm.

nuclear size and morphology, and reveals a lamina, nucleolar matrix, and internal NSK or endoskeleton, that corresponds with the main domains of the *in situ* nucleus and NSK.[18]

High magnification of resinless sections of plant NSK display an anastomosed network of branched filaments with dense aggregates of variable sizes. Two kinds of filaments are evident in these preparations: bare knobbed 15–25 nm filaments, showing a periodic organization compatible with that of IFs, and thicker filaments with associated aggregates, sometimes formed by filaments twisted around each other[19] (Figure 1A, C_6).

3. Protein composition of the plant NSK

Although the ultrastructural organization of the plant NSK is well established, information on its components is scarce. Homologues of most animal nucleoskeletal proteins cannot be identified in plants by sequence similarity. Nevertheless, the structural and functional similarities of the animal and plant nucleus and NSK, and the presence of immunologically related nucleoskeletal proteins in plants, suggest that plants have functional equivalents of the known animal nucleoskeletal proteins, which have diverged significantly from their animal counterparts. The NSK proteome of *Arabidopsis* is complex with up to 300 spots resolved, from which only 36 have been identified by mass spectroscopy. The fraction is enriched in proteins of the nucleolar domain.[20] The use of sera against animal nucleoskeletal proteins in plant nuclei revealed the presence of proteins immunologically related to lamins,[18,21,22] spectrin repeats-proteins,[23] NuMA,[19] actin,[24] nucleolin,[25] Sm proteins,[26] etc.

Two-dimensional electrophoresis-analysis revealed more than 200 distinct protein spots in onion NSK (Figure 1B). While its protein composition varies according to the extraction procedure, its basic organization does not.[18] Many proteins of the plant NSK have been characterised. They show a specific nuclear and NSK distribution. Some of them are components of the nuclear machineries for replication [as PCNA,[10] TOPOII,[18] transcription/splicing as Sm proteins[26] and ribosome synthesis and assembly (nucleolin,[25] TOPO I, fibrillarin,[18] UBF, ASE-1 and SURF-6); (F. Novillo, H. Li and S. Moreno Díaz de la Espina, 2000 unpublished results)]. Finally, others are involved in chromatin attachment, as the MAR-binding proteins[27,28]; others correspond to nuclear enzymes involved in posttranslational modifications such as protein kinase CKII[29] and disulfide isomerase[30]; or contain long coiled-coil domains, which are involved in the attachment of nucleic acids and other proteins to solid state components as NMCP1,[31] MFP1,[32] the lamin-like proteins[21,22,29] and NuMA[19] (Figure 1C, D).

4. Coiled-coil and IF proteins

The α-helical coiled-coil is one of the main oligomerization motifs in proteins. It is characterized by a heptad repeat pattern of primarily apolar residues that constitute the oligomere interface. Coiled-coils consist of two to five amphipathic α-helices twisted around one another to form a supercoil.[33] Coiled-coils are rather versatile protein folding motifs. They serve a variety of functions as motors (myosins, kinesins and dyneins), skeletal proteins (IF, laminin), or are involved in molecular recognition (SNARE), transcription of DNA (leucine zipper transcription factors), etc.[34]

IF proteins are major components of the cytoskeleton and the NSK of most eukaryotes, and they form the largest family of cytoskeletal proteins. All of them exhibit a typical tripartite domain organisation (Figure 2A). The building block of IFs is the central rod domain including four α-helical subdomains separated by non-helical linkers. They have diverging primary sequences, except for the two highly conserved sequences at both ends of the rod domain. Non-helical head and tail domains, of highly variable size and organization, which accounts for the heterogeneity of the family,[35] flank the rod domain. IFs are organized into five distinct gene families according to sequence identity, intron position and expression pattern. Lamins are canonical IF proteins that form the type V family, and are the only nuclear IF proteins. Lamins are distinct from cIFs by several features: a six unit heptad extension in the 1B subhelix, the short head region, and the long C-tail containing a nuclear localization signal (NLS) and a CaaX isoprenylation box[35,36] (Figure 2C). Lamins form lattices instead of filaments *in vivo*.[37] IFs of invertebrates are similar to nuclear lamins, indicating that modern IF genes have evolved from a lamin-like ancestral gene, by deletions of tail regions coding for the NLS and the CaaX box. Evidence for the existence of IF and lamins in yeast or plants[21,22] have not yet been confirmed by cDNA cloning.

IF proteins are only functional as oligomers. They assemble to form filaments, which constitute one of the three distinct cytoskeletal arrays of the cytoplasm: actin filaments, microtubules (MT) and intermediate filaments. The assembly of IF arrays occurs by oligomerisation of their rod domains. *In vitro*, tetramers associate laterally to form unit-length filaments (ULFs), which anneal longitudinally to form loosely packed filaments, which undergo internal reorganisation, to finally produce the mature filaments.[37] Although several IF proteins do self-assemble *in vitro*, *in vivo* most of the IF arrays are heteropolymers, and their formation involves the recruitment of specific IF associated proteins.[37–39] A hypothetical mechanism underlying IF dynamics, involves reversible interconversion between long filaments, short filaments and non-filamentous oligomers.[38]

IFs display a continuous exchange of protein subunits at the filament surface and stabilisation of soluble subunits by chaperone-type proteins. Many of them are involved in signal transduction, as serine and threonine kinases and phosphatases. These interactions not only lead to reorganisation of the IF cytoskeleton, but also affects the cellular distribution and properties of the signalling intermediates. Thus, IF proteins could play a specific role in the transmission of signals from the plasma membrane to the cell nucleus.[40]

The highly regulated phosphorylation/dephosphorylation of IF proteins also generates a soluble pool of subunits ready for re-polymerisation.[37,39,41] Similar modifications of IF proteins could also modulate the dynamics of the NSK *in vivo*. Coordinated phosphorylation of specific sites in lamins is a switch for the cell-cycle dependent assembly and disassembly of the lamina.[42] Phosphorylation also regulates the association of NuMA with insoluble cell components both *in vivo* and *in vitro*.[43]

5. Binding of IF and coiled-coil proteins to nucleic acids

IF proteins interact with nucleic acids. A domain of about 40 residues contains the nucleic acid binding site that probably loops out from the filament surface.[44,45] The repetitive arrangement of multiple DNA binding sites in the surface of a filament allows a strong cooperative multisite interaction with repetitive DNA sequences such as telomeric, centromeric and pericentromeric DNA.[44–46]

cIF proteins, transcription factors and also related NSK proteins contain α-helical rod domains and adjacent basic DNA-binding domains, that are responsible for nucleic acid binding.[47] Many transcription factors act not only as dimers, but also as tetramers or polymers, which expose multiple DNA-binding domains on their surface. They are able of interconnect distant *cis*-acting DNA elements, presenting a DNA-looping potential. The stability of the complex is enhanced by DNA supercoiling. They displace histone octameres from nucleosomes in the process of transcription.[45] Thus, the DNA binding function of IFs can play a role in the control of gene expression.[40]

6. IF-related proteins in the NSK

Besides lamins, other NSK proteins share basic structural and functional traits with cIF proteins, mainly the α-helical coiled-coil or other domains conferring oligomerization and the capacity to form filaments,[44] and DNA binding ability (α-satellite DNA and MAR sequences).[48] MAR-binding

proteins form polymers capable of multi-site interactions with either ssDNA or supercoiled DNA.[49]

The 10 nm core filaments forming the anastomosed network of the NSK are ultrastructurally indistinguishable from cIFs.[13,15,16] There is a tight interconnection of cIFs with structural elements of the NSK[44] that together with their ability to crosslink with MARs and genomic DNA sequences *in vivo*, suggest that cIFs could be complementary constituents of the NSK. Although several IF-type proteins have been identified in the plant NSK (Figure 1B) only a few, such as NuMA (Figure 1C) and lamin-like proteins (Figure 1D), have been characterized.

7. NuMA (Nuclear Mitotic Apparatus) protein

NuMA is a very long 200–240 kDa, coiled-coil protein conserved in vertebrates and *Drosophila*. NuMA is encoded by a single copy gene. Alternative splicing and extensive post-translational modifications, mainly at the C-terminus, generate multiple variants of NuMA, probably with different functions.[49] NuMA has a very long central α-helical rod domain with seven subdomains that constitutes the oligomerization domain (Figure 2B). It has sequence similarity with other structural coiled-coil proteins such as myosins, cytokeratins and lamins, and is flanked by globular head and tail domains of 24 and 45 kDa, respectively.[50] No functional domains are found in the rod domain of NuMA, except for the leucine zipper sequences and the overlapping site for the protein GAS41.[51] The C-terminus of the protein contains important functional domains such as a bipartite NLS involving the sequences RKR and KK; the overlapping LGN- and tubulin-binding domains; the protein 4.1R binding site; several consensus recognition sites for different protein kinases; and the last 130 residues that are important for proper nuclear reformation.[52] The C-terminus contains multiple alternative splicing sites capable of generating numerous isoforms of the protein. A calponin-homology domain in the NuMA N-terminus interacts with the actin-related protein 1 (Arp1) of the dynactin/dynein complex.[53]

7.1. SELF-ASSEMBLY OF NuMA

The assembly of NuMA involves at least three steps: first, in parallel and in register dimer formation, with a 207 nm long rod domain, and a hinge-like region in the middle of the molecule; second, C-terminus interactions of the dimers to form multiarm oligomeres, each arm corresponding to a dimer ending with a small globular head domain; and third, head and tail domain interactions of multiarm oligomeres to form lattices. *In vivo* experiments

with truncated NuMAs revealed that its C-terminus controls the oligomerization of dimers into arm oligomeres, while the N-terminus controls the assembly of the later into lattices.[50] The two first steps occur by self-assembly, but the third one probably needs posttranslational modifications by specific protein kinases and phosphatases, and also co-factors and binding proteins, that lead to more efficient and stable 3-D lattices. NuMA is a soluble protein in mitotic extracts that forms insoluble structures in non-mitotic ones while other NSK components, like the lamins, remain soluble. Overexpression of NuMA produces an ordered three-dimensional network in the nucleoplasm formed by quasihexagonal elements.[50]

Due to the flexibility of the molecule and its capacity to produce lattices, NuMA could perform a structural role in nuclear organization. In non-transfected nuclei, NuMA assembles in minilattices or aggregates, and forms islands whose association to the underlying network of internal lamins depends on an RNA.[17] Their interactions represent a critical factor in the stabilization of the internal NSK, the main components of which are RNP complexes and the B23 protein.[54]

NuMA is either a nucleoskeletal or cytoskeletal protein depending of the cell cycle stage. In interphase, NuMA is a component of the NSK, providing mechanical stability and compartmentalisation. The intranuclear NuMA is less phosphorylated, highly insoluble, and resists DNase digestion and high salt and non-ionic detergent extraction (Figure $1C_5$). However, it is solubilized by RNase digestion[17] and high urea concentrations (8 M). Solubility of the interphasic NuMA is probably influenced not only by RNase, but also by changes in its conformation, surface charge and polymerisation.[50]

7.2. NuMA PARTNERS

NuMA has recognition domains for several proteins, whose interactions are involved in the formation of multiprotein complexes (Figure 2B). For example, Arp1 (Actin-related protein 1) is a highly conserved protein sharing the basic actin fold. It mediates the association of NuMA N-terminus (calponin-homology domain) with the dynein/dynactin motor protein complex that directs the protein to MT minus ends.[55]

The 4.1R protein is a non-erythroid isoform of the cytoskeletal 135-kDa protein, which contains a 209 amino acid extension at the N-terminus, the head piece, that interacts specifically with NuMA in interphase, and also stabilizes the interaction among NuMA, dynein, dynactin and tubulin. The strong conservation of the interacting sequences of NuMA and 4.1R, and

the effects of overexpression of 4.1R in cells, reflect an important function in the organization of the nucleus and mitotic spindle.[52]

LGN, the mammalian Pins homolog, plays a role in spindle pole organization mediated by NuMA. The MT-binding domain of NuMA overlaps the LGN binding domain by ten amino acid residues, suggesting that LGN binding may inhibit MT association of NuMA through steric exclusion.[56]

GAS41 is a protein related to putative transcription factors conserved from yeast and *Arabidopsis* to humans that interacts with a domain in the C-terminus of the NuMA rod domain. This interaction provides the first evidence for an implication of NuMA in the regulation of gene expression, possibly by providing a framework for transcription.[51]

7.3. NuMA IN MITOSIS

In mitosis, the phosphorylated NuMA translocates to the spindle and interacts with the dynein-dynactin complexes. Depletion of NuMA or expression of deleted mutants prevents the anchoring of the mitotic kinetochores to the spindle poles, and cells become blocked in prometaphase.[55] NuMA is a component of the microtubule aster promoting activity, a multiprotein complex that induces spindle assembly in mitosis and is regulated by both the small GTPase Ran and importin B.[57]

The insoluble nuclear NuMA undergoes a first phosphorylation event at NE breakdown in the prophase-prometaphase transition, and a second one upon its association with the spindle,[43] contributing to the formation and maintenance of MT arrays. After NuMA phosphorylation, two dimers bind to a dynactin complex to form a multiprotein complex, comprising ten subunits of Arp1.[55] Protein 4.1 binds directly to a region of the NuMA tail corresponding to exons 20 and 21,[52] and mediates the binding of NuMA to Arp1. Then the minus end- directed motor protein dynein attaches transiently. NuMA is transported polewards by the multiprotein complex, and is incorporated into the spindle matrix that contributes to spindle organisation.[58,59] After completion of mitosis, phosphatases revert NuMA to its less phosphorylated state to be reimported to the G1 nucleus.

NuMA interacts also directly with tubulin, through a 100 amino acid domain at its C-terminus. The binding domain of the NuMA partner, LGN, largely overlaps with the tubulin binding site. Thus LGN prevents NuMA-dependent spindle organization *in vivo*, and could operate as a factor controlling mitotic spindle organization[56,60] (Figure 2B).

The binding of the tail domains of multiple NuMA dimers may stabilize the spindle poles in anaphase.[59] NuMA mutants that induce a dominant negative phenotype, or NuMA depletion with antibodies, result in the abrogation of kinetochore function.

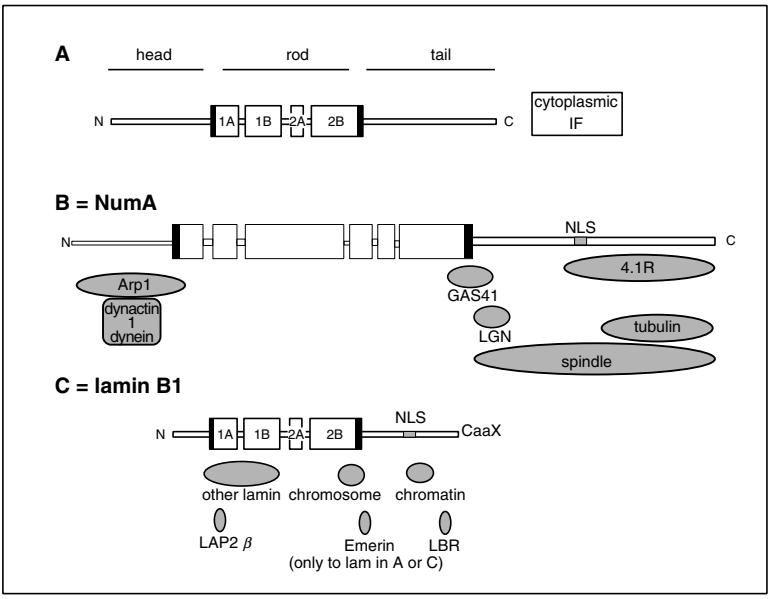

Figure 2. Schematic diagrams of the secondary structures of cIF (A), NuMA (B) and a lamin (C) represented at scale. Rectangles represent the α-helical segments of the rod domain, while their linkers as well as the head and tail regions are represented extended. The highly conserved ends of the rod domain in the three proteins are highlighted in black. The extension in coil 1B of lamins is represented as a black bar. The positions of the nuclear localization signals (NLS) in the tails of NuMA and lamins are represented as shadowed boxes. Lamin A and C types are not represented, as their structures are similar to that of lamin B1, except for the lack of the terminal isoprenylation box (CaaX) and the addition of a polyhistidine tail. The different partner proteins of NuMA and lamins are displayed as ovals below the corresponding interacting domains (see text). The interaction of emerin with lamins A and C is represented over the sequence of lamin B1.

7.4. NUCLEAR FUNCTIONS OF NuMA

The functions of NuMA during interphase are not well defined. The flexibility of the NuMA molecule, its capacity to form lattices and its ability to bind to different structural and/or regulatory proteins, and to MAR sequences *in vitro*,[61] made NuMA a good candidate to play a structural role in nuclear organization and regulation. NuMA defines nuclear shape and mechanical rigidity,[17] and it plays an active role in the reformation of the postmitotic nucleus. NuMA is associated with the NSK core filaments[49] (Figure 1C).

The silencing of the NuMA gene by RNAi in Hela cells produces an apoptotic phenotype.[62] Degradation of NuMA is an early process in

apoptosis.[63] NuMA is preferentially cleaved by caspase 3 *in vivo*, and specifically redistributes within the nucleus during early apoptosis, in association with specific changes in nuclear morphology (chromatin condensation and fragmentation). Their cleavage at the border of the coiled-coil and tail region, a region involved in organization of the NSK knotting centres, produces a 188 kDa form and could cause the collapse of the matrix and the rearrangement of the chromatin.

7.5. PLANT NuMA HOMOLOGUES

The basic mechanism of mitosis is conserved in eukaryotes, and homologues of most of the known proteins involved in vertebrate MT biogenesis are encoded in the *Arabidopsis* genome.[64] Spindle assembly is a highly regulated process requiring the coordination of plus- and minus- end directed motor proteins and non-motor proteins, such as NuMA and other MT-assembling factors, nuclear import factors and Ran GTPases.[65] MT motors are likely to be involved in spindle organization and chromosome segregation in higher plants, but because of the different structure of the acentric spindle poles in these systems, they would have a different function and distribution. The complex of NuMA, dynein and dynactin, is required for maintenance of focussed MT arrays, both in the presence or absence of centrosomes. The complex also organizes chromosome-bound MT within spindles.

By using a battery of antibodies against human and *Xenopus* NuMA, three homologues of 210, 220 and 230 kDa with different solubilities were identified in onion meristematic root cells[19] (Figure $1C_5$). The proteins show the same distribution as vertebrate NuMAs in a nuclear network in interphase (excluding the nucleolus), and in the mitotic spindle after NE breakdown (Figure $1C_{1,}$ C_4). Resinless sections demonstrate that the protein associates with NSK filaments[19] (Figure $1C_6$).

The potential of NuMA oligomers to form higher order structures, and the localization of AcNuMA in NSK filaments, suggest that NuMA plays a structural role in plant nuclear organization. However the distribution of the putative plant NuMA in the spindle differs from that in vertebrates. Plant NuMA does show a uniform distribution in the spindle matrix (Figure $1C_4$), where it probably contributes to the stabilization of the MT bundles or to segregation of the chromosomes.[19]

NuMA and its partner proteins are conserved in plants. Genes with similar sequences to hs- and XlNuMA, and also the protein GAS41, which co-localizes with NuMA in the nucleus, are present in *Arabidopsis*.[51]

8. Lamins

Lamins are the major components of the lamina, a fibrous proteinaceous meshwork of lamins and a complex set of lamin-binding proteins forming the exoskeleton underlying the inner part of the NE that links with the cytoskeleton through integral membrane protein complexes of the NE. Lamins also form stable complexes in the nuclear interior. At the NE, they form a well-organised lattice of near tetragonally oriented 10 nm filaments that attach directly to the spoke rings of the NPCs.[66] Lamins do not distribute homogeneously along the NE. High concentration of lamins co-localize with heterochromatin, while patches of lower concentration correspond with NPCs. Internal lamins concentrate in dynamic intranuclear regions containing either A-type or B-type lamins. The distribution of B lamins varies throughout cell cycle, while A-type lamins distributed in a diffuse pattern, forming a delicate framework.[17]

Lamins are type V intermediate filament proteins with a long α-helical coiled coil rod domain that mediates head to tail dimerization and higher order assembly; a short N- terminal head domain and a globular tail domain containing the NLS, an Ig fold and a terminal CaaX sequence, which is a target for isoprenylation and carboxyl methylation. They are classified as type A or B according to their sequence, tissue-specific expression, biochemical properties, cellular localisation and mitotic distribution. Type A are developmentally regulated, have neutral pI and a polyhistidine stretch in their C-tail, and become soluble during mitosis. Type B are constitutive, have acidic pI and localize to membranes in mitosis.[67,68]

Unicellular organisms and plants lack lamin genes. Invertebrates have single B-type lamin genes; except for *Drosophila* that also has an A-type gene.[69] Vertebrates have three genes for lamins that encode for seven proteins, belonging to types A and B that have different sequences, biochemical features and expression patterns, and that do not form heteropolymers. A-type lamins A and C are generated by alternative splicing of a single gene, have neutral isoelectric points, are soluble during mitosis and are incorporated into the reassembling lamina later than B-type lamins. B-type lamins B1 and B2 are essential, encoded by different genes, have acidic isoelectric points and remain membrane-bound during mitosis. Both types are translated as pre-lamins and undergo modifications of the C-terminal CaaX box including farnesylation, cleavage of the AAX tripeptide and carboxymethylation. A and B lamins have separate assembly pathways *in vivo*, but their specific architectural organization is unknown.[67,68]

Lamins from vertebrates and invertebrates differ significantly in their primary structures.[66,69] The two lamins of *D. melanogaster*, are more similar to each other than any other lamin, and share characteristics with both A-

and B-type vertebrate lamins. Like B-lamins, Dm0 is constitutive, has a CaaX box, and can associate to membranes; whereas lamin C does not have a CaaX box and is expressed in differentiated cells. Thus Dm0 and lamin C would be paralogues rather than homologues of the vertebrate A and B lamins.

8.1. SECONDARY STRUCTURE OF LAMINS

The main structural differences between lamins and IF proteins are: the six additional heptad-repeats in coil 1B of their rod domain, their very short (33 amino acids) globular domain head, and the C-terminal globular domain containing a NLS and a IgG fold and a C-terminal CaaX box (except for lamin C), which undergoes posttranslational modifications (Figure 2A, C). Like all IF proteins, lamins have strong homologies at both ends of the rod domain, which play essential roles in their assembly.[66] The C-terminal Ras-like CaaX box motif is subjected to a series of posttranslational modifications, including isoprenylation, proteolytic trimming and carboxymethylation of its terminal cysteine. Isoprenylation is essential for targeting lamins to the inner NE. In lamin A, the C-terminal 18 amino acids, including the isoprenyl group are removed by proteolysis.[70] The end domains are variable in the different lamins and play important roles in lamin assembly.[70] Lamins interact directly with each other, with lamina-associated proteins (LAP1 and LAP2, emerin, lamin B receptor, etc.), with NSK proteins like NuMA,[17] and with chromatin.[68]

8.2. LAMIN-ASSOCIATED PROTEINS

Lamins interact with many nuclear membrane and nucleoplasmic lamina-associated proteins (LAPs), forming polymers that constitute the lamina and confer mechanical strength to the nucleus.[66–68] The main ones are the lamin B receptor (LBR), the LAPs and emerin. They show different affinities for lamins. LAP1A and LAP1B interact with lamins A, B and C, whereas LAP2β specifically associates with lamin B and chromatin. They form multiprotein complexes with lamins, integral membrane proteins and different kinases.[66,67] LBR and LAP2 bind to chromatin and chromatin-associated proteins like HP-1, a protein component of heterochromatin. LBR and LAPs are phosphorylated *in vivo* and *in vitro* by kinases such as the CDK1 kinase.

Some of the LAPs are architectural partners that form stable complexes in interphase and have known structural roles. Amongst them are integral membrane proteins, including the LEM-domain proteins (LAP2, LBR, emerin, LEM2 and MAN1); the SUN-domain proteins; titin; actin that

interacts with emerin and spectrin; and proteins of the nesprin family with a modular structure containing multiple spectrin repeats and calponin homology domains for actin binding, that enable them to interact with different components The small isoforms of nesprin-2 bind to lamin A and emerin in the lamina. Others are chromatin partners such as histones and BAF; gene regulators including several transcription factors and repressors like MOK2 and pRb; and signalling proteins such as protein kinase Cα.[67,68] LAPs function as assembly elements for multiprotein complexes integrating structural and regulatory proteins, splicing factors, epigenetic modifiers and signalling molecules, indicating that the lamin-LAP complexes may serve as scaffold structures for nuclear architecture and nuclear reassembly after mitosis; large scale chromatin organization, gene regulation and signalling.[68]

LBR is the best characterized among them. It has a C-terminus with eight predicted transmembrane domains and a nucleoplasmic N-terminal domain that interacts *in vitro* with B-type lamins, HP1 and with chromatin itself, and also contains phosphorylation sites for PKA, CDK1 and an arginine serine (RS)-kinase.[66,67,71] The LBR-associated kinase keeps LBR phosphorylated at the serine residues of the N-terminus during interphase and mitosis. Dephosphorylation decreases the affinity of LBR for lamin B. LBR is the first component to bind to chromatin in telophase, and is required for proper NE assembly.

LAPs contain long N-terminal nucleoplasmic domains, a single transmembrane domain and a short C-terminal lumenal tail. LAP1A, LAP1B and LAP1C are produced by alternative splicing of the same gene product, as occurs with LAP2 protein isoforms. LAP2α has an extended unique C-terminal domain and is a part of the NSK, but the rest of the isoforms are type II integral membrane proteins.[71] Phosphorylation of LAP2 with an M-phase extract reduces its affinity for lamins, while that of LAP1 is not altered.

The inner nuclear envelope protein emerin is also a type II integral membrane protein and possesses the N-terminal nucleoplasmic LEM (LAP Emerin MAN) motif, common to several NSK proteins. Emerin specifically associates with lamin A or C *in vivo*.

8.3. POLYMERIZATION OF LAMINS

Lamins form dimers by parallel-unstaggered interactions of their rod domains, with two globular heads at the C-tail. Lamin dimers associate longitudinally to form polar head-to-tail polymers, in contrast to the lateral association of cIF dimers, which form antiparallel, unstaggered or halfstaggered tetramers.[66] Head to tail polymers associate laterally in an antipararell, quasi half-staggered fashion to form IF-like structures with a

distinct 24–25 nm beading repeat. *In vitro*, the beaded lamin filaments are not stable and further associate into fibres, and eventually, into large paracrystalline arrays with a transverse bending pattern of their 24–25 nm axial repeat. Overexpression of lamin mutants suggests that the two lamin types assemble differently *in vivo* and indicate that A-type lamins can form paracrystal-like fibres.

In cells, lamins polymerise as a 2D-array at the INE. The interaction must be specific, flexible and reversible to allow for nuclear growth, and later disassembly in mitosis. After targeting to the INE, their assembly into the lamina and further association is stabilized by integral membrane proteins. The lamina forms around B-type lamin dimers. These are tethered to the INE through association with LAP2β through coil 1B and an isoprene receptor, probably LBR, that maintains the lamin dimers lying parallel to the INE. The dimers form N-N half-staggered antiparallel associations that produce tetramers and head-to-tail oligomers to form higher order structures that lead to the assembly of 10–13 NSK filaments in an orthogonal flattened array adjacent to the INE.[67,71] A-type lamins are not required for the assembly of the basic lamina. Its association with the lamina occurs through the formation of hetero-oligomers with lamin B. Lamin A dimers form tetramers in the nucleoplasm by half-staggered antiparallel associations. The recruitment and stabilization of lamin A in the lamina is mediated by emerin and LAP1C. Lamin C associates with the lamina by the formation of antiparallel tetramers in the nucleoplasm that are incorporated into the lamina by head-to-tail association with the lamin B oligomers in the NE.

8.4. INTERNAL LAMINS

Lamins localize in intranuclear foci, containing either A- or B-type lamins, and in an intranuclear network.[17,72] The lamin foci form and disappear in a cell cycle-related way. They are most prominent in G1, where they are thought to represent pools of lamins, where intermediate assembly and/or posttranslational modifications of lamins (isoprenylation, phosphorylation, methylation and proteolytic cleavage) take place before their incorporation into the lamina. Immunolabelling of the endogenous A-type lamins reveals not only the foci, but also filamentous structures in the nucleoplasm. Lamin B-type foci do not co-localize with lamin A foci. They are most prominent during S phase and co-localize with replication sites. The internal pool of A-lamin has distinct solubility properties from that in the NE, and forms a thin fibrillar web with the morphological characteristics of the NSK core filaments.[17] The dimensions of their fibres correspond approximately to those of the octameric protofilament, the major block of the cIF tetrameric

protofilament (2–3 nm). A/C lamins form nucleoplasmic complexes involving LAP2α. They have been implicated in the regulation of gene expression, cell cycle progression and differentiation, by interactions with different partners such as BAF, transcription regulators, nuclear actin, and pRb.[72] Apparently, the interactions of the nucleopasmic lamin scaffold with RNA are critical in the stabilization of the endoskeleton. This interaction is probably required to maintain the lamin scaffold in a state of high molecular dispersion, and probably mediates the association of NuMA and other proteins, and protein complexes, with the lamin scaffold.[17]

The organization of lamins is dynamic.[42] The interaction of lamins with chromatin and the NE occurs very early in postmitotic reformation. Lamin B1 is quickly recruited to chromosomes during telophase and is stably re-assembled along with its binding proteins at the INE at early G1.[73] During S-phase, lamin B localizes in replication foci, and proper lamin organization is necessary for replication to proceed.[74] Lamin A enters the nucleus slowly after NE reformation and assembles in discrete foci, but only after several hours is it incorporated into the lamina.

8.5. ROLES OF LAMINS

Lamins have a sophisticated domain structure that allows multifunctionality in different contexts. They play a role in nuclear stabilization and growth, proper cell cycle regulation, cell differentiation and apoptosis, DNA replication and repair, chromatin organization, NE assembly and spatial arrangement of NPCs, and anchorage of NE proteins.[66,67,74] Mutations in lamins disrupt all these functions and cause genetic diseases.[68,71,72]

Lamins bind chromatin and MAR sequences, which anchor DNA loops to the NSK. MARs have a high A-T content and tend to unwind into ssDNA. The lamin rod domain is sufficient for MAR binding, provided it is assembled in oligomers larger than dimers. The rod domain is also sufficient for chromosome binding. A- and B-type lamins have a second motif for chromatin binding in the tail domain encompassing the NLS, that binds histones but not naked DNA. They bind core histones through a sequence of the tail domain adjacent to the rod domain. *Xenopus* lamins A and LII bind to minichromosomes by a sequence element in the C-terminal tail domain.

Lamins interact with DNA and chromatin proteins. Lamin associated proteins bind chromatin proteins and epigenetic modifiers such as BAF. These interactions are involved in heterochromatin formation, nuclear assembly and chromatin decondensation, and suggest a role for lamin complexes in the formation and epigenetic control of heterochromatin.[68] A-type lamins bind to multiple transcriptional regulators and affect

transcription by different mechanisms.[68] Lamin complexes provide a scaffold for the assembly of repair foci.[68]

Experiments with mutants demonstrate that lamins, but not lamin-associated proteins, are necessary for elongation of replication. B-type lamins probably provide a scaffold essential for the assembly of elongation complexes in replication foci. LAP2 or LAP2β interaction with HA95, an interphase protein between chromatin and NE, is involved in the control of initiation of DNA replication, but is dispensable for on-going replication.[75]

8.6. DO PLANTS HAVE NUCLEAR LAMINS?

Although no genes for lamins or their associated proteins have been found in *Arabidopsis* or rice so far, it is very unlikely that the lack of lamin orthologues means that lamin functions are lacking in plants. As lamins are involved in fundamental nuclear processes (see Section 8.5),[67,68] plants would have functional rather than structural homologues of the vertebrate lamins.

The architecture of the nuclei and nuclear matrices in plants includes a peripheral structure associated with the INE, ultrastructurally similar to the vertebrate lamina.[18,22] It contains IF–type proteins and shows cross-reactivity with antibodies against vertebrates lamins (Figure 1D).[18,22,29] Proteins sharing antigenic determinants with vertebrate lamins are present in several plant species (Figure $1D_1$).[21,22,29] They also have Mr, pI values and a nuclear distribution (Figure $1D_{2,3}$) similar to those of vertebrate lamins, and are specifically cleaved in apoptosis.[76] Although lamin-associated proteins are not conserved in plants, BY-2 tobacco cells transformed with an N-terminal fragment of the LBR that anchors the protein to the INM, direct the transformed protein to the NE. Binding mutants of the protein have altered targeting and retention, suggesting that the translocation mechanism for the LBR is conserved in plants that also have proteins weakly binding to it.[77]

Using A/C and B2 lamins as query sequences, several potential *Arabidopsis* proteins with moderate similarity were found. The alignment is predominantly with the α-helix rod domain, but some of the plant sequences also contain a homologue of the NLS.[78] The plant lamin-like proteins would have conserved domains necessary for essential lamin functions, but also other plant-specific ones and be functional rather than structural homologues of lamins. This occurs in coilin, the organizing protein of Cajal bodies, that in *Arabidopsis* is encoded by a distant coilin homolog and shows limited homology with the vertebrate proteins, though keeping the same nuclear localization and functionality.[79]

Besides lamin-like proteins, the plant lamina contains other plant specific proteins that could form part of this structure as nuclear intermediate filament proteins (NIFs),[80] NMCP1,[81] and NACs (nuclear acidic coiled coil proteins).[80]

At least three NIFs sharing antigenic determinants with chicken lamins, human keratins and IFs have been detected in the NSK of pea, *Arabidopsis*[80] and onion (unpublished results). They have Mr values of 65, 60 and 57 kDa, and display multiple isoelectric forms with pIs between 4.8 and 6.0. Peptide sequencing showed short regions of similarity with lamins B and A that would explain their crossreactivity, keratins and with *Arabidopsis* proteins containing long coiled coils, like NAC, FPP3 and MFP1,[80] although the last three proteins have predicted Mrs larger than the pea NIFs. The purified NIFs form 6–12 filaments *in vitro*. *In situ*, they show a peripheral and internal nuclear distribution.[80] Thus, plant NIFs although different from animal lamins could have lamin-like regions and be functional homologues of lamins.

9. Other IF components of the plant nuclear matrix: MFP1, NMCP1 and NMP1

Only a small number of long coiled-coil proteins have been identified in plants. The best characterized is MFP1. It is a conserved protein of the plant NSK with specific variability in the number of genes and proteins, and in its expression. The proteins sequenced from tomato, *Arabidopsis* and tobacco contain three domains: a N-terminus with two conserved hydrophobic domains; a central extended (about 85% of its sequence) coiled-coil rod domain; a C-terminal DNA binding domain, and many CKII phosphorylation motifs. Although the homology amongst species is not high (about 45%), some domains are highly conserved (about 90%), showing their relevance to protein functionality: among them are the two N-terminal hydrophobic domains and the spacing between them, two potential phosphorylation sites for CKII, and the C-terminal DNA-binding domain. MFP1 shares certain similarity with Hslamin A, NuMA and CENP-A.[32] One or two MFP1 proteins are expressed.[82] Onion cells have two antigenically related MFP1 homologues. Ninety kilodaltons-AcMFP1 has different phosphorylation stages and is a component of the NSK, while 80 kDa-AcMFP1 is not.[83] The association of MFP1 to NSK is regulated by CKII phosphorylation.[84] These proteins are highly expressed in proliferating cells, but their levels decrease when cells leave the cell cycle. MFP1 distribution, expression and the modulation of its binding to the NSK suggest that it is not likely a basic component of the expansible

nucleoskeleton but more probably is involved in the binding of chromatin to this structure (Figure 2F).[84]

NMCP1 is a 134 kDa-conserved protein of the carrot NE, which shows similarity to myosin, tropomyosin and IFs. It has a central coiled-coil domain, flanked by non-helical short head and long tail domains. The terminal domains contain potential NLS and many recognition motifs for protein kinases, including the CDK1 kinase and PKC. NMCP1 has a similar pI (5.6–5.8), although its size is almost twice that of lamins.[85] NMCP1 localizes to the NE, and behaves like a lamin during mitosis.[81] Putative orthologs of NMCP1 are also present in the *Arabidopsis* and rice genomes (accession numbers CAA 16682, BAC 19761). However neither the phylogenetic relationship between NMCP1 and lamins nor its polymerization abilities to form filaments are resolved at present. Though its association with integral nuclear membrane proteins, and its ability to bind DNA and chromatin have to be checked, the sequenced NMCP1 is a good candidate for a lamin-like IF protein in plants. Plants could have evolved strategies different from metazoans, including different nuclear structural proteins and binding partners, to localize conserved NE protein domains and function.

Another example of specific structural coiled-coil proteins in the plant nucleus is NMP1. It is a highly conserved α-helical protein of the plant NSK, with no homologues in animals or fungi. Its secondary structure is characterized by a large number of highly conserved short coiled-coils. NMP1 localizes to both cytoplasm and NSK. NMP1 lacks a classical NLS. Although, given its size (37.4 kDa), NMP1 could passively diffuse through the NPCs, the NMP1-GFP fusion protein that is significantly larger than the exclusion size for NPCs, also enters the nucleus. This suggests the existence of an active transport mechanism.[86]

10. FPP (*Filament-like Plant Proteins*)

The first family of plant-specific long coiled-coil proteins has been identified in the *Arabidopsis* genome. The FPP (Filament-like Plant Proteins) family is defined by the presence of two clusters of coiled-coil domains separated by a linker. All family members present four novel motifs, highly conserved in sequence and position. Seven FPP proteins are present in *Arabidopsis* and one homolog each has being identified in tomato and rice. All family members are expressed in a variety of *Arabidopsis* tissues. This family of proteins appears to be conserved in higher plants, but no convincing homologues were identified from yeast or animals by sequence similarity searches.[87] They represent an interesting field of

investigation with respect to their function and the evolutionary conservation of coiled-coil proteins in plants.

11. Concluding remarks and perspectives

In spite of the general conservation of genome organization, replication, division and expression, after a billion years of independent evolution, animal and plant cells have developed differences in the proteins involved in organizating their NSK.

Although a skeletal structure associated with the inner NE is evident in both systems, and proteins sharing antigenic determinants with lamins and IFs (Figure 1D, B) exist in plant cells, the orthologues of the genes encoding the main proteins organising this structure in vertebrates (including lamins, LAPs and LBR) have not been found in either the *Arabidopsis* nor in the rice genomes. An internal NSK of IF-type filaments is also evident in plants[18,19] (Figure 1A), and NuMA-like proteins are present in the NSK and mitotic spindle[19] (Figure 1C). Also a putative NuMA ortholog and the NuMA-associated protein GAS41, have been identified in *Arabidopsis*.[51]

Furthermore, plants seem to have evolved unique IF-like nuclear proteins such as NIFs, NMCP1, MFP1 and NMP1 with functional domains similar to those in animals, to solve the problems of nuclear architectural organisation and functioning. The challenge in this field is to clarify the molecular architecture of the plant NSK, and to determine the characteristics and functions of the specific subsets of proteins forming the lamina, and the NSK-filaments.

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PART 2

CYTOSKELETON AND DEVELOPMENT

MICROTUBULES AND THE CONTROL OF CELL ELONGATION IN *ARABIDOPSIS* ROOTS

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Abstract. A role for cortical microtubules, cellulose microfibrils and cell wall enzymes in the control of plant cell expansion has been suggested for several decades, but has not yet been proven in any system. *Arabidopsis* root epidermis is an ideal tissue to test this hypothesis, and consequently is the subject of many recent reports. Microtubules of epidermal cells are transverse to the root axis in the zone of fast elongation, but when cells enter the differentiation zone microtubules switch to a slightly oblique orientation in atrichoblasts and to a disordered distribution in trichoblasts. Factors that severely affect root elongation do so by acting on the process of fast elongation. Ethylene, auxin, water stress as well as reduced cellulose synthesis induce a similar response. Within minutes a new and much shorter final cell size is defined, and cells do not grow beyond this length. Eventually, fast cell elongation is totally inhibited. In cells that are longer than this new final cell size, cortical microtubules change their orientation in the time frame of 10–15 min and adopt orientations typical for the differentiation zone, while the orientation of cellulose fibrils is not changed in the walls of the affected cells. Microtubules, however, are not the causal agents: as fast elongation and its inhibition are insensitive to microtubule disrupting or

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stabilizing drugs. Control of cell size seems to be located at the level of cell wall metabolism. The profile of xyloglucan endotransglucosylase action changes only slowly, but a significant callose deposition in the walls of the underlying cortex cells accompanies the inhibition of elongation.

Keywords: Microtubules, fast cell elongation, cytoskeleton

1. Introduction

Anisotropic cell expansion is a cornerstone of plant development. It are changes in the cell wall mechanical properties that allow or restrict cell expansion. Although nowadays the complex of cellulose and xyloglucans is considered to be the load bearing network in the plant cell wall,[1] it is the orientation of cellulose fibrils that determines the mechanically anisotropic properties of the cell wall.[2,3] The strong correlation between the orientations of microtubules inside the plasma membrane and recently deposited cellulose microfibrils outside the plasma membrane have been stressed in many studies.[4] It has lead to the widely accepted cellulose synthase constraint model, that proposes that cortical microtubules in some way determine and constrain the trajectories that cellulose synthase complexes can follow in the plasma membrane while they add new cellulose fibrils to the inner side of the cell wall.[5] This model and the fact that perturbing microtubule dynamics or just eliminating microtubules impairs growth anisotropy[6] are the basis of a central dogma in plant cell biology. The dogma says that parallel microtubules, transverse to the cell axis, organize parallel and transverse microfibrils in the wall and thus lets the wall extend anisotropically along the cell axis. As a consequence a change in the direction of cell growth results from a change in microtubule orientation that induces a new cellulose ordering and finally the new axis of cell expansion. This dogma is widespread as it appears in standard textbooks.[7]

However the very strict sequential link between microtubules and microfibrils has been questioned for a long time.[8–11] Even so recent work on two Arabidopsis mutants showed extensive radial cell expansion despite no detectable changes in microtubule or microfibril orientation.[12]

It is clear to date both microtubules and microfibrils play a major role in establishing anisotropic cell expansion or elongation. Support for this is found in classic long term inhibitor experiments. But this does not imply that they act in concert as predicted in the dogma. Support for this is found in short term inhibitor experiments,[13] this report and consequently the link of the cytoskeleton with other aspects of wall metabolism as secretion and matrix restructuring, has been suggested.[13]

A missing link in the research on cell elongation or anisotropic cell expansion to date is the mechanism of control. All studies, also these with mutants, refer to the onset or the maintenance of elongation. Recently we have shown that the rate of elongation is continuously steered by the levels of endogenous hormones and that this control is exerted precisely in the zone of most rapid elongation.[14] Against this background of recent work on the matter we will reassess the matter of microtubules – microfibrils and cell elongation in roots of wild type Arabidopsis plants that are manipulated in their cell elongation activity.

2. Results and discussion

2.1. DEFINING FAST ELONGATION

As in many species of seed plants elongation activity is not evenly spread throughout the so called elongation zone in the root of Arabidopsis, which has lead to the definition of "subzones" in the elongating part of the root.[15] The classic method to describe the distribution of elongation activity is plotting values of the REGR (relative elemental growth rate) along the root.[16] In Figure 1 the distribution of REGR is shown for the root of 4–5 days-old *Arabidopsis* seedlings, used in our experiments. However some authors came to the conclusion that this way of representing elongation activity does not show the real picture and with new approaches in computational image analysis they made clear that elongation activity is regulated in a step-wise fashion.[17] These authors show that the zone of maximum elongation rate stretches from about 570–1,050 μm from the root cap – root junction (RCRJ). Recently we have introduced a method to monitor elongation activity on cell level.[14] The length of cells in the trichoblast cell files is an excellent parameter for kinetic analysis of cell elongation activity. It turns out that once these cells left the meristematic zone they elongate very slowly until they reach a length of about 35 μm but then elongate in about 2 h time to a length of about 135 μm, the mean length of cells that normally start to develop a root hair (LEH is the Length of the cell with the Earliest root Hair bulge). This very fast elongation happens in the zone between 570 and 900 μm away from the RCRJ. During root hair outgrowth elongation declines and trichoblasts reach a final length of about 157 μm at about 1,200 μm away from the RCRJ. The data obtained with the method of *in vivo* monitoring of cell length[14] coincide perfectly with the details revealed by computational methods[17] but additionally give details on level of the individual cell: fast elongation occurs between 570 and 900 μm, and elongation stops in the beginning of the differentiation zone.

2.2. THE CONTROL OF FAST ELONGATION

In a recent paper we have reported that the fast elongation is controlled by ethylene in a dose dependent way.[14] In the complete absence of ethylene (after AVG treatment) cells elongate considerably more than in normal conditions where there is some endogenous ethylene present in the root. At saturating conditions ethylene or its precursor ACC (1-aminocyclopropane-1-carboxylic acid) completely abolishes elongation and trichoblasts never elongate beyond the 35 μm limit.

As generally known, water stress results in a reduced root elongation. In Arabidopsis this effect also localizes to the zone of fast elongation. Seedlings were subjected to a mild hydraulic stress by adding mannitol at 10 g/L (inducing a change of –0.14 Mpa osmotic potential). Application of such an osmotic stress resulted in a rapid reduction of the elongation rate (Figure 1A). After 1 h the LEH was reduced to half the control value. The inhibition of elongation was practically instantaneous on cell level, as revealed by following individual cells. The maximal elongation rate in a root occurs in the cell located just before the first visible root hair bulge counting from root apex. In Figure 1C, the Cell A (highlighted by a white bar) was expected to reach the length of Cell B (highlighted by a black bar) within 30 min under normal conditions. When the root was exposed to osmotic stress, Cell A did not expand anymore during further development (Figure 1D). This cell was thus blocked in its elongation within less than 30 min. This dramatic decrease in LEH after 2 h treatment is illustrated by Figure 1B. The distance between the youngest root hairs is much shorter than in Figure 1C. In the period after 2 h the roots adapted to the osmotic stress as shown by higher growth rate after 3 h (Figure 1A, B) and illustrated by an increased spacing of the new root hairs in Figure 1E after 5 h.

Also auxin exerts its inhibitory effect on root growth in the elongation zone. At a concentration of 5 μM it induced a reduction of the LEH, similar to the effect of ethylene or its precursor ACC (Figure 1F). After 30 min 15% of inhibition on LEH was already found. After 3 h, the LEH was reduced from 127 ± 15 to 49 ± 7 μm. The changes in LEH induced by IAA are similar to these of ACC-treated roots (Figure 1F). As there is a clear crosstalk between auxin and ethylene in Arabidopsis[18] we controlled if the effect might be due to auxin-induced ethylene biosynthesis. The responses of wild type plants and the ethylene insensitive mutant *etr1-3* to exogenous auxin and ethylene were compared. *Etr1* is a mutant with a defect in the ethylene receptor.[19] *Etr1-3* did not react to ACC but had its cell elongation reduced by exogenous IAA (data not shown). These results indicate that the effect of auxin on cell elongation is not mediated through ethylene signaling.

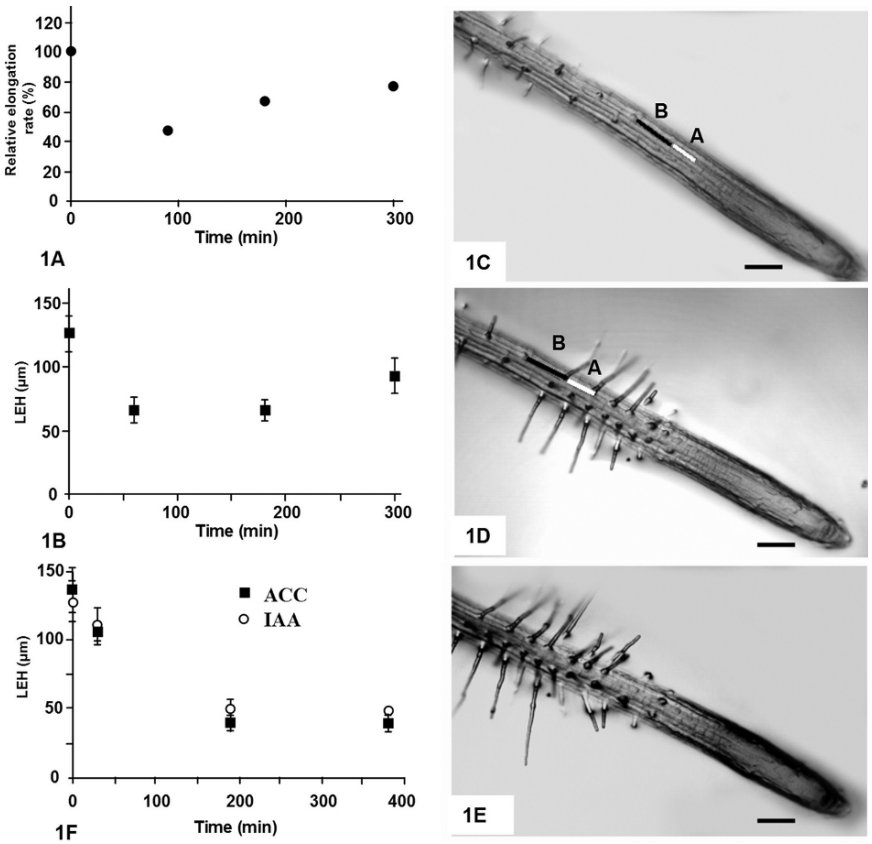

Figure 1. Effects of osmotic stress and auxin on root growth rate and cell elongation extent. A, Application of osmotic stress dramatically reduces root elongation rate. After 1.5 h, the root elongation rate reduces to around 50% of the control. B, Changes of LEHs. C, Before osmotic stress, Cell A has the elongation potential to reach the length of Cell B within 30 min. Cell A and Cell B are highlighted by a white and black bar respectively. D, After 3 h of stress, the cell elongation is dramatically inhibited in the same root. The failure of Cell A to reach the length of Cell B points to the rapid effect of osmotic stress on cell elongation within 30 min. E, After 5 h, the root exhibits recovery in elongation as indicated by the increase of the LEH. F, The effect of auxin and ethylene on cell elongation is comparable as revealed by similar changes of LEHs. Scale bar = 100 µm.

Mutations in genes coding for members of the cellulose synthase family not only reduce cellulose production but also reduce cell elongation.[20,21] This effect is mimicked by the cellulose synthesis-inhibiting drug 2,6-dichlorobenzonitrile (DCB).[13] When DCB is applied to the seedlings, it is also the fast cell elongation that is affected. Within 3 h (Figure 2) the LEH was reduced from 100 ± 14 to 43 ± 6 µm. DCB did not affect root hair initiation but slightly changed their morphology. The new root hairs had an enlarged base (inset of Figure 2).

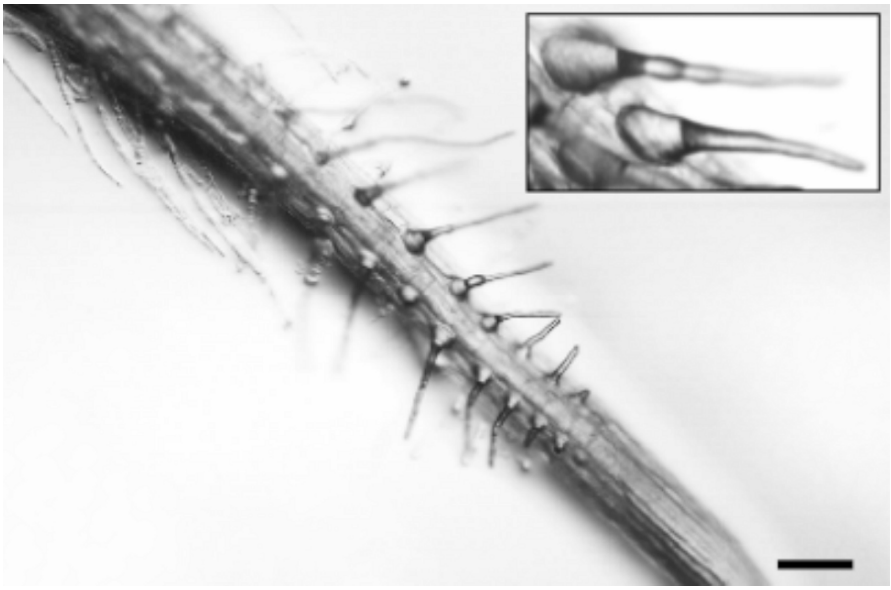

Figure 2. Effect of the cellulose synthesis inhibitor on root growth. Epidermal cell elongation is inhibited by 5 μM DCB. This is reflected in the shorter distance between two consecutive root hairs in a cell file. The root hair initiation is not affected in any treatment during the period of observation, resulting in similar numbers of newly formed hairs, but the root hair base appears swollen (see inset). Scale bar = 100 μm.

The zone of fast elongation is thus of particular importance for the normal development of the root. Cells enter this zone at a rate of one per 30 min (in the trichoblast cell files) and leave the zone about 2 h later. In this period of time their length increases by nearly a factor of four. This fast elongation is the target for many signals that affect root elongation.

2.3. MICROTUBULES AND MICROFIBRILS

Cortical microtubules and cellulose microfibrils have to date been widely accepted as main actors in the establishment of growth anisotropy or cell elongation in plants,[4] as many studies have revealed a strong correlation between the orientation of both components. During rapid elongation this is striking as both microtubules and microfibrils are transverse to the cell axis. The cellulose synthase constraint model proposes that cortical microtubules constrain the paths followed by cellulose synthases that are integral membrane proteins as they add new cellulose fibrils to the inner layer of the wall.[5] Although the role of both microtubules and microfibrils has never been formally denied, the precise role of both components in the control of cell elongation has been questioned since long.[8] The matter has recently attracted renewed attention.[13]

Therefore the orientation of as well microtubules as microfibrils was studied in the fast elongation zone during normal growth and during impaired elongation. Microtubule localization was studied as well *in vivo* as by using standard immunolocalization procedures.

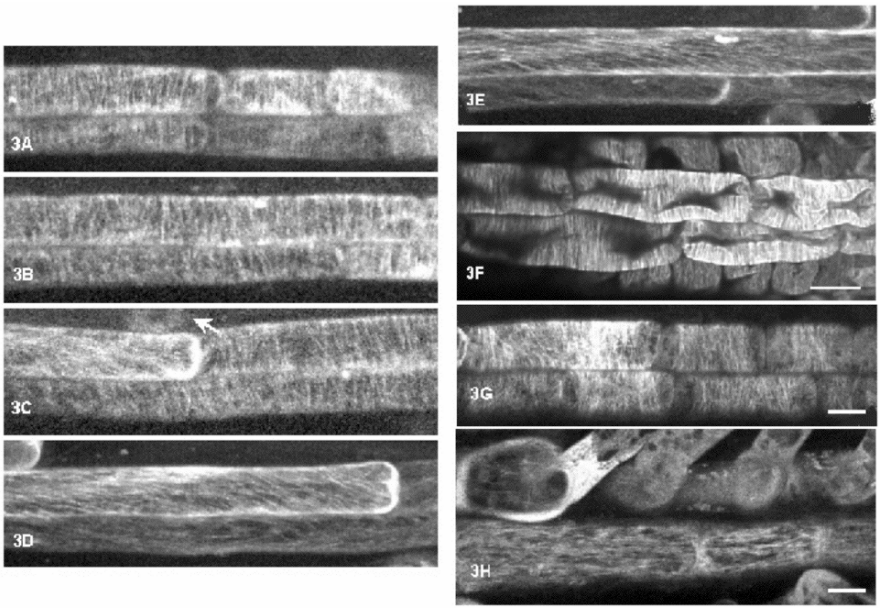

Figure 3. Microtubule orientation pattern in root epidermal cells revealed by GFP-TUA6 plants under normal grown condition and ACC treatment. A and B, Transversely aligned microtubules in the beginning (A) and the middle part (B) of the elongation zone. C, Microtubules start reorientation from transverse into oblique in atrichoblasts when a root hair bulge is forming from a neighboring trichoblast (indicated by arrow). D and E, In older atrichoblasts, microtubules are more oblique. F, Transverse microtubules in a GFP-TUA6 root labelled with the immunofluorescent approach. G, Transverse microtubules in the cells located in the elongation zone of an ACC-treated root. H, Longitudinal microtubules in atrichoblasts in the differentiation zone of an ACC-treated root. Scale bar = 15 μm.

GFP-TUA6 is a transgenic Arabidopsis plant with stable expression of the GFP-α-tubulin 6 fusion protein in hypocotyl and leaves.[22] We found weak microtubule fluorescence in roots. It was however sufficient to follow changes in orientation and to control the validity of conventional immunolocalization. The value of the latter is confirmed by comparison of Figure 3F showing microtubules after immunolocalization with Figure 3A and 3B showing GFP-microtubules *in vivo*, both in the elongation zone of the root. In normal growth conditions microtubules are transverse to the cell axis in the zone of fast elongation (Figure 3A, B). They switch to an oblique orientation in atrichoblasts at the end of the elongation zone, i.e. when the first root hair bulge appears in adjacent trichoblasts (Figure 3C, the arrow

indicates a root hair initiating). In older atrichoblasts, found in the differentiation zone where cell elongation has stopped, the microtubules finally adopt a longitudinal orientation (Figure 3D, E).

When the seedlings were exposed to ACC for 3 h, elongation was impaired like in wild type plants and differentiation started in cells much shorter than the traditional 135 μm. In the elongation zone microtubules remained predominantly transverse in cells up to a length of about 40 μm (Figure 3G). In the differentiation zone microtubules in atrichoblasts were longitudinal (Figure 3H). Mark the short length of the atrichoblasts (Figure 3H) compared to the size reached in untreated controls (Figure 3E, F).

The behavior of microtubules was also followed when a mild hydraulic stress was the cause of impaired elongation, now using immunolocalization. After 10 min of stress, the transverse microtubule orientation was not affected (Figure 4A). Disordered microtubules were found after 30 min of treatment, when cell elongation had been already arrested (Figure 4B). After 1 h of treatment, longitudinal microtubules were found in atrichoblasts at the end of the reduced elongation zone, where root hair bulges develop in trichoblasts (Figure 4C, D). Also under this treatment microtubules were still transverse in elongating cells up to a length of 35–40 μm (Figure 4D).

To visualize the mean cellulose microfibril orientation the wall matrix was first extracted with an acid treatment,[16] and then the Congo Red – polarization confocal microscopy method[11,23,24] was applied. The axiality ratio (AR) was determined for the outer epidermis cell wall at different places along the root (Table 1). The AR is a measure for the mean orientation of cellulose fibrils in the wall.[24] In the fast elongation zone and in the root hair initiation zone (cells 1–3 beyond the LEH) of control roots, the mean cellulose orientation was clearly transverse to the root axis, with AR values of 0.53 and 0.55. In the maturation zone (cells 10–13 beyond the LEH) the AR reached unity (1.07) meaning an isotropic situation without a clear preference in cellulose fibril orientation. Still further away from the root tip (cells 17–20 beyond the LEH) the AR had a value of 1.42, indicating that the mean orientation of the cellulose fibrils in the wall was now longitudinal. Treatment of the roots with 5 μM ACC for 2, 6 and 24 h did not affect the AR in the elongation zone. The AR values of 0.55, 0.56 and 0.57 are not statistically different from 0.53 in control roots (Table 1).

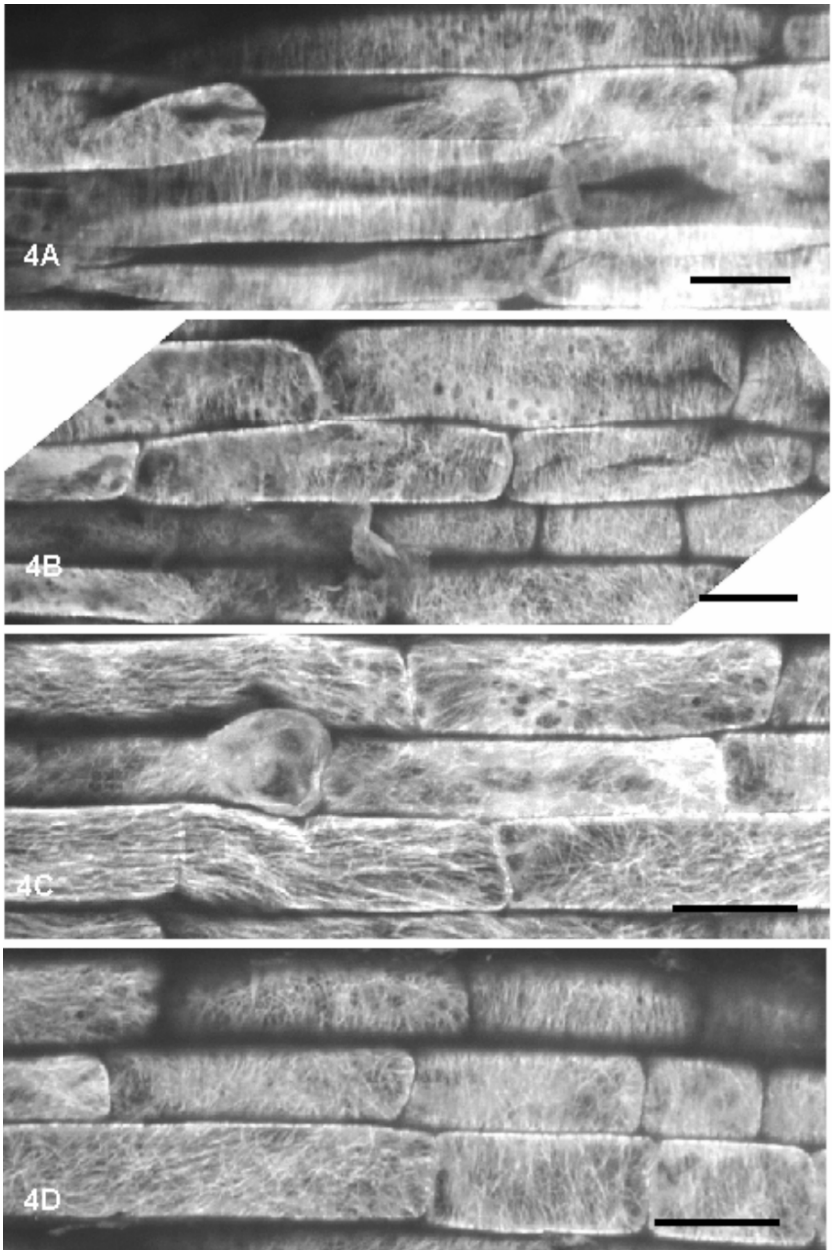

Figure 4. Osmotic stress induces dramatic microtubule reorientation after 30 min. A, The transverse alignment of microtubules is nearly not affected after 10 min of stress. B, After 30 min microtubules start the rearrangement from transverse into random orientations. C and D, At 60 min microtubule orientation is transverse in cells shorter than LEH, but longitudinal in atrichoblasts at the LEH border (root hair bulges being present in the adjoining trichoblast). Microtubules in short cells in the beginning of the elongation zone keep predominantly to a transverse orientation. Scale bar = 20 μm.

TABLE 1. ACC and mean orientation of cellulose microfibrils. Control and ACC-treated (5 μM) roots were pre-treated in boiling solution of acetic acid:nitric acid:water (8:1:8, v/v/v) for 30 min. After overnight staining in 1% Congo Red at 4°C, the mean orientation of cellulose microfibrils was determined with polarization confocal microscopy and is presented as axiality ratio[a] (average of roots ± SD).

Region	Control	ACC 2h	ACC 6h	ACC 24h
Elongation zone	0.53 ± 0.03	0.55 ± 0.07	0.56 ± 0.07	0.57 ± 0.05
Root hair initiation zone	0.55 ± 0.09	0.58 ± 0.05	0.52 ± 0.07	0.94 ± 0.20
Maturation zone 1[b]	1.07 ± 0.22	–	–	–
Maturation zone 2[c]	1.42 ± 0.16	–	–	–

[a]Axiality ratio is the ratio between the value of the Congo Red-based fluorescence intensity with the polarization vector of the laser beam parallel to the root axis and the fluorescence value with the polarization vector perpendicular to the root axis.

[b,c] Regions are respectively 10–13 and 17–20 cells away from the point of LEH.

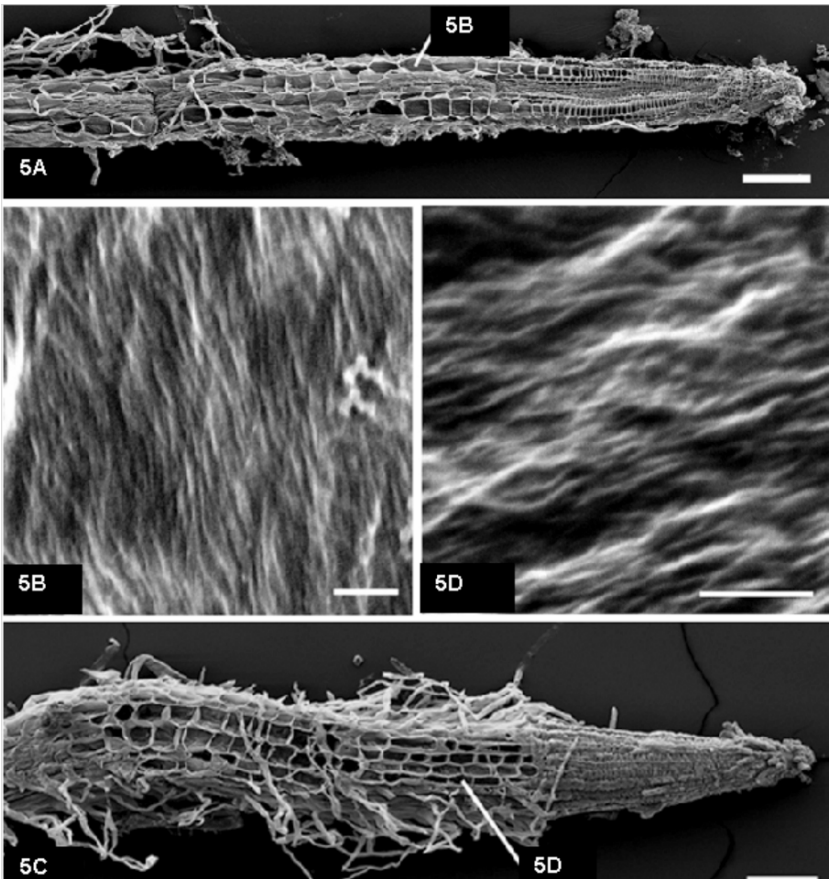

Figure 5. Effects of ACC on the innermost layer of cellulose microfibrils revealed by FESEM. A and C, Cutting faces of 12 and 24 h ACC-treated roots. B and D, Details of transverse and longitudinal orientation of cellulose microfibrils in epidermal cells in the root hair initiation zone. Scale bar = 100 μm in A and C; 250 nm in B and D.

Also in the root hair initiation zone ACC did not affect cellulose orientation during 6 h of treatment (Table 1). An increase of AR was only found after 24 h.

Besides this the innermost layer of cellulose microfibrils of cell walls was investigated using cryo-ultramicrotomy and field emission scanning electron microscopy (FESEM).[16] After 6 or 9 h exposure to ACC cellulose microfibrils were predominantly transverse in the root hair initiation zone (data not shown). Even after 12 h when cell elongation was clearly reduced (Figure 5A), cellulose microfibrils were still mainly transverse (Figure 5B). Only after 24 h of ACC treatment steep oblique or longitudinal cellulose microfibrils were found in the root hair initiation zone (Figure 5C, D).

It is clear that the severe reduction of elongation activity caused by ethylene, hydraulic stress or other factors, is accompanied by a quick reorientation of microtubules that is very obvious in atrichoblast cells. However, no changes in cellulose orientation could be found that related to the strongly reduced cell elongation.

2.4. MICROTUBULE REORIENTATION IS NOT INVOLVED IN THE CONTROL OF CELL ELONGATION

From the above experiments it was clear that in cells equal to or longer than the LEH, microtubules completely changed their orientation under the influence

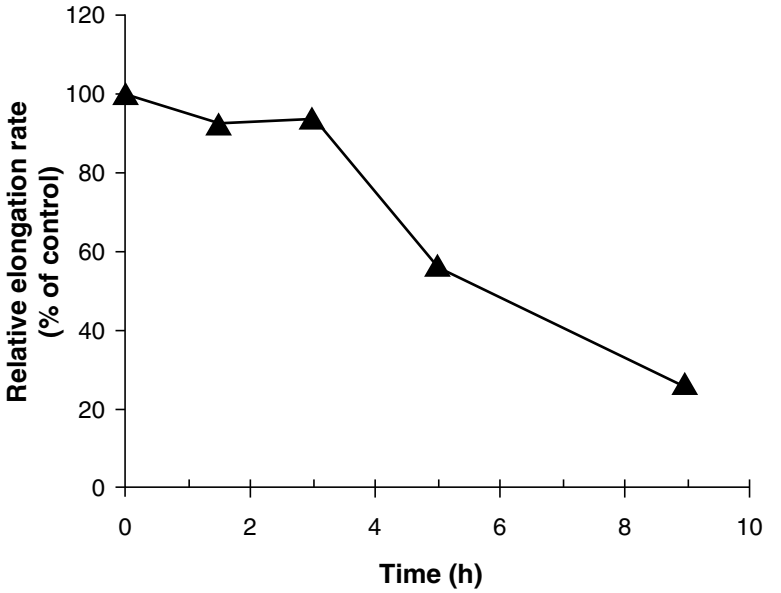

Figure 6. The effects of oryzalin on root elongation rate. During the first 3 h, the impact of oryzalin on root elongation rates is not detectable.

of ethylene or ACC within 1 h. To assess the importance of microtubules for elongation control, wild type seedlings were transferred to a medium supplemented with 5 μM oryzalin, an anti-microtubule drug, and the effect was monitored during 3 h.

After 15 min microtubules were not detected anymore in the epidermis by means of immunolocalization (data not shown). During the first 3 h the elongation rates remained normal (Figure 6). In this period of time, 6–7 new cells reached the LEH in each trichoblast cell file. At the cellular level, the epidermal cells had the same length and shape as in control roots (Figure 7A),

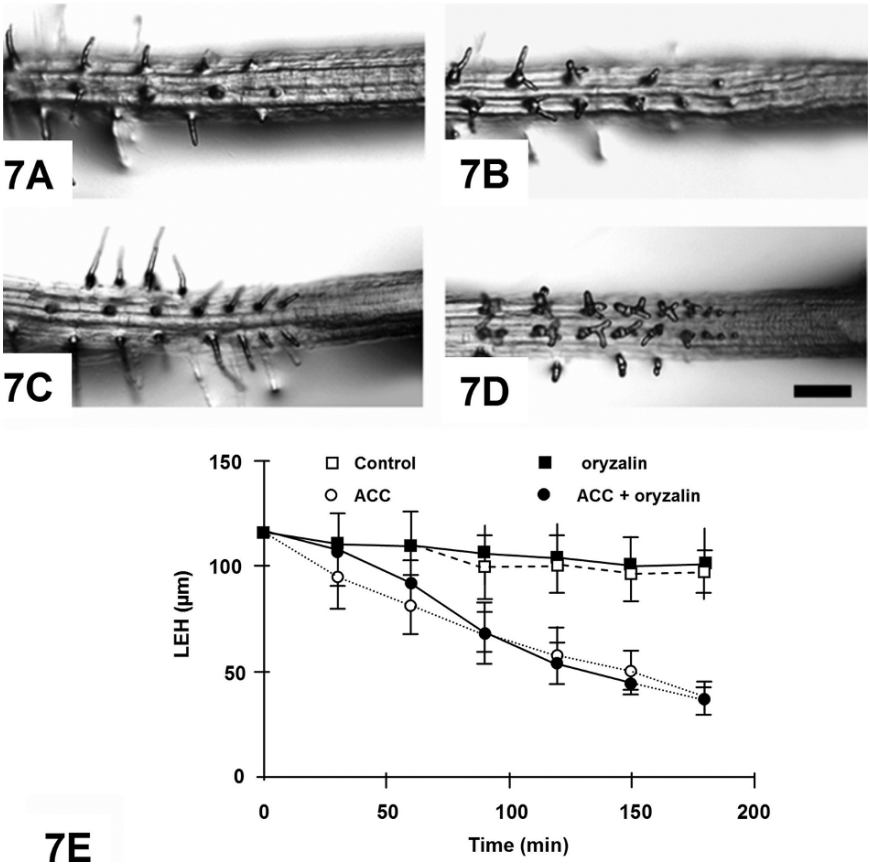

Figure 7. Effects of oryzalin and ACC on root growth after 3 h of treatment. A, Control root. B, Root treated with 10 μM oryzalin. The elongation of epidermal cells is not inhibited, whereas the shape of root hairs is obviously disturbed. C, Root treated with 5 μM ACC. D, Root treated with 10 μM oryzalin plus 5 μM ACC. Epidermal cell elongation is inhibited. This is reflected in the shorter distance between two consecutive root hairs in a cell file. Root hair initiation is not affected, resulting in similar numbers of newly formed hairs. Scale bar = 100 μm.

and consequently the distance between root hairs in the same cell file was normal and (Figure 7B). Also root hair initiation was not disturbed during this period. However branched root hairs were formed indicating the effectiveness of the oryzalin treatment (Figure 7B).

When 5 μM ACC was applied to the roots, the correlating LEH was established equally well in control as in oryzalin treated roots. In cells longer than the LEH, elongation was inhibited. After 3 h the effect was visually very striking as the shorter epidermal cells resulted in a shorter elongation zone and in a shorter distance between consecutive root hairs (Figure 7C, D). The branched root hairs in Figure 7D witness the effectiveness of oryzalin. The effect of ACC and the lack of effect of oryzalin on the value of LEH are confirmed by plotting the LEH values against course of time (Figure 7E). These experiments were repeated using taxol, a microtubule-stabilizing drug, yielding similar results (data not shown).

The data reported here on the relation between microtubules, cellulose microfibrils and cell elongation support the very recent findings[13] that microtubule orientation is not linked to cell elongation neither to cellulose orientation in the time frame of 6 h used in the experiments. Also the orientation of cellulose fibrils is not a measure or a guarantee for cell elongation. The long term effect (more than 6 h in this report, more than 8 h for wild type in reference 3) of microtubule destruction on cell elongation means that the drugs used are effective in cells 6–8 h prior to the onset of elongation. Thus microtubules play a crucial role preparing elongation in cells residing between the meristematic zone and the elongation zone. For maize this zone of the root has been described as the "transition zone",[25] pointing to the typical switch in activities and in direction of cell growth occurring in that part of the root.

2.5. THE CELL WALL AND CONTROL OF CELL ELONGATION

The cellulose-hemicellulose network is the major load-bearing component determining the mechanical strength of the wall.[3,26,27] It was suggested recently that arabinogalactan proteins and xyloglucans – the major hemicellulose – could be involved in maintaining growth anisotropy.[28,29] Focusing on a potential role for xyloglucans the xyloglucan endotrans-glucosylase (XET) action of the apoplastic XTH enzymes was localized and quantified in the epidermis using fluorescent xyloglucan oligosaccharides.[30] In untreated roots XET action was highest at the onset of fast elongation, and declined towards the end of the zone of elongation (Figure 8A). A treatment with 5 μM ACC for 2 h did not interfere with the location of high XET action along the root (Figure 8B) although the LEH was reduced and the elongation zone was much shorter. Using identical settings of the

confocal microscope (laser intensity, pinhole size, gain- and black level), XET-action related fluorescence intensity was quantified in control and ACC treated roots, and plotted against the position along the root (Figure 8C). Comparison of the fluorescence patterns and intensities revealed that ACC did not change the pattern of enzyme action, with the highest action at about 400 μm from the root tip. The site of root hair initiation had clearly moved towards the root tip, as shown by the arrow and the arrowhead that indicate the start of root hair initiation in ACC-treated and control roots respectively. After 6 h, the position of peak XET-action was clearly closer to the root tip in ACC treated roots than in controls (Figure 8D) as was the site of root hair initiation (arrow in Figure 8D).

Clearly an eventual involvement of XET-action in the control of cell elongation localizes to a part of the root that lies apical to the zone of fast elongation, a region where cells are preparing for elongation.

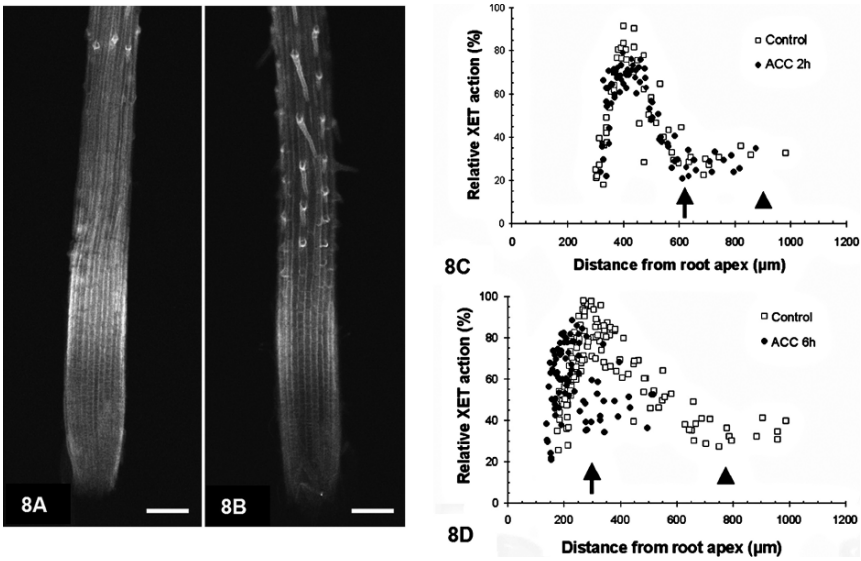

Figure 8. Effect of ACC on the spatial distribution of XET-action in the root epidermis.

A, High XET-action is localized in the initial part of the elongation zone in control plants. B, Treatment with 5 μM ACC for 2 h does not interfere with the location of high XET-action along the root, although the LEH was reduced and the elongation zone was much shorter. C, Relative values of XET-action are plotted against the distance from the root apex. Comparison of the fluorescence pattern and intensity reveals that ACC can slightly affect the XET-action but the treatments do not change the profiles of action along the roots within first 2 h. D, XET-action is still high after 6 h of ACC treatment, but is shifted towards the root tip compared with control roots. Arrows and arrowheads indicate the start points of root hair initiation of ACC-treated and control roots, respectively. Five roots were used for both treatment and control. Scale bar = 100 μm.

A change in cell wall composition inside the zone of fast elongation was however found using histochemical methods. Aniline blue is commonly used for callose staining[31] and even callose quantification.[32] Roots were fixed in 1% (v/v) glutaraldehyde for 30 min prior to staining, and the incorporation of aniline blue was recorded with fluorescence microscopy. Only a general background stain was observed in control roots (Figure 9A). Roots in which cell elongation had been blocked by an ACC or DCB treatment were studied using the same settings of the microscope and camera. There was no callose staining in the epidermis, but fluorescence was very prominent in the walls of the underlying cortex and endodermis cells. Figure 9B shows the result after ACC treatment.

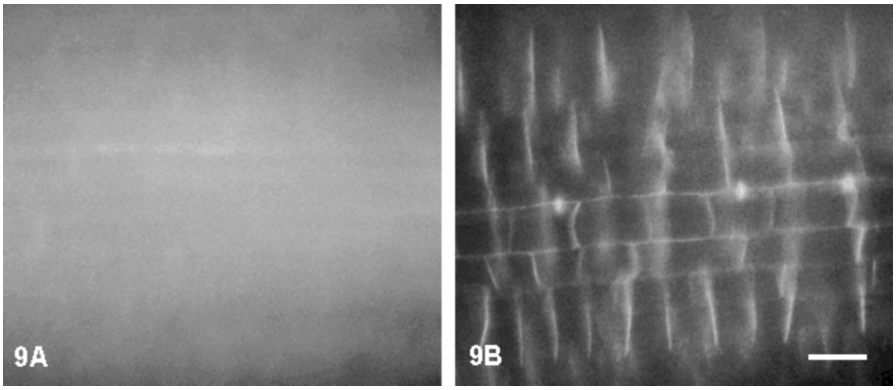

Figure 9. Callose enrichment in cortical and endodermal cell walls in fixed roots. A, Control root. B, Callose fluorescence highlights walls of cortical and endodermal cells on ACC-treated roots. Scale bar = 10 μm.

3. Conclusions

In conclusion cell elongation activity is restricted to a short zone in the Arabidopsis root. It takes each root cell about 2 h to proceed through this activity zone. During elongation the microtubules are all transverse to the cell axis. Factors quickly affecting root elongation act here. A stop in cell elongation is accompanied by a reorganization of the microtubules. They are however not involved in the elongation process itself or in its control, but they play a crucial role in the period preceding elongation, when cells are in transition between the meristem and the elongation zone. The data included in this paper are in agreement with other recent work[13] that points to a role for cell wall components as determinants in the control of elongation.

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REGULATION OF ROOT HAIR TIP GROWTH: CAN MITOGEN-ACTIVATED PROTEIN KINASES BE TAKEN INTO ACCOUNT?

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Abstract. Plant growth and development are complex processes modulated by multi-level regulation mechanisms that depend on both internal and

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external signals. Such signals with the capacity to alter the morphogenesis of plant cells are perceived at the cellular level. Based on mitogen-activated protein kinase (MAPK) cascades, signalling of diverse stress-related factors is responsible for the generation of cell- and tissue-specific responses, and can be expected to participate also in the signal-mediated growth of plant cells, like tip growth of root hairs. Investigating stress-induced MAPKs in growing root hairs revealed a new field of complexity in plant signalling networks, and thus, MAPKs supplemented the collection of tip growth-specific factors with putative regulatory functions. The emerging scenario appears to be more complex; MAPK signalling pathways can support the reorganization of root epidermal cells during formation of tip growing hairs, but can also participate in the harmony of root hair tip growth with the changing environment.

Keywords: Biotic and abiotic stresses, MAPKs, plant development, root hairs, signal transduction, SIMK, tip growth

1. Introduction

Tip growth is a special type of localised cell expansion based on the internal polarity of extending cells. Both internal polarity and tip growth are precisely regulated and balanced processes. Two well-studied examples of plant tip-growing plant cells are root hairs and pollen tubes. Physiological, molecular and genetic studies revealed a number of aspects regulating tip-growth machinery, but an understanding of the complex mechanism is still far from complete. In root hairs, several basic components involved in the successful formation of tubular protrusions from root epidermal cells were identified, particularly via modulation of cell differentiation at certain developmental stages using either specific inhibitors or stimulators of structural or signalling components. These investigations were greatly supplemented and accelerated at the genetic level by the generation of *Arabidopsis* root hair mutants.[1] Data concerning the involvement of particular genes have accumulated rapidly, and a multilevel scheme of sequential gene expression has been constructed.[1,2] Although it represents a direct approach, not all questions have been answered yet. A complete explanation of their role in the root hair growth mechanism is delayed because only sparse information about the function of the various proteins is available through studies of mutated genes. However, single- and double-mutant analyses have revealed a complex morphogenetic network in growing root hairs and thus, greatly reinforced our ideas about regulation levels of root hair developmental stages.[1–3]

The principle of tip growth machinery is based on precise regulation and maintenance of cell polarity. Polar distribution of crucial structural (e.g. actin) and regulatory molecules (e.g. Rop GTPases) is a guiding force for the construction of new, expanding cellular domains. Within the tip, the interaction of these components is restricted in space, but these are able to cooperate to maintain rate, efficiency and structural integrity of cell elongation. This interaction leads to a more or less stable shape for root hairs, where the growing tip is unstably dynamic in order to allow new cell wall and plasma membrane incorporation. Simultaneously stabilisation of the newly formed domains occurs in the flanks of the tip. These tubular extensions of the epidermal cells reach out into a new environment and, as a consequence, growing tips must "sense" their surroundings.

Taking together, the regulation of such a fast and effective growing system must be performed by regulatory mechanisms that integrate totally different growth conditions in the tip with the physiological status of the non-growing part of epidermal cell. In addition, the internal polarity must be correlated with external conditions. Due to these reasons the responses of roots and root hairs to a wide range of environmental stimuli are broadly used model systems for fundamental *in situ* studies of signal transduction and plant development. The search for proteins or protein complexes present in the growing tip with the ability to sense or transduce many different signals is thus a promising approach for the identification of regulatory components and mediators of the tip growth machinery. MAPKs (Mitogen-Activated Protein Kinases), transducers of extracellular signals, are localised to the tip of root hairs.[4] MAPK-based signal transduction cascades have been shown to operate during the proliferation of higher plant cells.[5] Upon signal perception, they mediate the transmission of those signals that control cellular processes including cell division, differentiation and stress responses. MAPKs involved in polarized cell growth of yeast and mammalian cells,[6,7] and in tip growing plant cells open a new area of plant cell and developmental biology. Interestingly, the possible impact of MAPKs and their signalling role in polarly growing plant cells will certainly extend our understanding of developmental processes in eukaryotic cells in general.

Here we address those developmental and cell biological aspects of root hair formation and elongation that could be taken into account within MAPK signalling-based regulations in plant cells.

2. Morphogenesis of plant roots

Developmental aspects of root hair formation are clearly based on the developmental programme of plant roots within the frame of plant

ontogeny. In contrast to the situation in animals, where all organs of a new individual are founded during embryogenesis, the generative cycle of flowering plants is temporarily divided into embryogenic and post-embryogenic phases. During the embryogenic phase, the future body plane of the plant is established, and during post-embryonal plant development tissue and organ differentiation takes place. Organogenesis is thus developmentally separated from the basic embryonal patterning, and cell morphogenesis together with cell differentiation are integral parts of the whole plant life.[8] Protomeristems are already set up in the embryo; they give rise to primary meristems, zones of continuous cell proliferation and differentiation in developing plants. The root meristem is derived from the embryo proper, and the pattern of cellular organization of future root development is already apparent in the stage of the mature embryo.[8–11] Structure and organization of the primary root are generated by the precise regulation of cell morphogenesis, giving rise to the shape of individual cells and whole organs. Cellular shape results from the regulated manner of the frequency and orientation of cell division, followed by the direction and extent of cell expansion.[8,12]

In plant roots, the longitudinal pattern of the developing apex determines the spatial zonation of cell morphogenesis. The meristematic zone is delineated by the population of dividing cells, the transition zone prepares cells for rapid elongation, the elongation zone encompasses expanding cells, and the differentiation zone starting after cessation of cell enlargement produces mature root tissues with specialized cell functions. This complex longitudinal pattern of the root reflects differences of root cells determined by their developmental stage such as tissue-specific and cell cycle-dependent responses.[13–15] The pattern of the root structure was broadly studied from cell biological and cell developmental points of view, and became especially interesting when the basic root structure and development of *Arabidopsis* were described. The *Arabidopsis* root has been set up as a model organ that allows direct implementation of all basic genetic approaches. Simple anatomy and pattern of cells arranged in regular files originating in meristematic initials in the root apical meristem, as well as a little variance in the position and number of cells of the primary root have made it an elegant system for the phenotypic scoring of developmental changes in genetic approaches.[16]

Developmental zone-dependent cell proliferation and subsequent cell differentiation is typical also for the epidermis layer. In *Arabidopsis* roots, the epidermis is differentiated very early into files of root hair cells derived from meristematic trichoblasts and files of non-hair cells derived from atrichoblasts. This developmental diversification is generated by periclinal cell division, giving rise to epidermal precursor cells. Subsequent anticlinal

cell divisions support the integrity of the two cell files with different fates.[17] Interestingly, developmental studies of *Arabidopsis* epidermal differentiation using the expression pattern of the *GLABRA2 (GL2)* gene revealed that the process of epidermal cell fate patterning operates very early. From the radial organization of the *Arabidopsis* root it is apparent that the pattern of epidermal cell differentiation is set up in a position-dependent manner. The *GL2* gene encodes a cell-type specific transcription repressor, and cell-type specific *GL2* expression is observed already at the heart stage of embryogenesis.[18] This is a clear indication that the patterning of the *Arabidopsis* root is a matter of tight genetic regulation well before germination. In addition, however, cell fate can reflect also external cues and position within the root, and with respect to environmental factors, is of particular importance in cell fate modulation.[11,12,19–21] Changes in position within the root can dramatically change the fate of cells, their developmental program and thus the pattern of the epidermis.[22] Therefore, the fate could be modulated relatively late in development utilizing signals from both the environment and neighbouring cells, which is important for root hair development.[21]

3. Root hair formation and tip growth

Root hairs are tip growing tubular extensions of root epidermal cells representing one of the most dramatic forms of terminal cell differentiation in plant roots. They play an integral role in root growth through water and mineral acquisition, and they are frequently used as a model of genetic, molecular and developmental studies. Root hair formation is initiated by the development of a bulge that extends from a well-defined area of the outer tangential cell wall of the trichoblast. Once polarity is established, elongation leads to the formation of a root hair as a long tubular extension of the epidermal cell.

3.1. ROOT HAIR INITIATION

The onset of root hair formation, encompassing a spatially well-defined outgrowth domain at the outer tangential cell wall of the trichoblast, is the morphogenic consequence of a sudden change in the growth axis of the trichoblast. It is supported and driven by intracellular redistributions of elements of the cytoskeleton and endomembranes.[23] In this process trichoblast elongation along the side walls is replaced by highly localised tip growth. These morphogenetic changes lead to alterations of cell size and shape. The development of this growing tip implies a complete reconstruction of the cell wall and the cell wall-plasma membrane interface.

Additionally, it requires rearrangement of the cytoskeleton (peripheral actin microfilaments, AMFs; and cortical microtubules, CMTs) as well as endoplasmic reticulum (ER) and the plasma membrane.[24] Cell expansion in this domain is based on integrated cell wall loosening and controlled deposition of new cell wall material.[25] Modifications of the cell wall at the site of bulge formation are based on the organisation and activity of the cytoskeleton regulated by signalling events. This cooperation is essential for defining of cell wall growing regions.[26]

3.2. CELL WALL

Rearrangement of the cell wall domain at the site of bulge formation requires a highly localized increase in the activity of cell wall-remodelling proteins, like xyloglucan endotransglycosylase (XET) and expansins. Localized high XET action is typically detected at the future site of bulge formation and in the bulge itself. In growing root hairs XET activity is no longer tip-focused, which would fit the restricted function of XET in reversible loosening of the cell wall only at the future bulge site.[27] Cell wall loosening in the initial stages of root hair formation might be supported by expansins that were found to be locally accumulated in the bulge.[28] This notion is strongly supported by a local and temporal decrease of the cell wall pH from 6.0 to 4.5 detected at this domain.[29] The 4.5 pH value is within the range that activates cell wall expansins in their wall-loosening activities.[30]

Hence, bulge formation is mainly a cell wall remodelling process. Distinct cell wall domains of the future bulge get thinner and therefore fail to resist internal turgor pressure. This weakening of the cell periphery complex[24,31] is associated with a local accumulation of expansins[28] as well as other potential cell wall loosening factors like osmiophilic particles[32] and arabinogalactan-proteins.[33] The whole process culminates in bulge formation, which is associated with, and depends on, the recruitment of the exocytotic machinery and endomembranes toward this subcellular domain.[28]

3.3. TRANSITION FROM THE BULGE TO HAIR ELONGATION

Bulge formation is followed by the installation of the tip-growth machinery within the outgrowing protrusion. Also in this case, rearrangements of the cell wall architecture must go hand in hand with the reorganization of the cytoskeleton.

The importance of the cell wall before and during tip growth of root hairs has been shown in a root hair mutant affecting the initial stage of tip

growth. The *kojak* (*kjk*) mutant of *Arabidopsis* is able to initiate root hairs, but they burst soon after bulge formation at the start of elongation. The *KJK* gene encodes a cellulose synthase-like (CSL) protein called (*At*CSLD3). This protein is required for the synthesis of non-cellulosic cell wall polysaccharides, a conclusion supported by the localisation of the GFP-fused protein to the endoplasmic reticulum.[34] Rupture of root hairs at the transition from bulge to cell elongation indicates that the missing component of the forming cell wall makes it unable to resist the pressure at the tip-forming domain, and clearly underscores the molecular diversity and independence of the two different developmental stages (i.e., bulge formation and tip growth).

The mechanism of local accumulation by polarized delivery is expected to act also for spatial expression of other structural cell wall proteins, like the extensin LeEXT1 in tomato from the family of hyroxyproline-rich glycoproteins,[35] or the proline-rich cell wall protein AtPRP3,[36] and the chimeric leucine-rich repeat/extensin LRX1 in *Arabidopsis*.[37] These cell wall proteins support specific properties of the newly formed cell wall in growing root hairs because their expression and activities are typical only for trichoblasts and expanding root hairs.

3.4. CELL ELONGATION BY TIP GROWTH

3.4.1. *Cytoskeleton*

The process of cell expansion has been shown to depend on the dynamic structure and arrangement of the cytoskeleton. In diffusely growing root cells the shape is determined by the position of the mitotic spindle and phragmoplast, both predicted by the preprophase band (PPB) during cell division, and by cortical microtubular arrays during cell elongation.[38–40] In addition, shape and growth of the cells are supported by the stage-specific arrangement of the actin cytoskeleton.[41] Also in growing root hairs, the cytoskeleton is of great importance. The configuration of actin in growing root hairs has been well documented to correlate with tip growth. Apical and sub-apical regions possess a highly dynamic, unstable fine actin meshwork and fine bundles of actin filaments reaching the basal part of the organelle-rich region where the nucleus is localised.[28,42–44] Basal parts of growing root hairs where active cytoplasmic streaming takes place contain thicker actin bundles. This actin configuration is typical for growing root hairs, but actin-rich domains are also present in late stages of bulge formation preceding the bulge-to-tip transition.[45] In non-growing root hairs dynamic F-actin disappears and actin bundles protrude into the tip.[42,44]

Depolymerization of the actin cytoskeleton with cytochalasins or latrunculins inhibits root hair growth.[28,42–44,46,47] Low concentrations destroy specifically the fine and dynamic F-actin in the sub-apical region; tip growth is arrested but cytoplasmic streaming is unaffected.[42] These concentrations also have influence on the position of the nucleus, thus indicating that the maintenance of the nucleus distance from the tip could be based on subapical dynamic actin.[48]

The microtubular cytoskeleton in growing root hairs consists of CMTs arranged in a network beneath the plasma membrane of the root hair tube.[49,50] Recent studies confirmed previous results that showed the vesicle-rich region in the extreme tip of growing root hairs is devoid of CMTs.[4,51] Around the nucleus and in the subapical region of growing root hairs of *Medicago truncatula,* in addition to CMTs an abundant meshwork of endoplasmic microtubules (EMTs) has been observed to correlate with tip growth. While CMTs were observed in all stages of root hair development, EMTs are present in the subapical region only in growing root hairs. EMTs functions were investigated by using the anti-MT drug oryzalin. EMTs were shown to be sensitive to low concentrations of oryzalin that did not inhibit cell viability or bulge formation. In addition, EMTs are highly dynamic. This indicates that EMTs play an important role in the orientation of growing root hairs,[51,52] as was also shown in other experiments where root hairs devoid of microtubules were still able to grow, but the growth pattern was irregular (e.g., wavy).[43,53] MTs therefore seem to have a similar function in the maintenance of the cytoarchitecture, as is generally accepted for the dynamic F-actin meshwork.

The involvement of the cytoskeleton in the regulation of root hair tip growth was recently documented by genetic studies with root hair mutants. The temperature-sensitive mutant *microtubule organization1* (*mor1*) of *Arabidopsis*, with an impaired HEAT repeat sequence of a microtubule-associated protein expressing aberrant cortical microtubules (caused a slightly wavy pattern of root hairs),[54] and the *Arabidopsis* mutant *deformed root hairs1* (*der1*) with an impaired locus encoding the major actin isoform ACTIN2, elucidated the actin function in root hair tip growth. Mutation of this locus prevents correct bulge site selection. In addition, tip growth was strongly affected.[55] A T-DNA insertion mutant in the *MRH2* gene,[56] renamed *ARMADILLO REPEAT KINESIN1* (*ARK1*),[57] caused wavy, and sometimes branched pattern of root hairs. It was shown recently ARK1 plays an important role in root-hair morphogenesis by promoting microtubule depolymerization, which is important to limit the excess accumulation of EMTs.[57] Mutation of the myosin *XIK* gene, a representative of class XI myosins in *A. thaliana*, impaired growth of root hairs by 50% compared to wild-type.[58] Mutation in the locus encoding for

the smallest subunit of the ARP2/3 complex caused considerable changes in the branching capacity of actin filaments, resulting in a "crooked" phenotype with aberrant root hairs.[59] These results prove a critical involvement of different cytoskeletal components in the correct progression of tip growth.

The cytoarchitecture of growing root hairs is designed to support the successful and efficient delivery of secretory vesicles to the growing apex. There membrane flow to and from the growing tip takes place through exocytosis and endocytosis. The secretory vesicles supply the growing tip with cell wall material and membranes, as well as with membrane proteins. In addition, membrane flow removes excess membrane material.[60] Incorporation of secretory vesicles is restricted to the growth area where root hair surface expansion occurs. This is an annulus surrounding the apical pole, as was shown by fluorescent microspheres marking the cell surface,[61] suggesting that the plasma membrane targets for the secretory vesicles are localized here.

3.4.2. *Calcium*

A decisive factor for the spatial regulation of exocytosis is the gradient of cytoplasmic calcium. Calcium influx, through plasma membrane calcium channels at the tip, leads to a localised increase of Ca^{2+} in the tip that is required for normal root-hair growth.[62,63] The role of calcium is closely correlated with the organization and function of the cytoskeleton. Selective depolymerization of dynamic F-actin in the sub-apex of growing root hairs induces broader tip formation as a consequence of a broader space for vesicle fusion.[43] MTs appear to play a similar role. The local application of elevated Ca^{2+} to non-growing parts of the cell leads to the formation of new tips only when MTs are depolymerised, whereas stable intact MTs prevent new growth.[64] Interestingly, *root hair defective2* (*rhd2*) mutants with defects in root hair tip growth have short root hairs and lack the localized elevation in Ca^{2+} at their tip.[65] Calcium thus could contribute to tip growth not only as one of its inducers, but also via stabilisation of the clear zone. The *RHD2* gene product was characterized in more detail. Using sensitive ratio-imaging and electrophysiological methods it was shown, that reactive oxygen species (ROS) like H_2O_2, and superoxide radical are specifically produced in the tips of growing wild-type root hairs, while in *rhd2* mutants the level of ROS in the tip is reduced.[66] The function of local ROS production in the tip of root hairs is correlated with the activation of hyperpolarization-activated Ca^{2+} channels and thus with localised Ca^{2+} influx. A single-channel examination, using excised plasma membrane, revealed that hyperpolarization-activated Ca^{2+} channels in root hairs, as well as in cells of the root elongation zone, which have significantly higher

activity than in mature cells.[67] Several lines of evidence indicate that the *RHD2* gene encodes a NADPH oxidase (RHD2/AtrbohC, Arabidopsis respiratory burst oxidase homologues), an enzyme catalysing ROS production. A defect in ROS production in the *rhd2* mutant is the reason for the lack of a Ca^{2+} gradient in the tip and for the termination of tip growth just after bulge formation. Together with the role of Rops (plant GTPases of the Rho class) in the modulation of actin cytokeleton, it is proposed that Rop proteins activate RHD2/AtrbohC to produce ROS, which in turn activate the hyperpolarization-activated Ca^{2+} channels responsible for generating the tip-focused Ca^{2+} influx.[66]

3.4.3. *Other ions and pH*

In addition to calcium, other ions take part in the electro-chemical gradients during tip growth, such as those of hydrogen, phosphorus and potassium, which are also the subject of nutrient exploration and uptake by the root hairs.[68] Balancing of the pH has been shown to precede root hair initiation. In the cell wall at the site of the future bulge, local acidification is required for root hair initiation in *Arabidopsis*. The local drop in pH at the cell wall is associated with localized alkalization of the adjacent cytoplasm, indicating local activities of a H^+-ATPase.[29] Intracellular redox control in general, as part of environment sensing, plays a positive role in root hair formation. High levels of thiols specifically enhanced *Arabidopsis* root hair elongation but, in addition, enhanced root hair density.[69]

Phosphorus regulates the growth rate and growth duration of root hairs. In the case of phosphorous deficiency one of the responses is an increase in root hair length,[70] but phosphorus as a limiting factor has also a large impact on the development of the root proper by increasing the number of trichoblast cell files.[71] This indicates a mechanism for the adaptation of the plant to the availability of phosphorous in the soil. Thus, nutrient uptake can be enhanced by increasing the absorptive surface area of the root.[72]

The findings that essential ions can be limiting factors and trigger developmental changes and root adaptation evoke questions about the signalling mechanisms of such changes. External changes in the immediate environment of the root can modulate the effectiveness of nutrient uptake by regulation of ion channels in the root hairs. Ion channels are indispensable for nutrient uptake, but in addition their distribution and the regulation of their activity generate a gradient and balance turgor pressure. Sensing mechanism can involve production of “morphogenic” compounds like ROS that have been shown to play a role in root response to some nutrient deprivation, in addition to the expression of several nutrient-responsive genes. Based on these findings it was hypothesised that root hair

cells in *Arabidopsis* contain a sensing system for nitrogen and potassium deprivation.[73]

Short root hairs and frequently more than one per trichoblast, are initiated in the *Arabidopsis* mutant *tiny root hairs* (*trh1*). The *TRH1* gene encodes a member of the AtKT (*A. thaliana* K^+ transport) family of potassium carriers. This potassium transporter protein is root hair-specific, because root hairs are unable to grow even when other functional potassium transport systems operate in root cells. Interestingly, the *trh1* mutant cannot be rescued by high K^+ concentrations in the medium, indicating that K^+ deficiency is not causing root hair growth arrest.[74] More recently, the link between TRH1 and auxin transport has been shown, indicating impaired transport of auxin under AtKT deficiency.[75]

3.4.4. *Plant hormones*

Hormones are important regulators in the root hair developmental program. Auxin is known to play a regulatory role in different root developmental events by its regulated local accumulation. In roots, auxin accumulates at the distal end of the root tip. Cell and organ polarity are controlled by auxin polar movement that is driven by polar localization of auxin carriers.[76] Also in root hairs, auxin plays a pivotal role for root hair initiation as well as for root hair elongation, as was shown in the mutant *auxin resistant2* (*axr2*), with a mutated gene encoding the transcription regulator of auxin-responsive genes. This mutant has only a few root hairs and the outgrowth of the hairs occurs in a more basipetal position (more distant from the root tip pole) on the trichoblasts.[77] The shift of the bulge position within the trichoblast indicates the loss of control over the selection of the initiation site. Thus, all the steps of successful root hair initiation work normally (i.e., accumulation of cell wall loosening factors, focused dynamic cytoskeleton and cell wall remodelling), but the regulatory proteins are probably not correctly distributed. It will be interesting to find the signalling background for the proper "anchoring" of protein complexes required for hair initiation at the right location in the cell periphery.

Ethylene is another plant hormone that affects the process of root hair differentiation, as has been characterized by the generation of ethylene-insensitive mutants and by the manipulation of ethylene action by precursors or inhibitors of its synthesis. The ethylene precursor aminocyclopropanecarboxylic acid (ACC) is able to induce ectopic root hair formation in wild type *Arabidopsis* seedlings while aminovinyl glycine (AVG), an ethylene synthesis inhibitor, prevents root hair formation.[78,79]

In general, the phytohormones auxin and ethylene promote polar hair initiation,[80] and they are also positive regulators of root hair elongation.[81] The study of ethylene mutants, which are blocked in ethylene response or

display a constitutive ethylene response in the absence of ethylene, further elucidated that both hormones support root hair formation by a combinatorial effect. In the absence of the ethylene response, endogenous auxin comes into play in root hair growth. The ethylene-insensitive mutant (*ein2*) and the auxin influx mutant (*aux1*) of *Arabidopsis*, both with reduced root hair length, can be rescued with 1-naphthaleneacetic acid (NAA).[82]

The phenotype of root hair mutants explains the direct impact of mutated genes on cellular shapes, on mechanical properties of the cell wall, and on the focal localisation and restriction of growth. Dramatic changes in the patterning of root hairs and tip growth are induced also by an unbalanced status of hormones, nutrients and some other external inputs. Reaction to these inputs requires an effective signalling, and changes in the activity of regulatory proteins and protein complexes. There is growing evidence that protein phosphorylation is an important event in the signal transduction pathway of external cues. The operation of MAPK signalling pathways in plants is well documented, and MAPK-mediated signal transduction of plant hormones might be directly related to hormonal regulation of root hair development.

4. Signal transduction and MAPKs

Growth and development of plants are affected by different stress factors. Plants must effectively react to, and avoid, negative consequences of the inhibition of basic metabolic and transport processes, destruction of membranes and dysfunction of the cytoskeleton. Such evolutionary relevant selective forces evolved a number of different response mechanisms protecting plants at the molecular, cellular, organ, whole plant and also the plant population levels. Cellular responses induced by environmental stimuli have a complex nature. The plants reaction is represented by a cascade of events from perception and molecular recognition of the external stimulus to the generation and transmission of the signal through signalling pathways, called signal transduction.[83,84] The result of these fast molecular reactions is the generation of a defined response protecting the cell against deleterious external factors by changing the cell physiology according to the external signal.

The information is transmitted by molecules that are described as messengers. One part of them is modulated by protein phosphorylation and dephosphorylation upon perception of an external signal. The status of protein phosphorylation is controlled either by protein kinases or by protein phosphatases.

One of the most conserved and best characterized protein kinase signalling pathways are mitogen-activated protein kinase (MAPK) cascades. Activation

of MAPKs is spatially and temporally performed in modules. MAPK modules are universal transducers of extracellular signals in all eukaryotic cells. The basic module of a MAPK pathway is composed of three functionally interlinked protein kinases (MAPKKK; MAPKK and MAPK). Depending on the amino acids that are phosphorylated during activation, MAPKs have been categorized within a large family of serine/threonine protein kinases. The activation of MAPK requires phosphorylation of both threonine and tyrosine residues in a so called T-X-Y activation loop in subdomain VIII of the catalytic kinase domain. MAPK phosphorylation is catalysed by a dual specificity MAPKK. The MAPKKs are first activated by phosphorylation of their threonine and serine residues by MAPKKKs. The dual mode of activation is unique and phosphorylation of both tyrosine and threonine residues is required for the full activation of MAPKs.[85,86]

The relationship between components within the MAPK module and other regulatory components, such as scaffold proteins, allows for the conversion of the transmitted signal. In this respect, MAPKK – MAPK interaction is characterized by high substrate specificity, allowing minimal variation of the downstream step of signal transduction. MAPKK can provide the specificity as well as an amplification step of the MAPK cascade. MAPKKKs, the most upstream components of the MAPK module, have different regulatory motifs and can be regulated differentially by a variety of convergent upstream activators.[87]

After perception of a signal at the plasma membrane, the cytoplasmic phase of the signalling pathway includes transmittance, intensification and targeting of the signal. MAPKs can activate a number of proteins with regulatory function. The activation of specific target proteins is the last phosphorylation step and it is obviously connected with the compartmentalization of the MAPK module within the cell. The substrate specificity of the activated MAPK pathway, as well as the existence of several synergistic and/or antagonistic MAPK pathways being activated upon different external cues, mediates an effective cell response. Effectory components of the cell are specific to a given signal through the activated MAPK pathway, where activation of transcription factors, cytoplasmic enzymes or cytoskeletal proteins may be involved. The response can be generated by fast and efficient control of gene expression, metabolism, cytoskeletal structure and dynamics, as well as by other cell components with regulatory functions.[87]

In principle, the MAPK pathway is a network of interacting proteins, interconnecting the plasma membrane with the nucleus and/or with other compartments in the cell by phosphorylation steps. Cells are faced with different extracellular cues, and signal transduction pathways must integrate diverse signals leading to co-ordinated cellular functions. Different extracellular cues may produce stimulus- or tissue-specific responses by utilisation

of one particular MAPK module. On the other hand some components of MAPK modules may have the ability to bridge different or related pathways in order to generate the proper and coordinated developmental response(s) of the cell.[84,86] MAPK pathways can act antagonistically to promote different developmental outputs, or MAPK pathways can be just the point of signal integration. The mechanisms of the specificity in different cell types and in response to different stimuli are partially based on proper localisation and accessibility of MAPKs within the cell. A special class of interacting proteins (anchoring and scaffold proteins) bring together specific kinases for selective activation, and they also control the subcellular localisation of signalling complexes within the cell.[87] The scaffolding mechanism is responsible for the specificity of MAPK signalling by creating multienzyme complexes composed of several signal molecules in certain compartment. This physical grouping favours rapid passage of the signal through the cascade and prevents unwanted crosstalk.[88] In addition, the scaffold proteins can promote the pathway specificity by presenting preferred substrates in locally high concentration, and discriminating between single pathways in that case, when pathways share common signalling components.[89]

The termination of the MAPK activated period is an integral part of a signal transduction wave triggered by one stimulus. The inactivation of MAPKs is catalysed by several serine/threonine protein phosphatases. The dephosphorylation of MAPKs is necessary for resetting the pathway to the basal level, when a new input can activate the pathway again. This feedback inhibition of the signalling cascade delineates the MAPK activation, usually, as a transient signal transduction process.[90,91] Protein phosphatases reversibly dephosphorylate MAPKs at their serine and threonine residues. They are categorized in two groups: type 1 (PP-1) and type 2 (PP-2A, PP-2B, PP-2C), according to their substrate specificity, ion requirements and sensitivity to inhibitors.[83,92]

Effective coordination of MAPK signalling cascades in the presence of functional protein phosphatases evokes questions about spatial and temporal independence and selectivity of both signalling components in the cells. In contrast to a relatively high substrate specificity of MAPKs, phosphatases seem to have a broader substrate specificity,[93] which could favor an easier access of phosphatases to other signalling components. This is reminiscent of questions about the specificity of one particular signalling array within the complex cellular signalling network. Like in the case of MAPK cascade coordination and localization by scaffold proteins, MAPK and protein phosphatase activities are regulated and coordinated by effective subcellular compartmentalization.[94]

Several protein kinases have been isolated and characterized so far, and their functions are well established in animal and yeast cells. In animal cells, archetypal MAPKs such as extracellularly-regulated kinases (ERKs) take part generally in signalling of developmental factors, while stress responses are mostly mediated by stress-activated protein kinase (SAPK) pathways. The members of these two protein kinase groups are structurally similar, but functionally distinct.[95,96]

4.1. PLANT MAPKS

Plants MAPK signalling pathways are analogous to those well defined in yeast and mammalian cells,[97] and a large number of serine/threonine protein kinases have been identified in plants.[5] With the completion and analysis of the *Arabidopsis* genome, a more complex view of MAPK functioning, homology and nomenclature in plants is possible.[98–100]

To date, all plant MAPKs are most-similar to the mammalian ERKs.[101] They participate substantially in sensing and adaptation to biotic and abiotic stress, as well as on the control of cell division and developmental processes regulated by hormones.

4.2. MAPKS IN PLANT PATHOGEN, HORMONE AND STRESS RESPONSE

Considerable progress in functional studies of the MAPK involvement in plant pathogen response comes from studies with bacterial or yeast elicitors. An example is the activation of MAPKs in *Arabidopsis* after treatment with bacterial flagellin peptide. Upon treatment, transient activation of AtMPK4, AtMPK6 and AtMPK3 was observed. The transient expression system allowed identification of upstream MAPKKs (AtMKK1, AtMKK4 and AtMKK5) and an upstream MAPKKK (AtMEKK1).[102–104] The activation of this MAPK module is induced by the flagellin receptor FLS2, leucine-rich repeat receptor kinase, and the downstream targets of activated AtMPK6 and AtMPK3 are WRKY transcription factors.[102] Interestingly, in addition to signalling functions based on the phosphorylation kinase activity, a structural role for MEKK1 in pathogenesis-associated signalling was suggested, which would be independent of its protein kinase activity.[105] MEKK1 is involved also in regulation of redox homeostasis in *Arabidopsis*. MEKK1-deficient plants accumulate reactive oxygen species and are altered in hormone signalling.[106] Triggering defence-induced MAPK signalling events has been described also in plant-*Agrobacterium* interactions. *Agrobacterium*-mediated T-DNA transfer induced the activation of MAPKs within the defence signalling pathway. In this study direct target of activated MPK3, transcription factor VIP1 was found.[107] Additionally, the

role of the MKK3 pathway in pathogen defence has been documented using *Pseudomonas syringae*.[108] In roots and nodules of *Lupinus albus*, after inoculation with *Bradyrhizobium* sp., salt stress-inducible and stress-activated MAPKs (SIMK and SAMK, respectively) were activated. Interestingly, maximal activation was found in the infection zone (the region of root hair emergence) where MAPKs could mediate the early events of rhizobial infection in response to a bacterial signal molecule. In this respect they could play a role in the establishment of symbiosis.[109]

Two of the best-characterized MAPKs in tobacco are salicylic-acid-inducible protein kinase (SIPK) and wounding induced protein kinase (WIPK). Their original activation specificity was experimentally tested, and it was found that SIPK and WIPK can be induced by various stresses. In an effort to find upstream activators, yeast two-hybrid screening identified SIPKK, a protein that interacts with SIPK but not with WIPK.[110] NtMEK2, another MAPKK characterized from tobacco, is an activator of both SIPK and WIPK.[111]

For the perception of the plant hormone ethylene, the signalling cascade was studied using a mutational approach. The *ETR* (*ETHYLENE RECEPTOR*) genes encoding putative ethylene receptors, and the *CTR1* (*CONSTITUTIVE ETHYLENE RESPONSE-1*) gene encoding a plant raf-like MAPKKK have been described.[112–114] Accordingly, *ctr1* mutants of *Arabidopsis* with constitutive expression of ethylene-inducible genes have more root hairs in ectopic positions.[114]

Auxin signalling in plants represents a complex process with a number of responses influencing different levels of plant development.[115] Investigations of MAPK signalling in mediation of auxin response, tested at the level of auxin-responsive gene expression, showed an interesting crosstalk between auxin-regulated activations of transcription and other MAPK-mediated signalling cascades. Expression of the constitutively active form of NPK1, the tobacco MAPKKK, specifically activated the MAPK pathway, but led also to suppression of auxin-induced gene expression in transfected mesophyll leaf protoplasts.[116] Similarly, *Arabidopsis* ANP1, an orthologue of tobacco NPK1, mediates H_2O_2 signal transduction under oxidative stress by activation of the two stress-related MAPKs, AtMPK3 and AtMPK6. In parallel, the same MAPKKK inhibited the auxin response.[117]

4.3. MAPKS IN DEVELOPMENTAL PROCESSES

The complex nature of MAPK signalling pathways has been shown by functional analysis of the *Arabidopsis* and tobacco MAPKKKs in the regulation of cell division. NPK1, the tobacco MAPKKK, regulates lateral expansion of the cell plate during cytokinesis,[118] and phragmoplast-associated kinesin-like

proteins NACK1 and NACK2 are regulators of NPK1 activity and proper localisation.[119] Similarly, the *ANP* family of *Arabidopsis* MAPKKKs are positive regulators of cell division[120] and cytokinesis-dependent activation.[121,122] Furthermore, phragmoplast-specific localisation was described for alfalfa MMK3 and tobacco Ntf6 MAPKs.[121,122] However recent developmental studies show much broader involvement of MAPK signalling pathways in plant morphogenesis. Multiple aspects of plant development are under the regulation of interconnected signalling networks where the whole collection of morphogenetic events are outlined by input signals transduced by MAPK modules and by outputs in the form of MAPK-induced gene expression. The impact of this functional segment on plant ontogeny was documented at the levels of MAPK,[123] MAPKK[124] and MAPKKK.[125] An excellent example is the patterning and development of stomatal cell complexes, which is regulated at the genetic level, but also deeply influenced by environmental signals. After identification of the MAPKKK gene *YODA* as the main player in the establishment of guard cell identity and pattern formation,[126] other components of the signalling pathway were identified in *Arabidopsis* (i.e., MPK3 and MPK6, as well as their upstream activators, MKK4 and MKK5). Their regulatory role in the asymmetric cell division during specification of stomatal cell fate was clearly demonstrated.[127] Importantly, the complex view of how cell fate specification during stomatal development is being regulated at the level of intercellular signalling events has been established, because a complete MAPK signalling cascade of these key regulators (YDA-MKK4/MKK5-MPK3/MPK6) has been identified.[127]

The range of external factors attacking plants in their environment and the evolution of effective response mechanisms, with the participation of MAPK signal transduction pathways, indicate that the impact of MAPK-mediated signalling on the generation of physiological reactions of plant cells has a much more complex nature than we can see today. The identification of different MAPKs, and the characterization of their functions in plants, represent a big challenge. Additional comprehensive descriptions of recent developments in this field are summarized in recent reviews.[5,98,128–134]

5. SIMK in *Medicago* root hairs

Plant roots are constantly being exposed to new external signals, which is especially evident in growing root hairs extending into ever-changing microenvironments. Tip growth of root hairs itself is driven and modulated by interaction of different internal and external factors. To understand the mechanisms of signal recognition and transmission to relevant responses

supporting effective tip growth, suitable candidates have to be expressed and activated under certain conditions in plant root cells. *Medicago sativa* is one of several plant species with well-characterized MAPK-mediated signal transduction pathways involved in the signalling biotic and abiotic stress factors. Progress in understanding plant responses to external stress conditions comes from the identification of several *Medicago* MAPKs and their upstream activators, MAPKKs. Activation of SIMK (alfalfa stress-induced MAPK) by moderate osmotic stress was documented,[135] although the downstream target components were not elucidated. However, extensive searching for upstream regulators of SIMK activity led to the identification of regulatory components. Yeast two hybrid screening identified an upstream MAPKK, SIMKK. SIMKK was shown to activate SIMK in response to salt stress.[136] SIMK activation by wounding was shown to be closely correlated with the wound-induced expression of MP2C, the *Medicago* type 2C protein phosphatase. MP2C directly inactivated SIMK after a period of activation, thus resetting the SIMK signalling pathway.[137]

In further experiments SIMK was found to be activated by various abiotic stresses and also by biotic stress factors such as fungal elicitors. The transient protoplast expression system further elucidated that SIMKK, activates SIMK exclusively in salt stress conditions, while both SIMK and MMK3, another *Medicago* MAPK, can be activated by SIMKK in response to pathogen signals.[138,139] This interesting phenomenon was shown to operate also for AtMPK6, an *Arabidopsis* orthologue of SIMK. AtMPK6 can be activated by diverse abiotic stress stimuli including oxidative stress.[117,129,140,141] A new field in the signalling of plant abiotic stresses has been opened by the problem of environmental pollution with heavy metals. Transduction of metal-induced signals by MAPK pathways was clearly established. In *Medicago*, copper or cadmium ions activated four distinct mitogen-activated protein kinases. Among them, SIMK was very rapidly activated by copper. This activation was specifically mediated by upstream kinase SIMKK.[142] Similar responses to copper ions have been demonstrated in rice, where three different MAPKs were activated including the OsMPK6, the orthologue of SIMK.[143]

Recent studies of different signal transduction pathways, with the possible involvement of *Medicago* MAPKs, shed more light on ethylene signalling in plants. It has been shown that the ethylene precursor, aminocyclopropane-1-carboxylic acid (ACC), activates distinct MAPKs. In *Medicago*, the ACC-activated MAPKs were SIMK and MMK3, and interestingly, as in the case of elicitor signalling, ACC-induced activation of SIMK and MMK3 was specifically mediated by SIMKK. As *Arabidopsis* AtMPK6 was also activated by ACC, further experiments were performed with transgenic *Arabidopsis* plants over-expressing SIMKK. These

transgenic lines have constitutive AtMPK6 activation and ethylene-induced target gene expression.[144]

SIMK is expressed in the root meristem of *Medicago*. The majority of SIMK is localised in the nucleus of root interphase cells, and cells in G2 especially have higher amounts of the SIMK protein.[145] Accumulation of SIMK in G2 may specifically correlate with the stage of the cell cycle, as was shown in alfalfa suspension cells, where transient accumulation of both mRNA and protein of SIMK in G_2 was observed.[146]

In root meristematic cells, SIMK localization changes during the cell cycle. With the onset of mitosis SIMK escapes the nucleus and associates with the spindle poles in early prometaphase. SIMK is excluded from the chromosome area until completion of nuclear division and only then enters the newly reconstructed nuclei. Thus the distribution of SIMK in the alfalfa root meristem can be considered cell cycle-dependent. Interestingly, salt stress, known to trigger specifically the activity of the SIMK, induces the re-location to other compartments of dividing cells [i.e., to microtubule structures like the preprophase band (PPB) and the phragmoplast].[145] Spatial and temporal pattern of SIMK localization, influenced by cell division stages, was further pronounced by tissue specific differences. SIMK protein was present in stele and cortex tissues, but more abundant in epidermis. Higher amounts of SIMK in the epidermis were detected in the root elongation zone.[145] It is believed that tissue-specific and cell cycle-dependent intracellular distribution of SIMK in root meristematic cells of *Medicago sativa* can be directly relevant to biological functions of SIMK in developmental phases of the root. In addition, the biological functions of SIMK in developing root tissues are under the regulation of environmental factors.

SIMK is strongly expressed in root cells and the SIMK protein accumulates within nuclei of both meristematic and postmitotic root cells of the transition zone, and in the nuclei of the elongation zone. This situation changes dramatically during root hair formation. SIMK redistributes from nuclei into the cytoplasm, and accumulates within outgrowing bulges and at tips of emerging root hairs. In growing root hairs the high concentration of SIMK protein in the tip is still sustained.[147] SIMK obviously takes part during the bulge outgrowth phase as evidenced by its distinct accumulation at the cell periphery of bulging domains. The amount of SIMK in the growing tip increases gradually with the decreasing signal intensity in the nucleus, indicating redistribution from the nucleus into the growing tips of the root hairs. During this redistribution, SIMK is activated.[4]

The polar localisation of SIMK in root hairs raises the question about its possible function as a molecular transducer in tip growth. In an effort to find relevant answers, root hair-specific accumulation and activation of

proteins should be addressed first. Site selection on the tangential cell wall, subsequent bulge formation and establishment of the tip growth are associated with and depend on polarized localisation of proteins with regulatory or effector functions.

Actin-binding proteins like actin-depolymerization factor (ADF) and profilin are polarly focused in the bulges and growing tips of root hairs. They are responsible for driving the assembly/disassembly of F-actin meshworks.[148] They act in a synergistic fashion[149]; while profilin enhances actin polymerization at the barbed ends of AFs,[150] ADF activates the pointed ends.[151] Effective enhancement of actin dynamics within bulges selectively recruits endomembranes and other components of the secretory apparatus that re-direct exocytosis preferentially towards these subcellular domains. In addition to G-actin itself,[152] profilin and ADF/cofilin also are linked closely to diverse signalling pathways.[153] Actin, ADF/cofilin, and profilins are often phosphorylated.[154–156] In that respect it is interesting the finding, that proper SIMK localisation in the tip of growing root hairs is closely connected to and depends upon the sustained dynamic properties of the actin cytoskeleton. Unbalanced changes in the structural or functional status of F-actin, caused by depolymerization or stabilization, induce tip growth arrest and SIMK misplacing.[4] Tip growth attracts many other components into apical and subapical domains of root hairs. RabA4b was detected at the tip of root hairs, where it can be involved in regulation of vesicular trafficking within the clear zone.[157,158] ARF1 is expressed in root hairs of *Arabidopsis* where it is essential for polar localisation and activity of ROP2.[159] GTPase-activating protein (ARFGAP) RPA has been characterized as a potential regulator of the *Arabidopsis* ARF1 in root hairs and pollen tubes.[160]

Other potential components of signalling pathways involved in the polarization of plant cell growth are RopGTPases, a plant–specific subgroup of the Rho GTPases. Plant Rops were shown to be localised specifically in the tip of growing root hairs and of pollen tubes.[161,162] Their function in cell polarity and tip growth is suggested to be linked to the actin cytoskeleton and tip-focused calcium gradients.[163,164] Manipulation of RopGTPase expression using specific *AtRop4* and *AtRop6* mutants compromise polarity and growth of *Arabidopsis* root hairs. Constitutive Rop activation prevents the formation of tip-focused calcium gradients and abolishes polarized tip growth. Loss of AtRop4 activity also caused a delocalisation of the normally tip-localised $Ca2^{+}$ gradient.[165] The most fitting scheme of RopGTPase function in the maintenance of polarity in tip-growing cells is the effective regulation of the actin cytoskeleton in a calcium-dependent manner. As localisation of RopGTPase to the tip was not sensitive to actin or microtubule destabilisation,[165] and a function for

RopGTPase in vesicle trafficking have been proposed,[164] the leading function of Rops in actin-and calcium-based membrane motility comes to the forefront. A proposed integrating function of Rop proteins in root hairs is the activation of RHD2/AtrbohC for ROS production. ROS stimulates the hyperpolarization-activated Ca^{2+} channels and thus facilitate calcium influx that is involved in the modulation of actin dynamics. All components cooperate together for execution of tip growth.[66] The main question is what is the key point for precise spatial localization of this complex. At the stage of hair outgrowth it seems to be the role of the polarized production of ROS.

Main aspects of spatial control of polar growth in root hairs were characterized in detail using *scn1* mutant of *Arabidopsis*. *SUPERCENTIPEDE1* encodes AtRhoGDI1 (Rho-GDP dissociation inhibitor), which negatively controls the activities of Rho-GTPases. Loss of function of AtRhoGDI1 affected both spatially regulated ROS accumulation and hair outgrowth. Instead of one root hair large outgrowths with multiple irregular bulges are produced in the mutant root.[166] Aberrant root hairs in *scn1* do not focus ROS to a single point at the tip, but ROS are spread diffusely into multiple bulges and hairs. Similarly, hair cells are unable to keep Rop activity focused as in the wild-type root hair tips, while expression of YFP-AtROP2 is delocalized to multiple sites of polarized growth.[166] Therefore, SCN1 controls both the position and the shape of hair outgrowths.

ROS are produced and concentrated at the tip from the early stages of tip growth and this polarized production and accumulation remain until the hair tip growth terminates.[166] ROS thus represent unique components of the tip growth machinery not recognized before. MAPKs may transduce ROS-generated signals through phosphorylation of target proteins.[167] *Arabidopsis* Ser/Thr protein kinase OXI1 (OXIDATIVE STRESS INDUCIBLE1), belonging to the AGC family of protein kinases, is activated upon ROS treatment. In addition, OXI1 is required for activation of the downstream MAPKs, MPK3 and MPK6, in response to active oxygene species.[168] Interestingly, OXI1 activity is required for root hair elongation because root hairs are shorter in *oxi1* mutant.[168] These findings suggest that root hair development mediated by ROS signalling is most probably based on the activation of specific MAPK pathways, where the interconnecting component could be OXI1.[167,169] Interestingly, OXI1 is activated also by phosphatidic acid.[170] One of the enzymes producing phosphatidic acid is phospholipase Dζ1 (PLD1). PLD1 is over-expressed in the *glabra2* mutant with ectopic and branched short root hairs.[171] Because phenotype of the *gl2* mutant with ectopic and branched root hairs was induced by over-expression of PLD1, it was suggested that GL2 acts as a transcriptional repressor of *PLD 1*.[171] Another Ser/Thr protein kinase, PINOID (PID) with

possible relations to intracellular signalling networks, has been implicated in root hair development. PINOID is a positive regulator of auxin transport. Because PID positively regulates cellular auxin efflux, and thus maintains optimal auxin levels for root hair formation, over-expression of PID suppressed root hair growth due to the decrease of auxin in the hair cells. This effect and normal root hair tip growth are restored by antagonists of PID kinase activity or by inhibitors of auxin efflux.[172]

Placing of the SIMK into that scheme is suggested by its activation and closely correlated with its tip-focused localisation. If SIMK activity is reduced by treatment with a MAPKK inhibitor, loss of tip-focused SIMK was induced. Interestingly, polar actin organization, targeted vesicle trafficking and polarized growth of root hairs was also inhibited. This is a clear indication that polarized tip-growth is dependent on MAPK activity as further documented by a SIMK gain-of-function mutation ectopically expressed in tobacco, where root hairs are longer and more resistant to MAPK deactivation by the inhibitor UO126.[4]

Data supporting the suggestion that protein kinases can play a role in the process of root hair formation are still accumulating. The mutation in the *IRE* (*INCOMPLETE ROOT HAIR ELONGATION*) locus of *Arabidopsis* considerably reduces root hair length. In contrast, mutation of another locus (*HY5*) induces a long root hair phenotype with root hairs about twice as long as the wild type. Protein products of these two loci are important in the modification of root hair growth duration. Interestingly, the *IRE* locus encodes a protein that includes a serine/threonine protein kinase domain.[173] Another interesting finding concerns the *SOS4* (*SALT OVERLY SENSITIVE 4*) gene of *Arabidopsis*. The *sos4* mutant, deficient in the synthesis of a pyridoxal (PL) kinase, is hypersensitive to the salt stress. The phenotype of mutant roots indicates defects in the root hair initiation, particularly the blocking of tip growth.[174] An important consequence of the expression of both *HY5* and *SOS4* in root hairs is the indication that their effects can be mediated by auxin or ethylene response pathways.

Root hair formation can be regulated not only through the positive actions of protein kinases, but also through negative regulators of their activity. A possible involvement of protein phosphatases, inactivating MAPKs through dephosphorylation, in root development, has been addressed by experiments with protein phosphatase inhibitors. Treatment of *Arabidopsis* roots with inhibitors of type-1 or type-2A phosphatases decreased root growth rate, arrested root hair growth and induced cell shape deformation in the elongation zone.[175] Although the decreased function of protein phosphatases induced changes not specific for root hairs only, the results indicate that both protein phosphorylation and dephosphorylation can have significant impacts on root hair tip growth.

Associations of SIMK with the actin cytoskeleton and vesicular trafficking seem to be a key aspect of root hair tip growth. Although the exact molecular mechanism of actin regulation by SIMK has still to be defined, one can expect such participation in tip growth because data from animal and yeast cells document distinct roles in the dynamic organisation of the actin cytoskeleton and in polarisation of cell growth in an actin-dependent fashion.

The most prominent examples of a MAPK controlling the actin cytoskeleton are p38 of animal cells and MPK1 of budding yeast. Both proteins are critical for polarization of the actin cytoskeleton. The p38 kinase also is responsible for migration of animal cells,[7,176] and MPK1 is involved in yeast polarized cell growth.[6,177] Localisation of MPK1 together with scaffold proteins (Sph1 and Spa2p) to sites of polarized growth is determined by binding to several components of this MAPK cascade.[178] Similarly, MAPK (called Fus3) together with scaffold protein Ste5 accumulate at pheromone-stimulated sites of polarized growth during the mating response of budding yeast. Accumulation of active Ste5 at the plasma membrane polarizes the actin cytoskeleton.[179] Recently, Afr1p, a protein regulating polarized localization of the Mpk1p in shmooing yeast cells, has been identified. Deletion of the *AFR1* locus caused mislocalization of Mpk1p at shmoo tips although over-expression of the scaffold protein Spa2p, which is localized normally in the tip, cannot rescue this defect.[180] These data clearly show the importance of recruiting, and scaffolding components placing correctly, MAPKs at the place of their expected action.

Establishment of polarized growth in yeast is mediated by a series of steps involving similar checkpoints like in tip-growing plant cell, i.e. weakening of the cell wall by digestive enzymes, polarization of the actin cytoskeleton, incorporation of new plasma membrane at the cell surface and synthesis of new cell wall.[181,182] Polarisome proteins play indispensable functions by scaffolding Cdc42p-containing complexes and MAPK cascade proteins. The main polarizing step is the recruitment of Cdc42p to growth sites. Here the GTPase activates effectors (most probably Ste20p) that signal to the actin cytoskeleton.[182,183]

The establishment of polarity and polar growth in yeast is orchestrated by a complex network of protein-protein interactions,[184] but Cdc42p, a member of the RhoGTPase protein family can be identified as an essential component in guiding polarized growth in yeast. The functions of Cdc42p in yeast polarity represents a highly promising basis for a comparative search for Rop GTPase functions in plant tip-growing systems. A polarized actin cytoskeleton plays an essential role in the localisation of regulatory molecules in mammalian cells by scaffolding them, as it has been shown in nerve termini.[185,186] In addition, the cytoskeleton itself could act as a track

for the delivery of the signalling protein complexes, located on endosomes in neurons.[187] Recently in plants, endosomal compartments also were established as signalling platforms in receptor-mediated signalling cascades.[188]

All these data from eukaryotic cells indicate that MAPKs are involved in the dynamic organization of the actin cytoskeleton. In addition to direct binding, which could be relevant for the regulation of actin cytoskeleton components by means of phosphorylation, MAPK activation can be positioned upstream of actin cytoskeleton rearrangement. For instance, a perception of mechanical signals can be generated and propagated by stretching or compressing pressure cues. In animal cells, imposing stretch-stress on the plasma membrane rapidly activates MAPKs.[189] Similarly in yeast, MPK1 is activated via local weakness of the cell wall.[190] In plant cells, weak walls are immediately challenged by high internal turgor pressure, implying that local stretch-stress may be imposed on the plasma membrane.[191,192]

In order to understand tip growth mechanism an important issue, in addition to the effort to characterize root hair-specific factors, is to identify common aspects within other tip-growing systems. Identification of all similarities and differences allows for a systemic approach to anticipate possible functions of orthologous and paralogous counterparts in different systems. Similarly, like the analogy of yeast and animal tip-growing objects leads to important progression in ideas, methodology and approaches, it is worth co-evaluating the two typical plant tip-growing systems, root hairs and pollen tubes. Although different circumstances are associated with their ontogenetic development, more and more data support the conclusion of a high degree of parallelism in their modes of growth.[193–195] An example of a convergent scenario unifying proposed theories in both systems is the finding that tip-localized ROS produced by a NADPH oxidases (NOX) are needed to sustain the normal rate of both pollen tube[196] and root hair tip growth.[66,166]

Importantly, there is recent evidence for a MAPK signalling pathway operating in growing pollen tubes. *In planta*, growing pollen tubes face unique conditions with respect to signalling that can modulate their growth. Particularly pollen-pistil interactions encompass the activities of many proteins with a variety of functions, regulating polar tube growth in a precisely temporal and spatial manner. Such interactions clearly involve signal reception and transduction.[197] This is particularly evident in the event of self-incompatibility (SI), a situation the root hairs never encounter. SI represents a recognition and rejection system for "self," or genetically identical pollen, preventing the production of "self" seeds.[197] This system triggers on a signalling network, resulting in the inhibition of tube growth of incompatible pollen. In *Papaver rhoeas*, the SI reaction rapidly reduced

viability of incompatible pollen and induced activation of a 56 kDa putative MAPK. Specific involvement of p56-MAPK in this reaction was proved by inhibition of MAPK activity using a MAPK cascade inhibitor that was able to prevent SI-induced arrest of incompatible pollen. Importantly, evidence that SI-activated MAPK signals can induce a caspase-3-like (DEVDase) activity was provided.[198] Based on the pattern of p56-MAPK activation, it is most likely that this rather late reaction in the whole process triggers a Ca^{2+}-dependent signalling network targeting the actin cytoskeleton or other cellular components leading to overall pollen tube inhibition via programmed cell death.[198] Possible interactions of MAPK signalling cascades with the actin cytoskeleton were tested in *Picea meyeri* pollen tubes after treatment with F-actin depolymerising agents. Among the proteins differentially expressed, MAPK-like protein was revealed by proteomic analysis. Interestingly, this protein was downregulated after actin disruption, indicating a possible interesting link between functional F-actin and MAPK signalling events in pollen tubes.[199]

It has been difficult to correctly characterize expression of genes with possible roles in root hair development in a complex way. Recently however, profiles of pollen and root-hair transcriptomes for complex comparison of the basal sets of genes specifically up- or down-regulated in tip-growing cells are available. Abundance of the signalling components in pollen tubes has been proven on the transcriptomic level. Within the range of genes encoding signal transducers and being upregulated in pollen, MAPK genes with increased expression levels were identified.[200] Broad transcriptomic analysis of the root-hair differentiation zone of wild-type *Arabidospsis* was performed in comparison to the *rhd2-1* mutant. The 'root-hair morphogenesis transcriptome' revealed a set of 606 genes with significantly greater expression in wild-type plants. Importantly, decreased activity of several MAPK genes was detected in the root hairs zone of the *rhd2-1* mutant in comparison to wild-type.[56]

Taking together all aspects of root hair formation, the pattern of spatio-temporal expression of SIMK in alfalfa trichoblasts during tip growth can be drawn. In the light of described MAPK functions in polarly growing mammalian and yeast cells, tip focused and activated MAPKs seem to be essential for sustained root hair growth. SIMK can be involved in root hair initiation, as well as in maintenance of subsequent tip growth, via putative cytoskeleton- and membrane-associated functions performed at sites of active exocytosis and endocytosis. Possible functions of SIMK include:

- Recruitment and polarization of dynamic actin and initiation of tip growth. Dense F-actin meshworks accumulate in early outgrowing bulges and remain associated with tips of emerging and growing root hairs.[28,45] Our analysis of the effects of actin inhibitors on SIMK

localization and activity suggests that SIMK is not only associated with the actin cytoskeleton but is also directly affected by the altered dynamics of F-actin.[4,131]

- Participation in putative membrane-associated functions via interactions with secretory endosomes, or lipid signalling components like PIP_2, localised in the tip of growing root hairs.[45,47] In this scenario SIMK might be involved in polarizing the exocytotic apparatus and in supporting abundant endocytosis, which is inherently coupled to exocytosis in apices of root hairs.[60,193]
- Sensing mechanically- and osmotically-unstable domains within the root hair tips. Remodelling of these domains is mediated by local accumulation and activity of expansins in the bulge,[28] tip-accumulated AGPs,[33] and cell wall degradation process responsible for cell wall thinning in the bulge.[31] Thus, subcellular compartments in the bulge and growing tip are faced with local osmotic and mechanical imbalances. The signal-induced nature of the sudden changes in the cell's growth mode suggest that SIMK is a good candidate for such putative molecules, which link polarity cues with protection against stress conditions.[201]

6. Conclusions

Root hair development is an important subject for plant biologist. A plethora of new results continuously supplies new molecular players for the developing scheme of root hair formation. Our knowledge is very incomplete about their regulations and interactions, as well as on their delivery, positioning and maintenance in selected cellular subdomains. Thus, we still have insufficient understanding of the basic principles of the tip growth. Emerging cell biological and molecular results support the notion that root hair formation and tip growth involve MAPK signalling pathways. Participation of SIMK and other MAPKs in the regulation of root hair tip growth in general is assumed to be an integral part of complex cell signal transduction pathways. MAPKs can phosphorylate and thereby regulate diverse targets including nuclear transcription factors, cytoskeletal components and other protein kinases. In this way MAPKs can participate in the transcriptional regulation of genes and in the post-translational modification of gene products including those that are directly involved in root hair development. The current state of knowledge validates involvement of MAPKs in the development of plant tip-growing cells. It remains to characterize in which extent and under which circumstances.

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CYTOSKELETAL CHANGES DURING SPERMATOGENESIS IN *CHARA* ANTHERIDIA

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Abstract. Cytoskeletal changes during spermatogenesis in *Chara* proceed from regular mitotic divisions to specific events associated with the formation of sperm cells. During mitosis the antheridial filaments show three microtubule systems: cortical, transverse microtubule arrays during interphase develop into mitotic spindle in prophase, metaphase and anaphase, and eventually form a phragmoplast during telophase. We document a quadripolar mitotic spindle during metaphase. The actin organization during spermatogenesis differ from that of the microtubules: During interphase, F-actin forms a network covering the nucleus and an actin plate develops adjacent to the cell walls between dividing cells and some actin filaments extend from the plate toward chromosomes during prophase, metaphase and anaphase; at telophase, F-actins is part of the phragmoplast. Cell differentiation begins with lateral movement of nucleus, development of manchette MTs along the nucleus and eventually flagella form at one end of the manchette MTs. The manchette MTs elongate and surround the nucleus, which then elongates to a crest-like structure before forming a helical shape. The last stages of maturation are characterized by the disappearance of manchette MTs but flagella remain. The susceptibility of MTs to oryzalin during spermatogenesis depends on the developmental stage. MTs during mitosis depolymerized by 5 μM oryzalin but the same treatment was ineffective in differentiating cells. During cell differentiation cortical MTs were deploymerized by 50 μM oryzalin, but manchette MTs and flagella were not affected. In mature sperm, oryzalin treatment stabilized manchette MTs and flagella.

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1. Introduction

Spermatogenesis describes a complex and carefully controlled process in all sexual biological systems. Spermatogenesis in the plants lineage has evolved from motile sperm to passive pollen dispersal. Thus, spermatogenesis is important for the assessment of cellular differentiation and illustrates phylogenic events (for review, see[1]). Examining spermatogenesis in the green alga *Chara*, which is thought to be most closely related to land plants,[2] is likely to reveal important events and cellular changes associated with the transition from aquatic to land environments.

The male sexual organ of *Chara*, the antheridium, is formed from divisions of a nodal cell. First, precisely oriented mitotic events produce antheridial octants; then, two periclinal divisions in each octant divide the antheridium into three different layers. The outer layer cells develop into shield cells, the middle layer forms the manubrial cells and the inner layer produces capitula cells. The antheridial filaments are derived from mitotic divisions of the capitula cells[3]. Thus spermatogenesis in *Chara* includes two processes: a large number of mitotic divisions produce antheridial filaments. Secondly, intracellular differentiation forms the characteristically shaped, flagellated and motile sperm. During intracellular differentiation, the nucleus of the cell condenses and elongates into a helix of about two and half turns.[4] The mature spermatozoids of *Chara* are biflagellated, contain little cytoplasm, and coil up within the cell wall.[5] Despite the long-known anatomical changes during the development of antheridia, spermatogenesis in *Chara* is an interesting topic for many additional aspects, for example:

1. How does a plant cell develop motility, which is characteristic of animals?
2. How does the nucleus attain the spiral form and what if any function does the cytoskeleton play during this process?
3. Is the organization and function of the cytoskeleton during mitotic divisions in *Chara* similar to that of higher plants?

The cytoskeleton is likely to control the complex morphogenetic processes during spermatogenesis. For example, depolymerization of microtubules by colchicines[6,7] or oryzalin[8] prevents nuclear and cellular shaping in early spermatids. In the green alga *Nitella*, disruption of microtubules by colchicine results in malformed nuclei.[9] Another cytoskeletal element, F-actin, plays important roles during spermatogenesis of the liverwort *Conocephalum* and the ferns *Ceratopteris*, *Equisetum,* and *Pteridium*.[10] F-actin affects cytokinesis, maintenance of cellular connections between

developing spermatids, shaping of nuclei and cells, organelle distribution and size.

The cytoskeletal organization during spermatogenesis in *Chara* was first studied by transmission electron microscopy, and microtubule patterns during cell differentiation were reconstructed from thin-sections.[4] Despite the groundbreaking significance of these studies, Pickett-Heaps' work was limited by the techniques available at that time. As a result no information on actin microfilaments during spermatogenesis is available as of now.

In this paper, we describe the organization of MTs during mitosis of the antheridial filaments and changes in the organization of F-actin during the spermatogenesis in *Chara* and report the presence of a transient quadrupolar spindle. The discussion addresses putative functions of MTs and F-actin during nucleation.

2. Materials and methods

2.1. CULTURE CONDITIONS

Chara contraria was grown in 20-L glass tanks containing soil from a local pond under a 3-cm layer of sand. The tanks contained tap water and were kept at room temperature (22–25°C) under natural window illumination. Thalli with 2–3 internodal cells with antheridia were dissected for immuno-cytolocalization studies.

2.2. CHEMICAL TREATMENTS

Antheridia were harvested and either fixed immediately (controls) or treated with oryzalin (Chem Service, West Chester, PA) at concentrations of 5 or 50 μM in artificial pond water,[11] for up to 5 h and then fixed.

2.3. IMMUNO-CYTOLOCALIZATION

2.3.1. *MT staining*

Whole tips with antheridia were fixed in 1.5% formaldehyde in PHEMD buffer (60 mM Pipes; 25 mM Hepes; 10 mM EGTA; 2mM $MgCl_2$; 5% v/v DMSO pH 7.0) overnight at 4°C.[12] After digestion of the cell wall with 1% cellulase Y-O and 0.1% pectolyase Y-23 (both Koamicho Corporation, Japan) containing 1% BSA, MT were stained with monoclonal antibody YOL 1/34 (Accurate Chemical & Scientific Corp.) followed by Alexa 488-conjugated secondary anti-rat antibody (Molecular Probes, Eugene, OR).

2.3.2. *F-actin staining*

The specimens were fixed in 1.5% formaldehyde in PHEMD buffer for 1.5–2 h at room temperature. Cell walls were removed by incubation for 5 min in 1% cellulase YC, 0.5% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) and 0.1% BSA (Sigma Chemical Co., St. Louis, MO), then washed with 5% glycerol in PHEMD buffer for 10 min and incubated in 0.1% Tritron X-100 in PHEMD buffer for 30 min. F-actin was stained with Alexa-phalloidin 488 for 5 h. The specimens were then washed with phosphate buffered saline and mounted in VectaShield (Vector Laboratories, Burlingame, CA).

2.3.3. *Nuclear staining and confocal microscopy*

Staining of nuclei was performed with 0.005% propidium iodide in phosphate buffered saline for 2 min. Images were taken with a confocal laser scanning microscope (BioRad MRC-1024) with Lasersharp 2000 software (v. 5.1). Individual experiments were repeated at least three times.

3. Results

3.1. MITOSIS OF THE ANTHERIDIAL FILAMENTS

3.1.1. *Microtubules*

The antheridial filaments of *Chara* are derived by mitosis of the secondary capitula. A large number of cell divisions cause extension of the antheridial filaments[4] (Pickett-Heaps, 1967). Mitoses are often synchronous or progressive. In some occasions, all mitotic stages could be seen in the same filament (data not shown) or in adjacent filaments (Figure 1a). The microtubules during mitosis of the antheridial filaments are organized into three distinct systems: hooplike cortical microtubule arrays during interphase, mitotic spindles and phragmoplasts. At interphase, the MTs are organized perpendicular to the longitudinal axis of the antheridial filament, and parallel to each other (Figure 1a). As chromatin condenses into chromosomes, cortical MT arrays gradually reorganized into mitotic spindles with two or more focal points at the periphery of the nucleus (Figure 1a) during early prophase. At this stage, the nuclei swell and become multi-angular in shape and are surrounded by mitotic spindles. During prometaphase, two independent MT spindles form. A large and a small, about half the size of the large spindle, may result from the division of centrioles. These spindles are organized perpendicular to each other (Figure 1b). As mitosis progresses, the spindles form a quadripolar mitotic scaffolding (Figure 1c). At this time

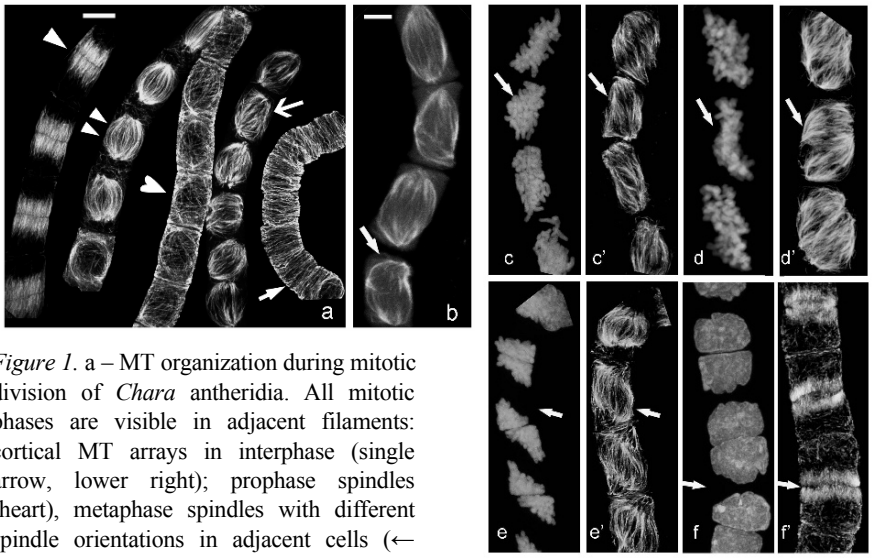

Figure 1. a – MT organization during mitotic division of *Chara* antheridia. All mitotic phases are visible in adjacent filaments: cortical MT arrays in interphase (single arrow, lower right); prophase spindles (heart), metaphase spindles with different spindle orientations in adjacent cells (← single arrow, upper right); telophase (double arrow heads); and phragmoplast (single arrow head); b – prometaphase, shows two independent MT spindles perpendicular to each other in the same cell (arrow); plain letters (c–f) show location and arrangement of nuclei; primed letters (c'–f') denote microtubule organization of the same cells. c – metaphase MTs show quadripolar spindles (arrow) but chromosomes align in a single plane; d – metaphase MTs are characterized by two laterally oriented spindles (arrow); e – anaphase MTs interlace chromosomes (arrow); f – during telophase the phragmoplast is characterized by the typical dense MT plate (arrow). Bar for a = 10 μm; b is the same magnification as c, d, e, f, bar = 5 μm.

chromosomes align at the equatorial cell plate. After the formation of this apparatus, one spindle reorients itself such that the two spindles are aligned side by side (Figure 1d) before merging into one bipolar, fusiform mitotic structure. The MTs in the spindle appeared organized into different sub-groups, and each with its own focus point at the spindle pole (Figure 1c). The polarity of the metaphase spindle is determined by the polarity of the prophase spindle, spindles with different polarity were found in the same filament (Figure 1a). During anaphase, the MTs shrink and cause the sister chromosomes to separate. As the separated chromosomes move toward the cell poles, the MTs nucleate at the interzonal spaces between the future nuclei and form an anaphase spindle. The interzonal MTs are attached to the two groups of chromosomes, and the MTs that originated from the cell poles shrink and finally penetrate the chromosomes (Figure 1e). As the cell progresses through telophase, the anaphase spindle gradually forms a phragmoplast (Figure 1f), which is composed by two groups of opposing MTs with a MT-devoid zone in between. As the phragmoplast expands centripetally, the cell plate forms between the two sister nuclei, and

randomly organized MTs develop at the surfaces of the two sister nuclei (Figure 1f). Divisions of the filaments are successive and the division plane is always perpendicular to the longitudinal axis of the filaments, despite the existence of diagonal and anticlinal spindles.

3.1.2. *Actin*

The actin organization during mitosis is different from that of the MTs: During interphase, F-actin forms a reticular mesh along the cell walls and extends around and eventually encloses the nucleus (Figure 2a). In some cases, the nucleoli are also surrounded by actin (Figure 2a). As the nucleoli disappear in early prophase, F-actin becomes more concentrated at the two intercellular poles and forms actin rings or plates and the nucleus is

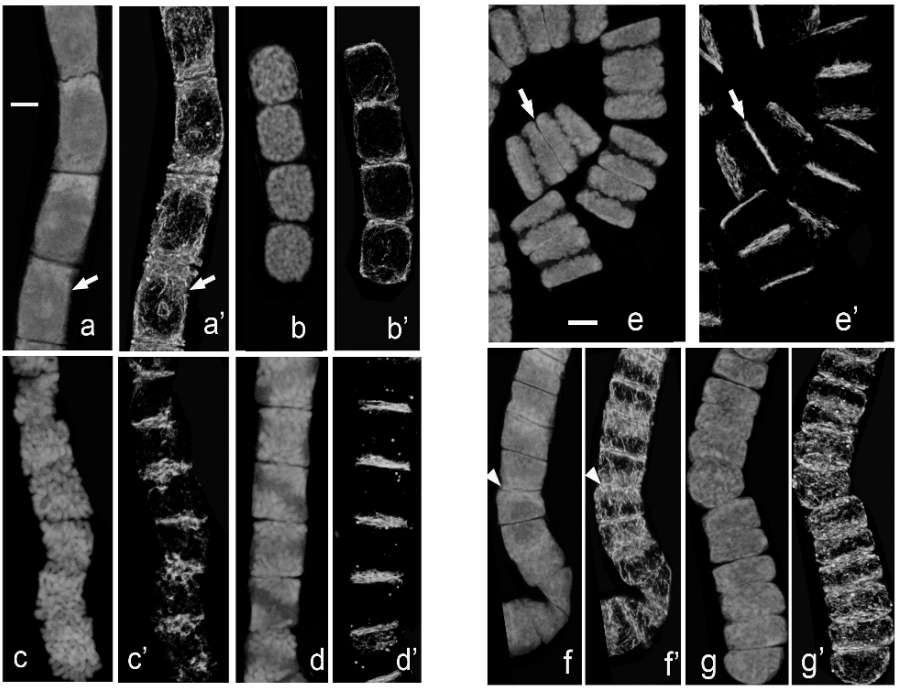

Figure 2. F-Actin organization during mitotic division of antheridia. Plain letters (a–g) show location and arrangement of nuclei; primed letters (a'–g') denote microtubule organization of the same cells. a – During interphase, F-actin forms mesh that encloses the nucleus (arrow). b – During prophase chromatin condenses into chromosomes and F-actin is more concentrated at the apical and basal cell wall. c – metaphase is characterized by more prominent F-actin concentrated at the cell plate. d – separation of sister chromosomes during anaphase typically move diagonally or anticlinal. The interzonal region is devoid of F-actin. e – telophase exhibits F-actin aligned at the cell pate (arrow). f – during late telophase F-actin locates to the phragmoplast (arrowhead). g – at the end of the mitotic cycle F-actin reorganizes into a reticular network that surrounds the nucleus (progresses to [a]). Bar = 5 μm. same magnification for all images.

embedded in an actin network (Figure 2b). During metaphase, the actin plates at the cell poles become more obvious, and some actin filaments extend from the opposing pole actin plates into the chromosome matrix and attach to the chromosomes (Figure 2c). During anaphase, F-actin that is attached to chromosomes, shorten and eventually join the actin plates at the cell poles (Figure 2d). Unlike MTs, actin filaments are not prominent in the interzonal region between two separating daughter chromosomes (Figure 2d), perhaps a result of very fine filaments or low affinity to phalliodin. At late anaphase to pre-telophase the cell plates are lined with F-actin (Figure 2e, arrow) but the space between the sister nuclei is devoid of actin (Figure 2e). At late telophase and during early cytokinesis, F-actin becomes a prominent part of the phragmoplast, fills the space between sister nuclei and is distributed similar to the MTs (Figure 2f). The nuclei become cylinder-shaped, the propidium iodide staining of the nucleus becomes irregular and indicates a reticulate pattern of F-actin (Figure 2g). During this stage F-actin reorganizes into a reticular network that surrounds the nucleus, leading to the distribution shown in (Figure 2a).

3.2. DIFFERENTIATION

3.2.1. *Microtubules*

As mitosis progresses, the cell volume decreases. After the final division, the nuclei move to one side of the cell against cortical MTs and often in different directions in the same filament (Figure 3a). This event starts cell differentiation but the signal that determines the direction of nucleus movement is unknown. During and after migration of nuclei to the cell wall, chromatin accumulates along the periphery of the nucleus, and large clusters of dense chromatin form within the nucleus. Most of the MTs are organized into typical interphase cortical MT arrays, few become oblique to the longitudinal axis of the cell. Later, the MTs at the nucleus side bifurcate into groups. The manchette MTs (Pickett-Heaps, 1968) develop from the side of the nucleus that is adjacent to the wall (Figure 3b) and eventually form the flagella. As the manchette elongates along the nucleus, the nucleus begins to elongate, the chromatin at the periphery of each nucleus develops three to five finger-like ingrowths into the nuclear plasma (Figure 3c). During this time cortical MTs remain present and focused at one end of the nucleus (Figure 3c). These MTs extend toward the surface of the nucleus. Later, the cortical MTs disappear (Figure 3d). The manchette MTs continue to elongate and extend through the two ends of the nucleus, and cause the formation of a crest (Figure 3e). The nuclei elongate with the manchette MTs and form a helix with two and half turns (Figure 3f). As differentiation progresses, the manchette MTs disappear and only the flagella remain (Figure 3g).

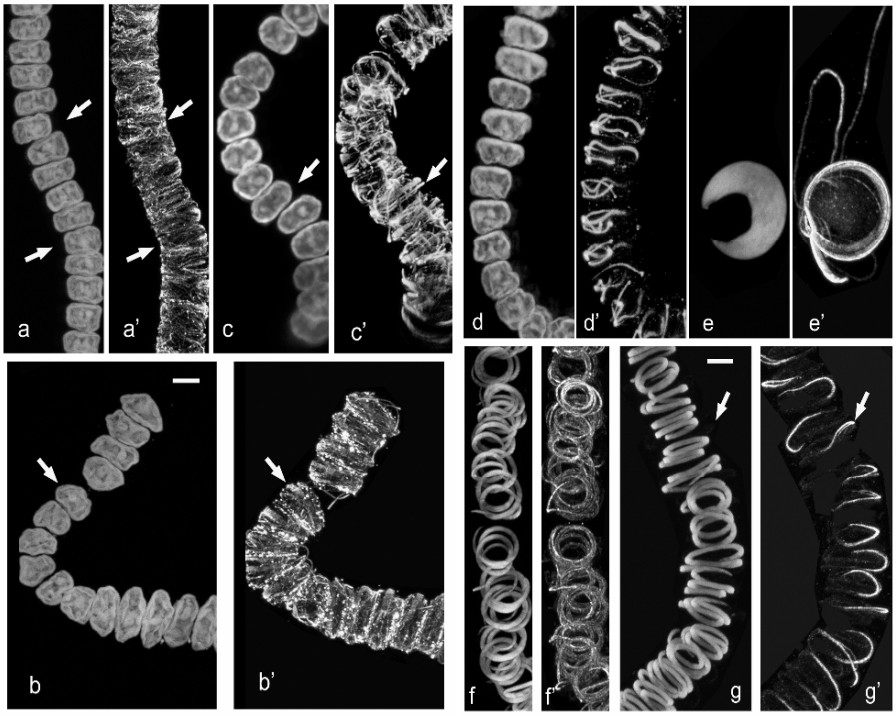

Figure 3. MT organization during cellular differentiation of antheridia. Plain letters (a–g) show location and arrangement of nuclei; primed letters (a'–g') denote microtubule organization of the same cells. a – The beginning of cellular differentiation is characterized by nuclei that move to one side of cells (arrows); MTs exhibit typical interphase arrays. b – the formation of the manchette MTs starts from the side of the nucleus adjacent to the wall (arrow). c – the manchette MTs elongate along the nucleus and flagella (arrow) form parallel to the manchette MTs, the chromatin at the periphery of each nucleus develops finger-like ingrowths into the nuclear plasma. d – cortical MTs disappear, and the manchette MTs and the flagella surrounding the nucleus remain. e – the nucleus condenses and elongates along the manchette MTs assuming a crest-like shape. f – Nuclei with 2½ turns form with manchette MTs still present. g – mature sperms in a filament, manchette MTs disappear but two flagella remain (arrow). Bar = 5 μm, same magnification for all images.

3.2.2. *F-actin*

When the antheridial filaments reach their final length and mitosis stops, F-actin reorganizes. The actin plates between cells become less obvious, and the nucleus is surrounded by an actin network. When the nucleus begins to elongate, this network develops one or two focal points on the surface of the nucleus (Figure 4a). Then, F-actin concentrates at one side of the nucleus, and extends from the focal point toward the opposite side of the nucleus, eventually covering the nucleus (Figure 4b). As the nucleus moves to one

side of the cell, one end of the nucleus becomes F-actin free. This stage initiates elongation of the nucleus toward the focusing point (Figure 4c). The nucleus condenses and elongates in the direction of F-actin (Figure 4d) and finally forms a spiral form (Figure 4e). At the spiral stage, some remaining long actin filaments coil around the nucleus (Figure 4f).

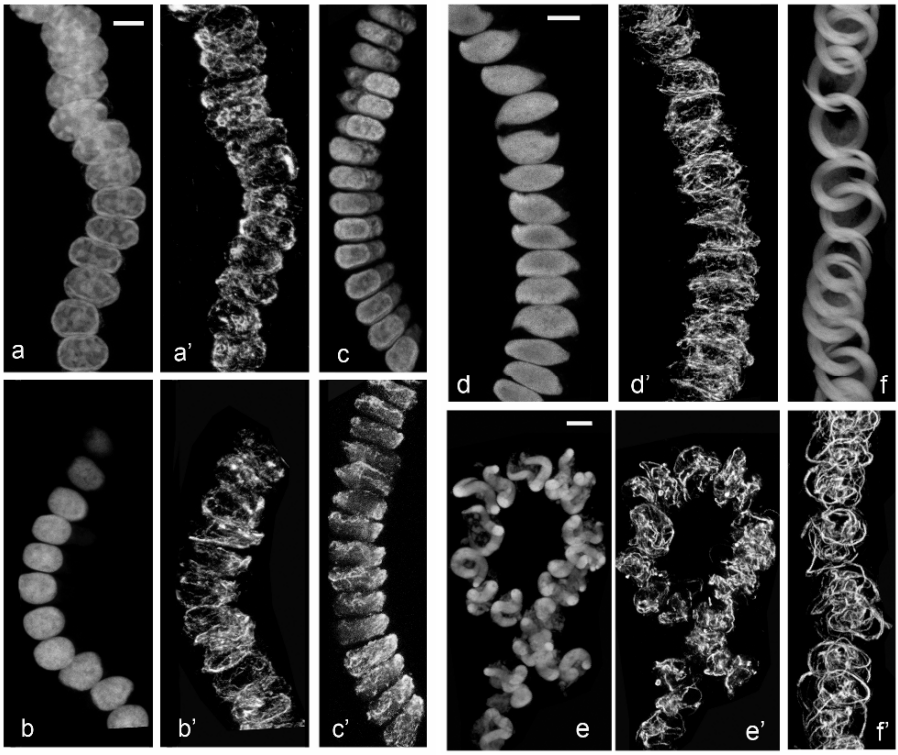

Figure 4. F-actin organization during cellular differentiation. Plain letters (a–f) show location and arrangement of nuclei; primed letters (a'–f') denote microtubule organization of the same cells. a – F-actin concentrates at one or two points at the surface of the nucleus. b – F-actin concentrates at one side of the nucleus and extends from the focal point toward the opposite side of the nucleus. c – Nuclei flatten and elongate toward the focus points of F-actin. d – Nuclei condense and continue to elongate in the direction of F-actin mesh. e – Nuclei form spirals. f – At the spiral stage, remaining F-actins coil around the nucleus. Bar = 5 μm, same magnification for all images.

3.3. ORYZALIN TREATMENT

The dinitroaniline herbicide oryzalin binds to tubulin dimers and induces MT depolymerization.[13] As shown above, *Chara* spermatogenesis is a MT-

dependent process and application of oryzalin is likely to shed light on the function of MTs during spermatogenesis. The sensitivity of MTs to oryzalin in *Chara* antheridial filaments depends on the developmental stage. During mitosis, 5 μM oryzalin applied for 5 h caused the typical oryzalin effect and depolymerized MTs completely (Figure 5b, d, f). However, the same treatment had no effect on MTs during cell differentiation (data not shown). The wall MTs during cell differentiation depolymerized by 50 μM oryzalin after 40 min, but flagella and manchette MTs remained intact (Figure 5h).

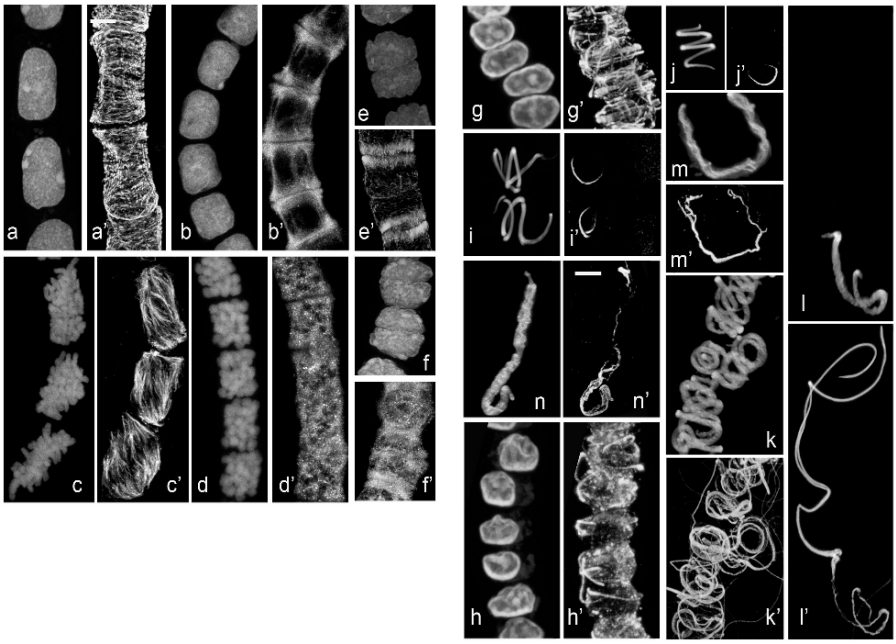

Figure 5. Effects of oryzalin on the MT organization during the mitosis of antheridial filaments. Plain letters (a–l) show location and arrangement of nuclei; primed letters (a'–l') denote microtubule organization of the same cells. a – cortical MTs during interphase (a) were completely disrupted after 5 h treatment of 5 μM oryzalin (b). Spindle-shaped MTs (c) depolymerized after oryzalin treatment without affecting the nuclear structure (d). In controls, the strong staining of MTs at the phragmoplast stage (e) faded after oryzalin treatment, the high concentration of stain at the cell plates suggests little transport of tubulin and possibly indicates the onset of MT stabilization during differentiation (f). Differentiating spermatozoids (g) required exposure to 50 μM oryzalin (40 min) to depolymerize cortical MTs (h); however, manchette MTs and flagella were not affected (h). Nuclei in the helical stage, in non-released sperms (i) and released sperms (j) did not exhibit manchette MTs only short flagella. However, after 5 h in 50 μM oryzalin, sperm showed strong manchette MTs and long flagella indicating MT stabilization (k, l). Both in the control (m) and oryzalin-treated (n) samples, some cells contained straightened nuclei with narrower than usual manchette MTs. Nuclei in oryzalin-treated cells showed granular structure (l, n). Bar = 5 μm, same magnification for all images.

The shape of nuclei deviated from untreated controls. As soon as the spermatozoids form helical nuclei, the response to oryzalin changed. In controls, both the unreleased (still in the antheridial filaments, Figure 5i) and released sperm cells no longer contain manchette MTs (Figure 5j), just two short flagella. However, after oryzalin treatment (50 μM, 5 h), the majority of sperm retained manchette MTs around the nuclei and long flagella (Fig 5k, l). Thus, oryzalin stabilized MTs rather than dissolving them. Both controls (Figure 5m) and oryzalin-treated (Figure 5n) samples contained some cells with straightened nuclei. In these cells, the manchette MTs were less prominent. However, after oryzalin treatment the manchette MTs were discontinuous, and the spiral nuclei developed a granular pattern (Fig 5l, n).

4. Discussion

The cytokinesis of antheridial filaments is successive and three distinct MT systems are recognized during mitosis of the antheridial filament cells: cortical MT arrays, mitotic spindle and phragmoplast. No preprophase bands (PPB) of MTs were observed. In higher plants, the division plane of a cell is directed by either of two MT cycles. One is typical for meristematic cells: the border between future cells is marked by a PPB of cortical MTs arrays. As the separation of the sister chromosomes progresses, the phragmoplast forms at the site of the future cell wall and expands centripetally. The alternative MT cycle lacks both interphase cortical MT arrays and PPB, cell wall formation is determined by a radial MT system. These MTs define a cytoplasm domain and control wall deposition at its perimeter. This MT cycle takes place in the reproductive phase in higher plants and the differentiation of syncytial endosperm.[14] In the present study in *Chara*, mitotic spindles perpendicular to the longitudinal axis of the antheridial filaments were observed. However, the cell division plane is always perpendicular to the long axis of the filament. Unlike higher plants, no PPB or radial MT systems are present in *Chara*. Therefore, division is determined only by the phragmoplast or an as of yet unknown signal before metaphase determines the division plane of the cell.

This is the first report of a quadripole microtubule system during *Chara* spermatogenesis: two independent spindles develop and then fuse during the transition from prophase to metaphase. At this time the two spindles join and form a quadripole spindle apparatus. Finally the quadripole spindle transforms into a bipolar spindle. Quadripolar microtubule system (QMS) was reported during the monoplastidic cell division in sporocytes of lower land plants[14,15] where it predicts the orientation of the future division plane. In early meiotic prophase the plastids derived from division of single

plastids migrate to positions so that either the tips of two plastids or the four plastids resulting from a second division are located in separate domains. Microtubules that radiate from the plastid tips or from individual plastids in the spore domains interact with MTs in the future division planes and give rise to the QMS. In the present study, the origin and function of the QMS is unknown. It may develop from the prophase spindle and the division/ migration of centrosome at prophase. No similar structures were reported during spermatogenesis in Charophyta, the QMS in Chara may not be related to division polarity, because the division plane is always perpendicular to the longitudinal axis of the antheridial filament and independent of the orientation of the four focal points.

There are four types of microtubule organizing centers (MTOC) involved in land plants mitotic spindle formation[16]: the centrosome (only in final division of spermatogenesis), the polar organizer (PO), the plastid MTOC, and the nuclear envelope MTOC. In our study, the MTs in prophase spindle are clearly focused on two points at the cell poles, which is characteristic of centrosome MTOC. When cells proceed to metaphase, for the quadripolar spindle, each pole is sharply focused. Bipolar spindles typically have characteristic centrosome MTOC. However, a small proportion of spindles with poles more like PO are also observed[17] they have broad and discrete poles, each of which is composed by several minipoles from which subsets of spindle MTs emerge. Future studies need to explore the nature of the MTOCs of the mitotic spindle in *Chara* spermatogenesis.

The manchette MTs, which appear during cell differentiation, closely match the condensed/elongated nucleus and they may play a role in the shaping of the nuclear spiral. Manchette MTs linked to nucleus envelope was also found in rodents[18] and abnormal manchette MTs resulted in deformed nuclear shapes during the spermatogenesis of azh/azh mutant mice.[19]

This is the first description of F-actin organization during spermatogenesis in *Chara.* During the mitosis of the antheridial filaments, F-actins form unique plates beneath the cell walls. This structure may affect the division, but this concept awaits further confirmation. We found that F-actin participated in the late telophase phragmoplast, similar to the phragmoplast of higher plants. During cell differentiation, F-actin and MT organizations are similar and suggest interactions between actin and MT similar to the one observed in higher plants.[20] These two cytoskeletal elements control cell differentiation and result in the spiral shape of the nucleus, similar to the involvement of F-actin in cytokinesis and nucleus shaping during spermatogenesis in lower land plants.[1]

The response of MTs to oryzalin changed with the developmental stage: MTs during the differentiation phase of spermatozoids were less susceptible

to oryzalin. It is likely that manchette and flagellar MTs do not form tubulin dimers that are accessible for oryzalin. The observation that oryzalin exerts a protective function for specialized MTs is novel and requires further investigation. The higher sensitivity of mitosis MTs to oryzalin may stem from a rapid turnover of these MTs.

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PART 3

CYTOSKELETON AND SIGNALLING

PLANT TUBULIN PHOSPHORYLATION AND ITS ROLE IN CELL CYCLE PROGRESSION

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Abstract. Review of the literature reveals that plant tubulins can undergo extensive phosphorylation by different types of protein kinases, and that phosphorylation on serine/threonine residues as well as at tyrosine residues can result in the generation of a high level of polymorphism of plant tubulin. Biochemical evidence is presented to demonstrate that phosphorylation of plant tubulin is controlled by different types of protein kinases. Because the phosphorylation sites can be located along the whole length of both the alpha and beta tubulins, it is possible that this type of post-translational modification in plants can participate in modulating the tubulin-tubulin interaction, interactions of tubulins with other proteins (including MAPs and proteins of other cytoskeletal structures), with the plasma membrane and in regulating the functional stability/instability of microtubular arrays. The possible effects of different types of tubulin phosphorylation on cell cycle progression in higher plants are discussed.

Keywords: Plant tubulin, microtubules, phosphorylation, protein kinase, protein phosphatase, cell cycle

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1. Introduction

Microtubules are necessary for a wide spectrum of cellular functions, which include cell division, intracellular transport, cell wall deposition, organelle positioning, cell motility, and the generation of cell polarity.[1,2] Distinct types of microtubular arrays are formed transiently in higher plant cells as they proceed through the cell cycle. The most prominent of them are the interphase network of cortical microtubules, the preprophase band, the mitotic spindle and the phragmoplast. The major component of microtubules is tubulin – a heterodimeric protein where the molecular mass of each subunit (α- and β-) is about 50 kDa. Microtubules are formed by self-assembly of tubulin heterodimers. Actually, tubulin exists in cells as a mixture of polypeptides differing in their isoelectric points. Such multiple tubulin isoforms were found in plants using a variety of polyacrylamide gel electrophoretic techniques.[3–5]

As a rule, multiple plant tubulin isotypes originate from the expression of a whole gene family that encodes different subunits of tubulin.[3–6] The small genome of *Arabidopsis* contains at least six expressed α-tubulin genes[7,8] and nine expressed β-tubulin genes.[8,9] The large-size genomes of some other plant species apparently contain even bigger families of tubulin genes.[2,6] However, a greater heterogeneity of isoforms of both tubulins is a result of different post-translational modifications. It is well established that post-translational modifications of animal tubulins give rise to a high number of α- and β-tubulin isoforms.[10–12] This may result in generation of tubulins with different properties and, accordingly, different populations of microtubules with different functional capabilities.

In animal cells multiple post-translational modifications described for α-tubulin include: reversible removal of the C-terminal tyrosine,[13] acetylation,[14] polyglutamylation,[15] removal of the penultimate glutamyl residue at the C-terminus of a molecule leading to the formation of Δ2-tubulin,[16] and polyglycylation.[17] For β-tubulin, phosphorylation,[10] polyglutamylation[15] and polyglycylation[17] have been described. Except acetylation, all these post-translational modifications occur in the C-terminal hypervariable regions of both subunits. These post-translationally modified forms of tubulin may be detected by means of specific antibodies.[17–19]

Although multiple tubulin genes have been described in higher plants (for review see[2,5,6]), our knowledge of post-translational modifications of plant tubulin was limited until quite recently.[4,5,20] By the end of the 1980s there were only two reports that α-tubulin of meristematic onion cells[21] and of carrot suspension cells[22] was recognized by antibodies against animal tyrosinated tubulin, as well as a short communication on the identification of an acetylated form of α-tubulin in interphase microtubules, the preprophase

band and phragmoplast of rye root cells.[23] In preliminary experiments from the early 1990s we found that [γ-^{32}P]-ATP and [^{14}C]-acetyl-CoA can be used as precursors for plant tubulin phosphorylation and acetylation, respectively.[24,25] This prompted us to further investigate the mechanisms and functional role of post-translational modifications of plant tubulin.

2. Plant tubulin- and microtubule related protein kinases

2.1. CYCLIC NUCLEOTIDE- AND CALCIUM-DEPENDENT TUBULIN PHOSPHORYLATION

It is known that phosphorylation of animal tubulin can be involved in mitosis-dependent reorganization of microtubules and the regulation of their interaction with other cellular structures (e.g. plasma membrane).[11] It has been found that animal tubulin can be phosphorylated by different systems of cyclic nucleotide-dependent (cAMP- and cGMP-dependent) protein kinases, Ca^{2+}-dependent protein kinases (including Ca^{2+}-calmodulin-dependent and Ca^{2+}-dependent, phospholipid-stimulated types of enzymes), casein kinases and tyrosine kinases.[10,11]

In different experiments with phosphorylation inhibitors it was established that their use results in the increasing stability of cortical microtubular networks to cold,[26] and also leads to delayed formation and disappearance of the preprophase band.[27,28] The treatment of *Tradescantia* stamen hair cells with okadaic acid and other protein phosphatase inhibitors caused changes of the metaphase transit times and the pattern of sister chromatid separation.[29] It was shown by another group that treatment of *Arabidopsis* shoots with inhibitors of serine/threonine protein phosphatases (okadaic acid or calyculin A) provoked the destruction of root morphology, which can be explained by the influence of these compounds on the function of cortical microtubules.[30] It also was found that in the presence of calyculin A or okadaic acid, pollen tubes of *Lilium longiflorum* grew very slowly, branched frequently, and contained isolated, randomly oriented, curved actin bundles and microtubules.[31] It was proved that phosphatase inhibitors, as well as protein kinase inhibitors, destroy not only root morphology in *Arabidopsis* shoots but that cortical microtubules also become disorganized after exposure to some types of inhibitors.[32] In particular, these effects were characteristic of phosphoprotein phosphatases such as calyculin A and cantaridin. The protein kinase inhibitor staurosporine also had this effect, though in some experiments such results were not detected.[33–35]

On the other hand, it has been shown that calmodulin in the presence of Ca^{2+} is capable of effectively depolymerising cortical microtubules in lysed carrot protoplasts.[36] Thus, the literature indicates that phosphorylation and

dephosphorylation represent a part of the molecular mechanism responsible for both the organization of the cortical microtubular networks and of mitotic function.

More-or-less simultaneously with our first results on the discovery of α- and β-tubulin phosphorylation in cells of *Nicotiana plumbaginifolia*[24,25] the proofs of phosphorylation of the two tubulin subunits in carrot cell suspension cultures were obtained.[37] For this reason we conducted several experiments to identify the types of protein kinases that can be involved in the phosphorylation of plant tubulin. To identify the possible influence of specific activators of different types of protein kinases (cAMP, cGMP, 1,2-diacylglycerol and calmodulin) on tubulin phosphorylation, a system involving permeabilised *N. plumbaginifolia* protoplasts was developed.[20,25] After preloading with [γ-^{32}P]-ATP, permeabilised protoplasts were incubated with the activators in the presence or absence of Mg^{2+} and Ca^{2+} (Table 1).

TABLE 1. Reaction conditions for testing endogenous tubulin phosphorylation in *N. plumbaginifolia* protoplasts.

Type of activator (and its concentration)	Presence of activator(s) in reaction medium				
	1	**2**	**3**	**4**	**5**
Mg (5 mM)	+	+	+	+	+
Ca^{2+} (2 mM)	-	+	+	+	-
1,2-Diacylglycerol (18 μM)	-	-	+	-	-
Calmodulin (15 μM)	-	-	-	+	-
cAMP (10 μM)	-	-	-	-	+

Then cytoplasmic fractions enriched with microtubules were resolved by SDS-electrophoresis followed by drying, autoradiography and scanning densitometry of the gels. As a result, it was shown that both α- and β- tubulins were intensively phosphorylated during activation of cAMP-, Ca^{2+}-, Ca^{2+}-calmodulin-dependent protein kinases and Ca^{2+}-dependent, phospholipid-stimulated protein kinase (so-called protein kinase C) (Figure 1). No changes in phosphorylation levels of both α- and β-tubulins were noted during treatment of protoplasts in the presence of cGMP.

Recent works have shown unequivocally that cyclic nucleotides are present in plant cells,[38,39] and that they play a key role in regulation of plant physiology.[40] With an understanding of the protein domains that can bind cyclic nucleotides, another group of authors scanned the completed genomes of *Arabidopsis thaliana* and *Oryza sativa* for the effectors of these signaling molecules.[41] No biochemical or bioinformatic evidence was found

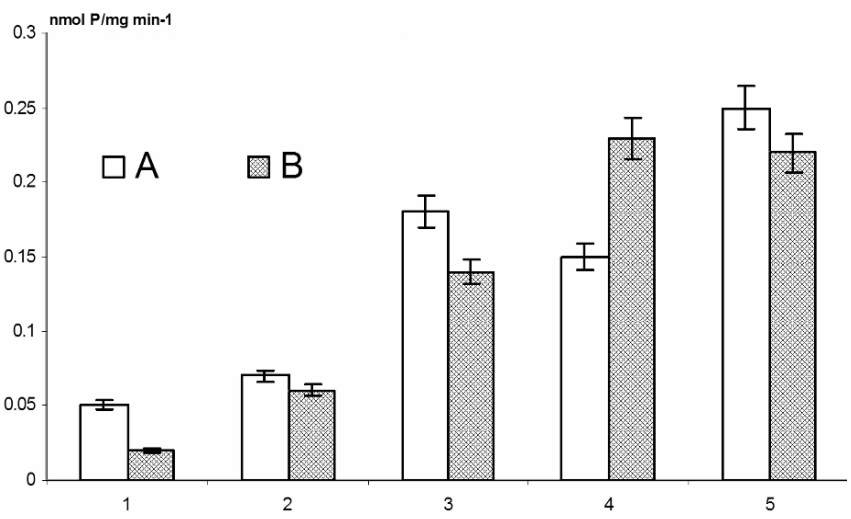

Figure 1. Incorporation of ^{32}P in α- and β-tubulins in *N. plumbaginifolia* protoplasts: (A) α-tubulin; (B) β-tubulin. Reaction conditions as indicated in Table 1.

for a plant cyclic nucleotide regulated protein kinase, suggesting that cyclic nucleotide functions in plants have evolved differently than in mammals. It is possible however, that plants have evolved entirely novel domains, which can be modulated by these second messengers.[41] It will be interesting to compare this *in silico* analysis with future biochemical data regarding the direct effectors of cyclic nucleotide signaling in plants.

For more detailed analysis of the functional role of this modification of tubulin in plant cells several specific regulators of different types of protein kinases were used in our experiments.[42] Dibutyryl-cAMP (10 μM in combination with 100 μM ATP), as an activator of cAMP-dependent phosphorylation, and polymyxin B (5 mM) and trifluoperazine (5 mM), as inhibitors of the protein kinase C and Ca^{2+}-calmodulin-dependent protein kinases, were investigated with regard to their ability to affect microtubule dynamics and to induce structural changes of microtubules. The root tips of 3-day old *Allium cepa* seedlings were treated with these compounds. The effects of these regulators of protein kinases on the structural reorganisation of interphase and mitotic microtubules were studied after exposure of plant material to cAMP (12 and 24 h) and polymyxin B or trifluoperazine (6 and 12 h). The monoclonal antibody TU-01 (against α-tubulin) and TU-06 (against β-tubulin) (kindly provided by Drs. V. Viklicky and P. Draber, Institute of Molecular Genetics, Prague, Czech Republic) were used for visualising microtubules in onion meristematic root tip cells by immunofluorescence microscopy.[42]

Immunofluorescence analysis of microtubules showed that treatment by dibutyryl-cAMP causes the disruption of both interphase and mitotic microtubules, and accumulation of depolymerised tubulin around the nuclei. The treatment of onion cells by trifluoperazine causes the reorganization of microtubules, and change of their spatial organization from a transverse to a longitudinal orientation and formation of thick longitudinal arrays. Treatment of *A. cepa* cells with polymyxin B caused the same effects on microtubular organisation as trifluoperazine. It was found that these changes in the spatial organisation of microtubules after treatment by dibutyryl-cAMP and the protein kinase inhibitors led to disturbances of cell cycle progression.[42] Recently, qualitative and quantitative changes in phosphoproteins of *Nicotiana tabacum* suspension cells during elicitation by bacterial lipopolysaccharides were analysed using two-dimensional electrophoresis in combination with a phosphoprotein-specific gel stain,[43] Trypsin digested phosphophoproteins were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and nano-electrospray-ionization liquid chromatography tandem mass spectrometry. Among 27 proteins, a cytoskeletal tubulin was identified as one of target proteins which phosphorylation process has $Ca(^{2+})$/calmodulin-dependent character.[43] It was found that α-tubulin from tea (*Camellia sinensis* (L.) O. Kuntze) contains four protein kinase C phosphorylation sites.[44]

In parallel with the data about the role of cAMP,[45,46] Ca^{2+}-calmodulin[47] and 1,2-diacylglycerol,[48] which confirm the participation of cyclic nucleotide- and Ca^{2+}-dependent protein kinases in the regulation of structural and functional features of plant microtubules, it is impossible to ignore a likely role in these processes for such protein kinases as cyclin-dependent[49] and mitogen-activated protein kinases.[50,51]

2.2. ROLE OF CYCLIN-DEPENDENT KINASES IN PLANT MICROTUBULE ORGANIZATION

In plants, the orientation of cell division is thought to be important for normal development, and in many tissues it is stringently controlled.[52] There must be points in the cell cycle where the effectors of microtubule dynamics are activated or inhibited. Studies in animals have implicated cyclin-dependent kinases (CDKs) in regulatory pathways that regulate MAP activities,[53] particularly at the entry to S phase and into mitosis. In plant cells, it is not yet known whether some of the identified microtubule-stabilizing or motor proteins could be substrates of cyclin-dependent kinases. However, considering the data obtained over the past few years, it appears likely that the cyclin-dependent kinases may have similar roles in plant cells.

Based on structural similarities, plant cyclin-dependent kinases have been classified as cyclin-dependent kinase A (CDK-A), cyclin-dependent kinase B (CDK-B), etc.[54] Canonical plant cyclin-dependent kinase, or CDK-A, is highly conserved at the amino acid sequence level and is most similar to the yeast Cdc2/CDC28 or animal CDK1-3 proteins. All CDK-A proteins contain the conserved cyclin interaction or PSTAIRE motif and have been shown to functionally complement yeast cdc2/CDC28 mutations.[54] CDK-A mRNAs are expressed through the cell cycle, and their protein kinase activity becomes increased during the transition from G_1- to S-phase as well as from G_2- to M-phase.[55] The overexpression of CDK-A in a kinase-negative mutant of *Arabidopsis* slows down or blocks cell cycle progression in both G_1- and G_2-phases, further indicating its multiple roles in the cell cycle.[56] Similarly, specific inhibitors (roscovitine, oloumicine and bohemine), drugs with a high specificity for canonical cyclin-dependent kinases, block both G_1- to S-phase and G_2- to M-phase transitions.[57] CDK-A members were found to bind some of the D-type cyclins.[58] They interact with the A2-type cyclin in the yeast two-hybrid assay,[59] but it is not known if CDK-A associates with B-type cyclins.

A second class of cyclin-dependent kinases is found only in plants,[60] with members containing the PPTALRE or PPTTLRE motifs – the so-called CDK-B class.[54] These cyclin-dependent kinases appear to have specific functions in S-, G_2-, and M-phases, as suggested by their phase-specific expression and activity.[61,62] However, their exact roles are not well understood.

Many recent experiments on immunolocalization of CDK-A proteins, using antibodies against the conserved PSTAIRE domain or against the C terminus, have confirmed that kinases, in particular cdc2, indicate the presence of this antigen at multiple locations: in the nucleus, on the mitotic chromosomes, and associated with several microtubular arrays, including the preprophase band, the spindle, and the phragmoplast.[55,63–66] Moreover, it was recently shown that P34 (cdc2) kinase is associated with cortical microtubules from higher plant protoplasts.[67] Another cycle-dependent kinase, CDK-B, is also found on mitotic structures.[62,68] For instance, it was found that in rice the fluorescence signal of CDKB2; 1-GFP was tightly associated with chromosome alignment, as well as with spindle structure, during the metaphase. During telophase, the signal was localized to the spindle midzone and the separating sister chromosomes, and then to the phragmoplast.[62]

In higher plant cells, the division plane is predicted by an enigmatic microtubule array called the preprophase band. It has been established that cdc2 controls the development and destabilization of the preprophase band (see review[65]). First, microinjection of active cdc2 in *Tradescantia* cells causes rapid depolymerization of the preprophase band and induces a

premature breakdown of the nuclear envelope.[69] In contrast, the inhibition of cdc2 by specific inhibitors leading to an arrest of the cells in G2, stabilizes the preprophase band.[28,57] In addition, the co-localization of cdc2 with the preprophase band has been demonstrated in various cell types.[63,66] It is not known whether cdc2 inhibits the activity of a MAP or activates a destabilizing factor. TKRP125, which localizes at the preprophase band, has a cdc2 phosphorylation site[70] and is therefore a candidate substrate for this kinase.

During mitosis, the role of cyclin-dependent kinases in microtubule organization remains unclear. Cdc2 is localized within the spindle.[66] The inhibition of cdc2 activity induces abnormalities in the bipolar spindle structure[57] whereas an increase in cdc2 level does not affect the process of mitosis.[69] Therefore, cdc2 may keep MAPs inactive and hence maintain microtubules in a highly dynamic state or regulate the activity of microtubule motors involved in spindle bipolarity. Further studies are needed to support these hypotheses, which are not necessarily mutually exclusive.

There is accumulating evidence that protein phosphorylation during cytokinesis may play an important role in the formation of the cell plate, which divides the cell into two. Indeed, cyclin-dependent kinases and kinases involved in signalling pathways have been found associated with the phragmoplast and/or the cell plate. Cdc2 is co-localized with the phragmoplast[66] and MAPKs (mitogen-activated protein kinases) show a transient localization to the cell plate in late anaphase and throughout telophase.[50,71] However, the reported localization patterns are complex and sometimes contradictory, depending on the source of the antibody and even the fixation conditions used.

Recently, a functional GFP fusion with CDK-A (Cdc2-GFP) was made to investigate its subcellular localization during the cell cycle in tobacco cells.[72] During interphase, the Cdc2-GFP fusion protein was found in both the cytoplasm and the nucleus, where it was highly resistant to extraction. In premitotic cells, a bright and narrow equatorial band appeared on the cell surface, resembling the late preprophase band. Cdc2-GFP was not found on prophase spindles but left the chromatin soon after this stage and associated progressively with the metaphase spindle in a microtubule-dependent manner. Arresting cells in mitosis through the stabilization of microtubules by taxol further enhanced the spindle-localized pool of Cdc2-GFP. Towards the end of mitosis, Cdc2-GFP was found at the midzone of the anaphase spindle and phragmoplast. Eventually, it became focused at the midline of these microtubule structures. In detergent-extracted cells, the Cdc2-GFP remained associated with mitotic structures. Retention on spindles was prevented by pretreatment with the COK-specific inhibitor roscovitine and enhanced by the protein phosphatase inhibitor okadaic acid. Furthermore, it

was demonstrated that both the endogenous CDK-A and Cdc2-GFP co-sedimented with taxol-stabilized plant microtubules from cell extracts, and that Cdc2 activity was detected in the polymeric tubulin fraction.[72] These data provide evidence that A-type cyclin-dependent kinases associate physically with mitotic structures in a microtubule-dependent manner and may be involved in regulating the behaviour of specific microtubule arrays throughout mitosis. Therefore, cdc2 may maintain microtubules in a highly dynamic state by regulating the functional state of MAPs and the activity of microtubule motors involved in spindle bipolarity. Simultaneously, this protein kinase can phosphorylate tubulin molecules.

Interestingly, MAPK activity was correlated with the timing of phragmoplast formation. The possible role of MAPKs in the construction of the phragmozplast is still speculative. MAPKs may regulate microtubule stability, microtubule-based motor proteins and the transport of vesicles along the phragmoplast by plus-end-directed motor proteins, or may be involved in the fusion of these vesicles at the cell plate.[50,51] It is not yet known whether cdc2 is active or not during telophase. One might speculate that the inactivation of cdc2 is necessary for phragmoplast formation. In animal cells, the initiation of cytokinesis correlates with the inactivation of cdc2 kinase. Indeed, when the inactivation of cdc2 is prevented (by the expression of non-degradable cyclin B), cytokinesis is blocked and this effect is likely to be due to a lack of interzonal microtubules.[73] The anaphase events are not inhibited. Because the turnover of phragmoplast microtubules is slower than that of mitotic microtubules, a relative stabilization of microtubules by MAPs could be necessary to construct the phragmoplast structure.

2.3. PUTATIVE ROLE OF TYROSINE KINASES IN PLANT MICROTUBULE PHOSPHORYLATION

Recently, the potential effects of tyrosine phosphorylation on the morphology of *Arabidopsis thaliana* primary roots and microtubule organization, using different types of protein tyrosine kinases inhibitors (herbimycin A, tyrphostin AG 18 and genistein) and inhibitor of protein tyrosine phosphatases (sodium orthovanadate) was studied.[74] Effects of inhibitors were examined *in vivo* on *A. thaliana* expressing GFP-MBD using confocal laser scanninh microscopy. It was established that inhibitors of tyrosine kinases cause microtubules disorientation and disruption in epidermis and cortex cells of the elongation and differentiation/maturation zones, and this leads to disruption of normal root hair growth and development as compared to untreated roots. While enhanced tyrosine phosphorylation after treatment by sodium orthovanadate causes microtubules disruption in meristematic cells and changes microtubules orientation from transverse to longitudinal in

epidermis and cortex cells of both elongation and differentiation zones, it also leads to intense induction of root hair development and growth.[74]

Additionally, the possibility of plant tubulin phosphorylation on tyrosine residues was analysed preliminary by high-resolution isoelectric focusing.[20] After electrophoretic resolution of a purified tubulin fraction from *N. tabacum* suspension cells, mouse monoclonal antibodies P-TYR 10/E8 against phosphotyrosine (SUNBIO, Czech Republic) reacted with several protein bands. The majority of them were recognized also by antibodies TU-01 and TU-06 specific for α- and β-tubulin, respectively. After separation of tubulin by high-resolution isoelectric focusing, 11 charge variants of α-tubulin and 11 charge variants of β-tubulin were recognized by P-TYR antibodies. The possibility of phosphorylation on tyrosine residues of maize α- and β-tubulin was demonstrated, too.[75] Therefore, it was established that tyrosine phosphorylation, in addition to phosphorylation of serines and threonines, of both tubulin subunits on tyrosines can be involved in the regulation of plant microtubule functions. Thus, we suppose that tyrosine kinases and protein tyrosine phosphatases can be involved in the processes of dynamic microtubules reorganization in living plant cells through tubulin phosphorylation.

3. Interaction of serine/threonine protein phasphatases with plant microtubules

Also of particular interest are the molecular studies of phosphatases that act at different steps during mitosis to maintain microtubule-interacting proteins in active or inactive states. Phosphatase PP1 is required for entry into and exit from mitosis, whereas PP2 is necessary to maintain short mitotic microtubules.[64,76] In plants, it is difficult to speculate further on the phosphorylation changes of the microtubular proteins occurring during mitosis.[64] Indeed, even though recent molecular cloning studies have revealed the presence of two structurally distinct families of plant protein serine/threonine phosphatases (the PP1/PP2A family and the PP2C family[77,78]) their activities still remain to be demonstrated during the cell cycle. Recently, immunological and biochemical evidence was obtained for an interaction of maize protein phosphatase PP2A holoenzyme with the tubulin family.[79] Affinity chromatography of the maize PP2A preparation on immobilized tubulin revealed two peaks of phosphorylase *a* phosphatase activity. Both catalytic and constant regulatory subunits of PP2A were co-immunoprecipitated from a maize seedling homogenate by an anti-α-tubulin antibody.

An inhibitor of type 1 and 2A protein phosphatases promoted microtubule depolymerization in maize root cells,[80] indicating that cortical microtubule stability is regulated by protein phosphorylation. A recent genetic

approach identified a putative protein phosphatase 2A regulatory subunit to be involved in the control of cortical microtubule structures.[81] A genetic screen for mutants defective in microtubule functions provided further information on the control mechanisms of plant microtubules.[82] There were isolated many *Arabidopsis* mutants that are more sensitive to microtubule-destabilizing drugs than the wild type. It was shown that a novel phosphatase is defective in one of the drug-hypersensitive mutants and maybe involved in phosphorylation cascades that control the dynamics of cortical microtubules in plant cells.[82]

4. Conclusions

The combined data demonstrate that plant tubulin can undergo extensive phosphorylation by different types of protein kinases, and that phosphorylation of serine/threonine residues, as well as tyrosine residues, is involved in the generation of a high level of polymorphism of plant tubulin. Because the phosphorylation sites can be located along the whole length of both tubulin molecules, it is possible that this type of post-translational modification in plants can participate in modulating the tubulin-tubulin interaction and interaction of tubulins with other proteins, including MAPs, proteins of other cytoskeletal structures, the plasma membrane and in regulating the functional stability/instability of microtubular arrays. All these facts taken together clearly demonstrate a very important functional role of different types of tubulin phosphorylation in regulating the plant cell cycle.

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PLANT MYOSINS: DO THEY HAVE ROLES IN GRAVI- AND MECHANOSENSING?

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Abstract. Dynamic actin cytoskeleton and myosins, especially of the plant-specific class VIII, emerge as the most relevant candidates for plant gravi- and mechanosening as well as transduction of received physical signals into multitude of biological processes. Root apices are extremely sensitive to mechanical stimuli and show adaptive responses optimalizing root behaviour for their searching/avoidance and exploratory growth modes. Recently, the actin cytoskeleton and myosins have been proposed to participate in plant gravi- and mechanosening also via their roles in endocytosis and vesicle recycling, processes which are essential for the polar transport of auxin. Mechanosensitive endocytic vesicle recycling might act as an effective gravisensing system with a power to unite the two conflicting theories, *Statolith-Starch Theory* and *Cholodny-Went Theory*, under one umbrella – the unifying concept of gravisensing plant synapses.

Keywords: Actin cytoskeleton, gravisensing, mechanosensing, myosins, root cap

1. Introduction

Dynamic arrays of cytoskeletal elements represent mechanical devices for cellular polarity, development and function in all eukaryotic cells, a concept known as Inbger's tensegrity concept.[1–3] Recent advances in studies on cytoskeleton revealed that its dynamic properties are tightly linked to diverse signalling cascades monitoring extracellular and intracellular cues.[4,–6] Actin polymerization is typically induced by diverse signals received and transduced

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at the plasma membrane.[7,8] Moreover, local assembly of F-actin mechanically deforms membranes,[9,10] and mechanotransduction regulates the actin polymerization.[11] Dynamic actin cytoskeleton was proposed to act as an electronic-like integration device specialized for noise-to-signal enhancement allowing amplification of coherent signals and reduction of random noise.[12] Similarly to actin, tubulin polymerization is also often signal-mediated and related to mechanotransduction and gravity forces.[13] In addition, several molecular motors, especially myosins, interact abundantly with signal perception and transduction pathways related to mechanosensing.[14,15] Space shuttle flights revealed that microgravity conditions enhance degradation of rat myosin heavy chain suggesting that myosin abundance is also regulated via mechanotransduction pathways.[16]

While non-plant organisms are mobile and can escape from unfavourable environments, plants are sessile organisms for which the only survival imperative is adaptation. Dynamic actin cytoskeleton, tightly linked to signal perception and transduction via stimulus-responsive actin-binding proteins, is critical for effective adaptation to diverse environmental challenges. Therefore, it comes perhaps not as a surprise that actin and the two most important actin-binding proteins with respect of its dynamicities, profilin and ADF, are represented by several isovariants in plants, while mammalian cells can do with one-two isoforms of these proteins.[5,6] Within the context of the extreme variety of isoforms of plant actin and actin-binding proteins, it appears that the dynamic plant actin cytoskeleton serves as an essential buffer for a wide range of complex biological phenomena, including integrated signal transduction pathways.[17] The dynamic cytoskeleton is constantly monitoring and controlling internal balance of forces,[18] cellular architecture, as well as intracellular motilities. Co-expression of functionally distinct multiple isovariants of actin, profilin, actin depolymerising factor (ADF), and other stimulus-responsive actin-binding proteins allows effective buffering of cellular responses to continual fluctuations of environmental parameters.[5,6,17]

Gravisensing belongs to the general category of mechanosensing processes which encompass also the perception of sound and pressure in animals, and touch and osmosis in plants. The most outstanding feature of mechanosensory perception is the extreme velocity of the process, having latencies of less than one millisecond.[19] By this characteristic, the mechanosensing process is faster than any other biological process transforming environmental stimuli into diverse cellular responses, including photosensing.[20] This special situation is explained by the idea that mechanical forces might be directly transduced at mechanosensitive ion channels of the plasma membrane (e.g., in Sachs[21]; Spencer et al.[22]) via cytoskeletal elements (e.g., in Glogauer et al.[23]) thereby bypassing a need for special receptor molecules.[19] The best investigated system related to mechanosensing are hair cells of the

inner ear where actin filaments, myosin motor molecules, unknown linker proteins and a single calcium channel in the plasma membrane are proposed to form a basic module of mechano-electrical transduction complex.[24,25]

2. The cytoskeleton in root gravisensing

Growing root apices are very sensitive towards weak mechanical[26] as well as gravitational[27,28] stimuli. In spite of some interesting recent reports on gravisensing outside of the root cap,[29,30] cells of the central columella identified as statocytes are still accepted as the master site for transducing the gravity stimlus.[31,32] These cells are unique under several aspects. Ontogenetically they originate from suspensor cells[33,34] which are in close contact with the mother plant during embryogenesis and therefore are distinguished from any other cell type. They show polar organisation,[35] which is actively established, are maintained under the regime of cytoskeletal proteins,[27,28,36,37] and they seem to be depleted of prominent F-actin bundles and dense microtubules.[38,39] On the other hand, there are several reports for localisation of actin[40–42] and myosin[4,43] in central columella cells, typically in close vicinity to amyloplasts acting as statoliths. In particular experiments under microgravity conditions, in parallel with application of actin filaments affecting drugs, clearly showed that the position of these sedimentable particles depends on the external gravitational force and an interenal force exerted by cytoskeletal proteins.[28,41,42,44]

There is, however, still a controversial debate concerning the role of cytoskeletal proteins in particular of intact actin filaments in plant gravisensing.[45] Studies of gravirepsonses under pharmacological substances like latrunculin B dissecting actin filaments in its globular subunits and/or very tiny filaments clearly demonstrated increasing responses in comparison to untreated controls.[46,47] Using clinostats the authors have shown that even gravisensitivity can be increased by this drug. As we reported earlier, less polymerized cytoskeleton of columella cells enhances sedimentability of their statoliths allowing very sensitive gravisensing.[38,39] Thus, plausible interpretation of above data would be that latruculin B facilitates more effective gravisensing of root cap statocytes via effective transformation of longer F-actin elements into oligomers. Dramatic advancements in our understanding on the graviresponse of root apices has been obtained in studies focusing on auxin transport machinery. PIN3, an auxin efflux regulator from the PIN family, localizes to endosome-like vesicles as well as to the plasma membrane throughout statocytes.[48] After gravistimulation, this molecule rapidly relocalizes from the lower transversal part (apical) of the plasma membrane to the lower longitudinal parts of statocytes. Similarly to PIN1,[49] PIN3 localizes also to recycling pectin-enriched vesicles that

continuously cycle between the plasma membrane and peripheral endosomes in F-actin-dependent manner.[48,50] Intriguingly, also ARG1 DnaJ-like protein[51] and structural sterols[52] perform the same brefeldin A-sensitive recycling pathway driven by the actin polymerization and regulated via ARF-GEF protein GNOM.[49]

Involvement of vesicle recycling in perception of gravity and mechanotransduction of this signal were first reported in studies on endodermal cells underlying shoot gravitropism.[53–56] Firstly, it was suggested that this is a specific-feature of shoot endodermis-based statocytes that, in contrast to root cap statocytes, are highly vacuolized. However, as mentioned abovely, a later study[48] revealed that root cap statocytes also employ vesicle recycling for gravisensing. Moreover, the ARG mutant is known to have abnormal gravitropism in both roots and shoots[57,58] and recent a study revealed that ARG1 is recycled in actin- and BFA-dependent fashion between the plasma membrane and endosomes in root cells.[51] All this strongly suggests that exocytosis, endocytosis, and vesicle traffickings are either inherently linked with, or driving mechanoperception/mechanotransduction. In accordance with this notion, endocytosis in pollen tubes was shown to be inhibited by hyper-g conditions while microgravity stimulated endocytosis.[59] Surprisingly, results related to the PIN family brought about the stimulating idea that vesicle recycling at plant plasma membranes might occur in a similar way and with similar effects as in neuronal synaptic regions.[60,61] In this sense, the root apex can be considered to act as an integrating control centre, resembling neural tissue (e.g., brains) of lower animals, for interactions of plants with their environment.[62]

3. Differential expression of critical cytoskeletal molecules in the root cap

Immunolocalisation of several proteins, in particular cytoskeletal proteins like actin, myosin and profilin, indicate the unique status of root cap cells (Figures 1 and 2, compare also Baluška et al.[63]). Labelling by antisera against two myosins (from classes VIII and XI, for specific domains see Figure 4), and actin shows remarkably low expression of these proteins in root cap cells (Figure 1A, F, K) in particular in the central cells (columella cells = statocytes) in comparison to peripheral cap cells as well as cells of the meristem. There is a similar situation concerning HDEL proteins (Figure 2B). In contrast, a totally different picture was scored for profilin that showed homogenous labelling over the whole root apex (Figure 2A). With anti-myosin XI antibodies, distinct dots are visible within the

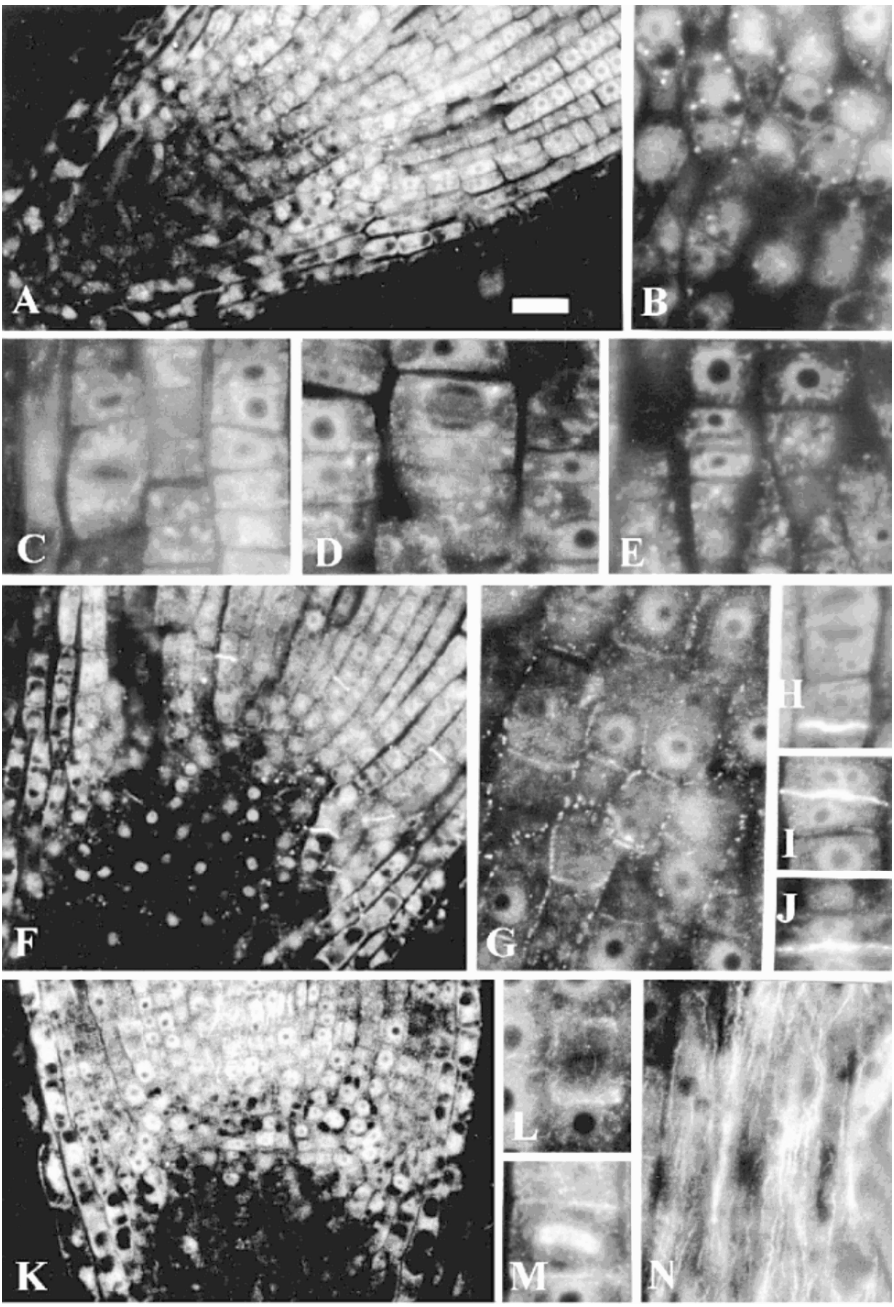

Figure 1. Immunolocalisation of myosin XI (A–E), myosin VIII (F–J) and actin (K–N) in cells of root apices from Medicago sativa. Columella cells (A, F, K) in the root cap show in general less intensive labelling than neighbouring secretory cap cells and in particular cells of the root meristem. For details compare the text. Bar in A represents: 35 μm for A, F, K and 15 μm for B, C, D, E, G, H, I, J, L, M, N.

cytoplasm of cap cells at higher magnification whereas in meristematic cells irregular spots can be observed (Figure 1B–E). Anti-myosin VIII (AtATM1) antibody, however, shows distinct dots specifically at the plasma membrane, in particular at newly formed cell plates (Figure 1F–J, see also Reichelt et al. 1999[72]). Concerning the role of myosins related to gravi- and mechano-sensing, of special interest is labelling of statoliths (amyloplasts) in statocytes (central columella cells). For both sera, fluorescence is clearly localised at amyloplast surfaces (Figure 3A–C). Several experimental treatments, in particular under mechanical stimulation and by osmotic stress, induce relocation of AtATM1 molecules from amyloplast surfaces towards the plasma membrane of transverse cell walls.[64] These results suggest the rapid intracellular shuttling is a typical feature of this plant-specific motor molecule.

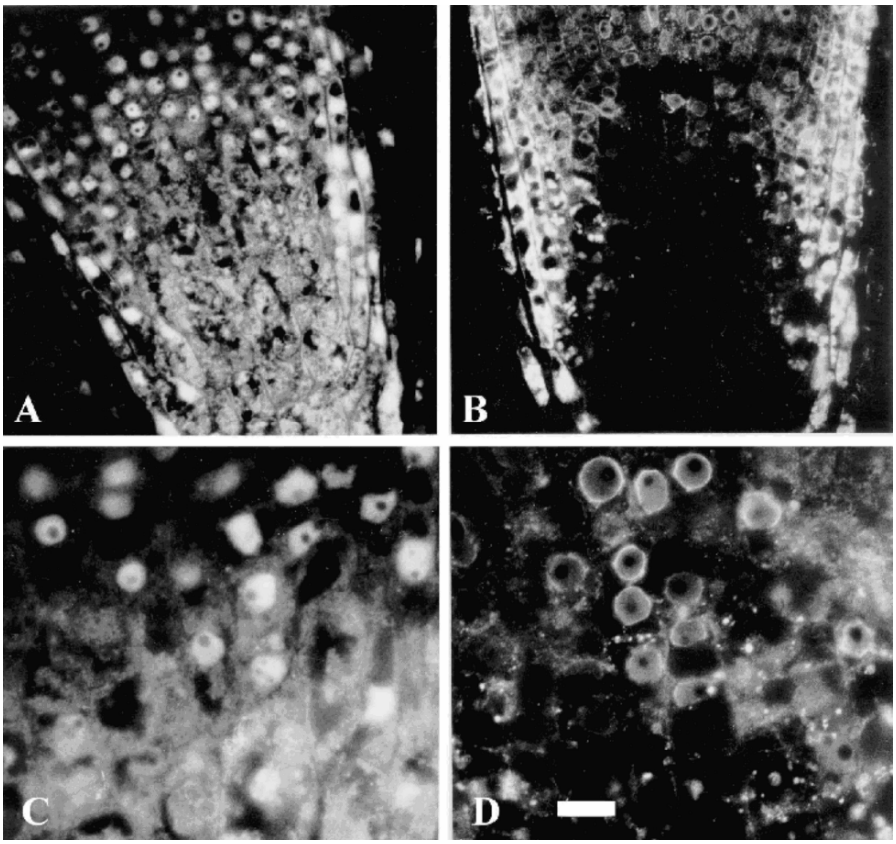

Figure 2. Immunolocalisation of profilin (A, C) and HDEL-based ER proteins (B, D) in root cap cells of *Medicago sativa*. Bar in D represents: 30 μm for A, B and 12 μm for C, D.

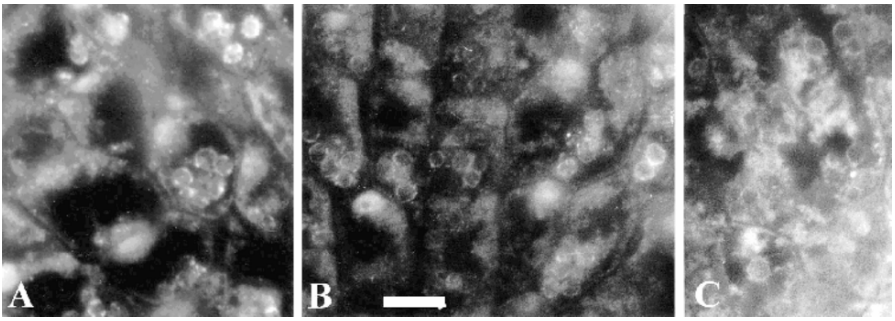

Figure 3. Immunolocalisation of myosin VIII AtATM1 (A) and myosin XI (B and C) in root cap cells (statocytes) of *Medicago sativa*. Statoliths show labelling at their peripheries with both anti-myosin VIII and anti-myosin XI antibodies. Bar represents 15 μm for A, B, C.

4. Domain organization of unconventional plant myosins

Molecular motors of the myosin family are characterized by the highly conserved C-terminus, also known as the head region, which is responsible for movement of the molecules in general, and velocities in particular, with binding sites for actin and ATP, respectively. Classification of myosins into their individual families is accomplished by their highly variable N-terminal tail region defining intracellular sites of activities like vesicles or membranes. Detailed database search for AtATM1 and other myosins of class VIII and class XI indicates differences for both types of molecules concerning head as well as neck and tail regions (Figure 4). At the head, AtATM1 is distinguished by a downstream located PEST sequence[65] responsible for cleavage and degradation processes of the molecule.

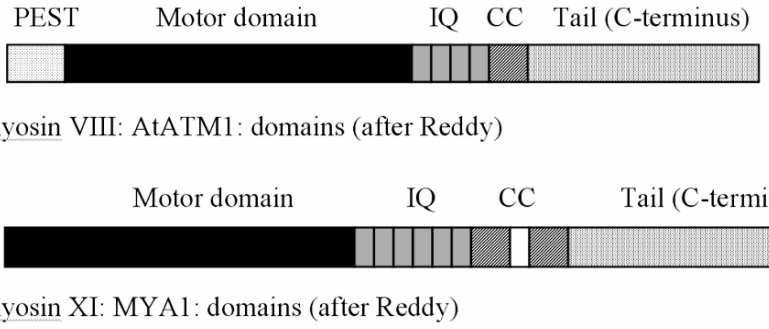

Figure 4. Domain organization of myosins of the class VIII and XI, after Reddy and Day.[71] The neck region shows differences in the number of IQ motifs, as well as in the size and location of coiled coil (CC) domains. Finally, at the N-terminal region, AtATM1 is distinguished by putative binding sites for endomembranes. For AtATM1, several phosphorylation sites have been indicated (PEST).

5. Conclusions and outlook

Recent advancements in cellular and molecular biology of myosins suggest that these motor molecules are situated at the cross-road of cytomechanics and signalling. This feature makes these molecules for an ideal candidate to perform both sensing and transducing of mechano- and gravity-based signals which are essential for the sessile life-style of higher plants. Root caps are highly specialized sensor organs[32,66] that are specialized for both sensing gravity and for transducing certain signals into appropriate growth responses. Despite some criticism, sedimenting amyloplast-based statoliths of root cap statocytes are still considered to represent the sensor for gravity. Localization of both plant myosins as well as of the actin-binding protein profilin to the surface of statoliths strongly suggests that these molecules are very good candidates for sensors of gravity. Importantly, they are in a position to transduce this information further towards effector molecules leading to root curvature. Among these effector molecules, F-actin is one of the most attractive as it can alter subcellular distributions of recycling proteins that can be ion channels and the collumela auxin transport facilitator PIN3.[48] As myosins also seem to participate in this recycling process, especially via their intervention with endocytosis,[67,68] one could propose also a concept in which the recycling process as a whole would act as a mechanosensing device optimized for transducing this signal into altered distribution of auxin.[69,70] This attractive concept is strongly supported by the study revealing that endocytosis reacts sensitively towards hypergravity and microgravity, when the latter situation stimulated endocytosis in pollen tubes.[59] Unique properties of AtATM1, which can rapidly redistribute within plant cells,[64] suggest that this class of plant myosins[71,72] is extremely interesting as they could participate both in gravistimulation via sedimenting amyloplasts[73,74] as well as via vesicular recycling of critical molecules like PIN3.[48] Further studies should shed more light onto this dynamically evolving field of plant cell biology. Mechanosensitive vesicle recycling acting as the gravisensing system has the power to unify, finally, two conflicting thories (the statolith-starch and the Cholodny-Went theory[75]) under one general concept of gravisensing plant synapses.[69,70]

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THE ROLE OF THE CYTOSKELETON IN PLANT CELL GRAVISENSITIVITY

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Abstract. This chapter highlights current ideas on the role of the cytoskeleton in plant cell gravisensing. It is important to distinguish between cell graviperception and cell gravisensing. The first implies the active perception of a gravitational stimulus by cells which are specialized for gravity perception, and the second refers to cell structure and stability in the gravitational field and their changes in response to microgravity. Special attention is given to the rearrangements of actin microfilaments and tubulin microtubules in multicellular organs as well as in the tip-growing plant cells experiencing microgravity, under clinorotation and gravistimulation. It is assumed that the cytoskeleton takes part both in plant cell graviperception and gravisensing and helps to provide growth stability for plants. The perspectives of cytoskeleton research are outlined.

Keywords: Cytoskeleton, plant cell, gravisensing, microfilaments, microtubules

1. Introduction

The discovery of plant cell gravisensitivity, which is one of the fundamental achievements of modern biology, is based on the metabolic and structural rearrangements of cells specialized and not specialized for gravity perception

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in microgravity.[1–4] For purposes of discussion, it is useful to distinguish between cell graviperception and cell gravisensing: the first implies the active use of a gravitational stimulus by cells, specialized for gravity perception (e.g., root and shoot statocytes; Figure 1). Gravity perception results in the normal spacial orientation of plants, growth and vital activity (gravitropism, gravitaxis). While the term "gravisensing" means the structure and metabolic stability in the gravitational field for the cells which are not specialized to graviperception.[5]

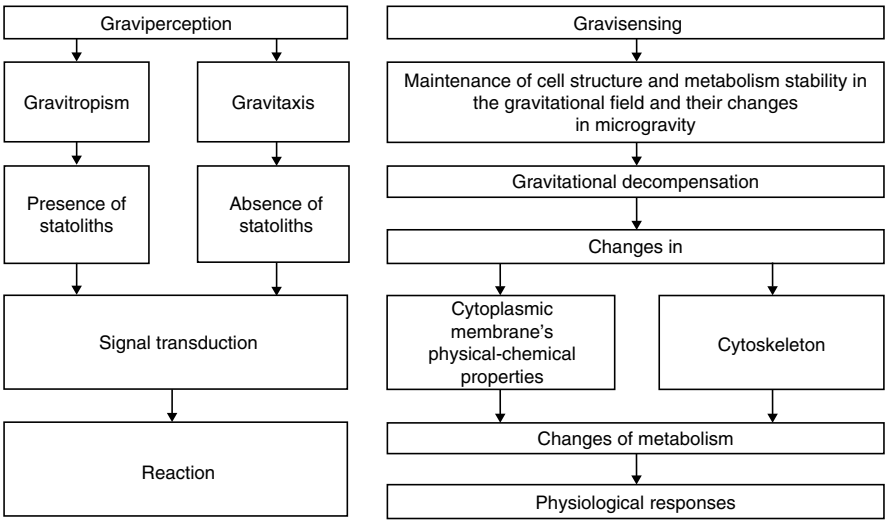

Figure 1. Difference between the terms "graviperception" and "gravesensing".

The structure of graviperceptive cells is diverse, but gravisensors are well known. These are statoliths of different types that change their position in the direction of the gravity vector and thus, initiate subsequent steps of the gravitational response.

A higher plant gravitropic reaction is a result of three main phases: perception of a gravitational stimulus, signal transduction and differential growth of graviresponsive organs. Several types of gravitropism have been distinguished in higher plants: positive gravitropism of roots growing in the direction of the gravity vector, negative gravitropism of shoots growing in the direction opposite to the gravity vector, and plagiotropism of leaves and root hairs which are disposed more or less perpendicularly to the direction of the gravity vector. In a root cap, a gravitational signal is perceived by highly specialized graviperceptive cells (statocytes), which show structural

polarity characterized by the position of a nucleus in the proximal part of the cell and amyloplasts (statoliths) and endoplasmic reticulum (ER) membranes in its distal part. In higher plants, the sites of graviperception (statocytes in root cap and in shoot endoderm) and realization of a gravitropic reaction (the elongation zone of roots) are spatially separated, while in tip-growing cells like *Chara* rhizoids or apical cells of moss protonemata, perception and response to gravity occur within a single cell.[6,7] In gravitropically responsive parts of *Chara* rhizoids, a statolithic function is ascribed to special compartments filled with crystallites of barium sulphate.[6] An apical cell of moss protonema displays a certain internal polarity, and amyloplasts are believed to perform the function of statoliths.[7,8]

Upon gravistimulation, statoliths of different types sediment to a distal location along the "lower" wall of the statocyte in the direction of gravitational vector and thus, initiate the signal reactions leading to gravitational response.[6] So called, starch-statolith hypothesis was proposed at the beginning of the last century and now it is the most accepted among the hypotheses explaining graviperception. Plants grown in microgravity, where gravitational vector is partially absent, develop a root cap but their graviperceptive apparatus does not function in the absence of a gravitational vector: amyloplasts-statoliths do not sediment in the distal part of a statocyte and spread over the entire volume of its cytoplasm.[4,9,10] Thus, the data obtained in space-flight experiments confirm amyloplast statolith function in root statocytes.

2. Cytoskeleton in graviperception

The cytoskeleton is suggested to be widely involved in modulation of cell gravisensitivity. It is a highly dynamic network of filamentous proteins traversing the cytosol, and anchored at or associated with the cell membrane and/or cortical ER. The major cytoskeletal components, tubulin microtubules (MTs) and actin microfilaments (MFs), are extremely sensitive to the variety of environmental and endogenous factors; thus, enabling plants to react quickly to changing environmental conditions.

The actin cytoskeleton is implicated in plant gravitropic reaction as a major component of signal transduction chain. Data obtained by immunocytochemical labeling have shown that root cap statocytes are uniquely organized with respect to the actin cytoskeleton; namely, they are depleted of prominent actin bundles, endoplasmic MT arrays and ER in their internal cytoplasm.[11] Observations of diffuse actin labeling surrounding statoliths suggest the presence of a population of short MFs and G-actin. Gravisensitive *Chara* rhizoids also contain thin bundles of MFs in apical and subapical zones of the cell while the basal zone contains thicker ones. Labeling of myosin in both higher and lower plants revealed the presence of

this motor protein around the statoliths (cress, maize and *Chara* rhizoids).[12] The polar arrangement of organelles is achieved and maintained by means of the cytoskeleton.[6,13,14] Because actin network participates in movement of cellular organelles and is found to surround statoliths it is proposed that MFs together with myosins facilitate statolith displacement upon gravistimulation. Such a conclusion was proposed following observations that disruption of actin MFs by cytochalasin D caused falling of the statoliths to a distal position in cells of *Chara* rhizoids and moss protonemata.[6,15] Therefore, it has been suggested that amyloplasts are anchored to elements of the actin cytoskeleton and the position of these statoliths in statocytes depends on the balance of two forces: the gravitational force and the counteracting force mediated by MFs.

Amyloplast sedimentation caused by gravistimulation could induce changes of tension within the actin network, leading to the activation of signaling reactions resulting in the gravitropic growth response.[6] However, understanding the ultimate role of the actin cytoskeleton in gravitropic growth response is still far from complete, and further study provided new but contradictory data on its involvement in gravitropic reactions. For example: in many experiments application of actin-disrupting drugs, such as cytochalasins during gravitropic stimulation of maize and cress did not inhibit gravitropic root bending.[6,16] But at the same time, it was reported that cytochalasin B (CB), cytochalasin D (CD) and another actin modulator latrunculin B (LatB) significantly inhibited the bending response of snapdragon (*Antirrhinum majus L*) flowering shoots. Other studies also show that CD inhibits or delays the gravitropic reaction in agaric fruit bodies and *Phleum* roots,[17] as well as impairing hyper-polarization of the cytoplasmic membrane in cress root cap cells.[18] In trying to explain these discrepancies, one should account for the possibility that CD could fragment rather than depolymerize MFs and partly disorganize ER, thereby, creating a massive accumulation of membranes, which slows or prevents the fall of amyloplasts and therefore, inhibits the graviresponse.[17]

The picture with the use of actin-disrupting drugs becomes even more complicated after considering a set of experiments where LatB caused promoted gravitropic curvature in *Arabidopsis* hypocotyls and inflorescence stems.[19] Enhanced curvature response was reported also for *Zea mays* roots and *Oryza sativa* coleoptiles after CB treatment.[16,20] LatB also enhanced graviresponse in *Z. mays* roots in spite of its inhibiting effect on root growth. It was noted that nanomolar concentrations of LatB caused even stronger promotive effect on maize root gravitropism.[21] Such low doses of LatB are suggested to specifically disrupt the fine and dynamic MFs in the statocytes.[12] As a result, amyloplasts “fall” with greater velocity, causing increased

gravitropic response. The localized disruption of this actin network by sedimenting amyloplast could facilitate gravity sensing in roots.[22]

It should be pointed out that, in all reports of the promotive effect of actin disruption on gravitropism, these observations were made after continuous gravistimulation (i.e., 90° horizontal reorientation). Based on this understanding, the conclusion could be made that MF disruption does not prevent the persistence of a signal established by a previous gravistimultion. Therefore, it is suggested that the actin cytoskeleton can control the duration or amplitude of an early signaling event induced by gravistimulation.[21] That was confirmed by a recent study showing the induction of extensive curvature in roots with disrupted MFs in root cap cells, even after extended clinorotation.[21]

Difference in actin cytoskeleton organization in *Arabidopsis* columella cell files could explain the discrepancies between effects of altered gravity conditions on the localization of plastids.[23] Thus, random-positioning machine and flight experiments had the greatest effect on plastid position in the flanking cells compared with the central columella cells. Such a difference could be caused by the fact that flanking cells may be more susceptible to perturbations in the gravity field because they have a more "restrained" organization of their cytoskeleton.[11] It is assumed that the cytoskeleton in these cells disrupts more readily and this lead to the greater dispersal of amyloplasts. Other experiments in *Lepidium* support this idea because they show that the flanking columella cells recovered quicker from centrifugal acceleration than did the central cells.[24]

Taken together, data on the role of the actin cytoskeleton in graviperception suggest that although amyloplasts can sediment without the actin cytoskeleton, as in a case of LatB treatment, under normal conditions they possibly move along MFs to reorient their position.[15]

When analyzing the data, including discrepancies and bearing in mind the experimental conditions, in particular the penetration of actin-disrupting drugs into root, or specific influences of actin modulators on different subpopulations of MFs causing their partial disruption and subsequent impaired graviresponse. At the same time, it might be that differences in the actin cytoskeleton organization among different plant organs, like root and stem statocytes, may determine the difference between organ gravisensitivity.[25] Thus, it can be concluded that actin cytoskeleton is a key part of a rather complex mechanism of gravisensing in both specialized and unspecialized graviperception cells.

Another component of the cytoskeleton, MTs, generally are believed to play no significant role in statolith positioning or movement in statocytes as, for example, has been shown by the use of tubulin-disrupting drugs in

Chara rhizoids.[26] At the same time, however, depolymerization of all MTs enhances the gravity-dependent mobility of statoliths in inverted cress root cap statocytes.[27] This might happen because root cap statocytes possess prominent MT arrays only in the cortical cell region.[11] The dynamic actin network surrounding statoliths, where MFs interact with putative myosin, could be relevant for the transmission of stimuli towards the cortical MT cytoskeleton and the cytoplasmic membrane.[10,11]

Another aspect of MT involvement in a gravitropic reaction is their participation in gravitropic growth response. Studies on rhizoids of characean algae and moss protonemata, as well as roots and shoots of maize, also confirm that MTs do not contribute directly to gravitropic bending.[28] However, it has been revealed that gravitropic curvature of plant organs such as stems and coleoptiles are accompanied by the reorientation of cortical MTs in epidermal cells. The same is true for gravitropically stimulated roots where MTs change their orientation in epidermis and cortex cells from transverse to longitudinal, which inhibits local growth and causes root bending.[29] The data correlate with reports showing that the outer cell layers are responding and transporting auxin, which regulates the graviresponse, and that outer cortical layers control growth inhibition leading to gravicurvature.[29]

Auxin regulation of gravitropism was primarily postulated by the Cholodny-Went theory, wherein asymmetric auxin concentration between the upper and lower parts of a root induces differential cellular growth leading to bending. Taking this into account, it is suggested that auxin depletion causes a longitudinal orientation of MTs in the lower flank of a root and that leads to inhibition of (re-)growth while non-inhibitory auxin concentrations maintain transverse MT orientation and growth in the upper flank of the bending root. However, in the same way, MTs can reorient as a result of mechanical stimulation. Therefore, it is suggested that auxin-induced MT reorientation is caused by mechanical stretching of epidermal cells during elongation, rather than by auxin action.[30] It should be mentioned, that for the gravitropic response of maize roots, MT orientation was found to be slower than gravitropic bending.[31]

Studies with tubulin-disrupting drugs have indicated that MTs are not necessary for the differential growth response to gravistimulation.[28] Application of several MT modulators (oryzalin, trifluralin, paclitaxel) had no effect on the gravitropic bending of *Antirrhinum majus* shoots.[17] However, for maize coleoptiles it was shown that gravitropism is strongly reduced by the use of another MT-disrupting drug ethyl-N-phenylcarbamate.[32] In addition, studies on gravitropic coleoptile stimulation[33] provided further evidence that MTs respond to stimulation directly and not as a result of

changes in cell growth induced by gravity. Therefore, MT reorientation seems to be a primary part of the gravitropic response, and not merely a secondary effect of the strains produced by bending.[34] Furthermore, Himmelspach and Nick[33] concluded that MT orientation could be uncoupled from growth, and that changes in growth rate are not sufficient to mimic the effects of gravistimulated MT orientation. It could be explained again by the redistribution of auxin in the epidermal cells, which can induce MT reorientation by a mechanism not involving changes in the rate of cell elongation.[33] Results of Takesue and Shibaoka[35] support this hypothesis by providing the evidence that in azuki bean epicotyls auxin can induce MT reorientation that does not change the rate of cell elongation. In support of the concept that MTs participate in gravitropic reaction directly evidences data from some ground-based experiments (see below). In the light of the Cholodny-Went theory, one can conclude that plant disorientation in relation to gravity vector can alter auxin redistribution and it also could cause MT reorganization. Nevertheless, it is quite evident that there is a complex correlation between the gravitropic growth response and MT organization.

2.1. GRAVITROPIC REACTION IN MOSS PROTONEMA

Protonemal growth is a result of elongation and division of its apical cell. The apical cells are good sensors of different exogenous factors. The moss protonemata are autotrophic structures but they can grow on medium supplemented with 0.2% glucose in the dark.[36] In white light, the protonemata grow plagiotropically and forms mats with radial symmetry. In darkness, protonemata grow negatively gravitropically forming bundles of parallel filaments.[37]

Apical cells of *Ceratodon*, *Funaria*, *Physcomitrella*, *Pottia* and *Pohlia* protonemata are known to have a specialized zone of amyloplast sedimentation.[8,38–40] In this zone, which is located behind the apical dome, amyloplasts sediment towards the lower wall of horizontally reoriented cells (lateral sedimentation). Amyloplasts can also sediment along the length of the cell (axial sedimentation), as shown by comparing the upright and inverted cells.[38,41,42]

It has been shown that MTs longitudinally extend through all zones of an apical cell, from its tip to the most distal region.[43] In some cases, the MTs seemed to converge in the apical dome, but whether these MTs continued around the dome or ended in the tip zone could not be determined. MTs appeared to be closely associated with the interphase nucleus and plastids in cells, as revealed by immunofluorescence. Often single MTs or groups of MTs were present below partially or completely sedimented plastids.[43]

In caulonemal tip cells of *Cerotodon* there are axially oriented MF-bundles exclusively located in the peripheral cytoplasm. The central cytoplasm and the vesicle-enriched dome are free of F-actin.[44] It is assumed that actin bundles are essential for polarized vesicle transport[45] and therefore, cause a directional exocytosis resulting in vectoral growth. The functional co-operation between MTs and MFs in accurately determining the position of organelles during apical cell growth is interesting, but more information is needed.[46]

2.2. POSSIBLE LINKS BETWEEN LIGHT AND GRAVISENSING

Light, especially red illumination, is a mediator operating with MF alignment in the apical dome and can shift the centrally located gap towards the illuminated apical flank before growth reorientation. This process is marked by an enhanced F-actin turnover in the tip, in which local degradation of bundles goes hand in hand with the synthesis of reoriented MFs. An elevation of the G-actin concentration could serve in return for rapid synthesis of the orientated MFs which immediately follows the partial destruction of actin and myosin. This new formation has its origin in pre-existing bundles that might help in the rebuilding of the collar-like structure.[44]

Participation of phytochrome in spore germination was indicated by experimentation with a photoreversible red-light/far-red-light system. At the same time, it was observed that the response of germinating spores to a photo-stimulus depended on other factors. For example, the phototropic effect of red light was shown to be much weaker under clinorotation than in a stationary position, though no essential differences were there in the initiation of spore germination.

2.2.1. *Microtubules reorganization during red light mediated phototropism*

Immunofluorescence showed that MTs have a predominantly longitudinal orientation throughout the apical cell (Figure 2a). Two o'clock red light illumination of the protonemata provoked depolymerization of MTs. The high (0.1–1.0 μM) concentration of oryzalin produced analogous results.[40] The changes in cytoskeleton structures caused by depolymerization of cell MT under an action of red light is suggested to be readily reversible. Thus, no tubulin structures can be seen in apical cells illuminated by red light for 2 h (Figure 2b). Some tubulin structures are seen, however, in the basal region of 4 h illuminated apical cells. Like oryzalin, red light activated apical cell sprouting i.e. forming the additional growth point at the lateral cell wall. It is remarkable that sprouting caused by red light takes place only in the illuminated region of the growing cell apex (Figure 2c).

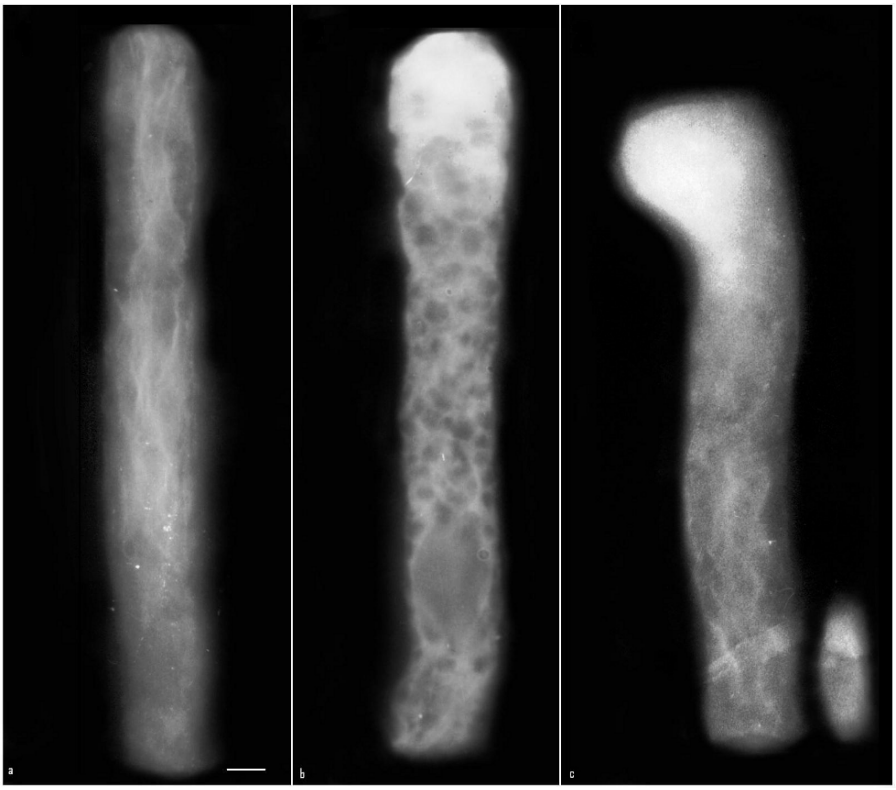

Figure 2. Microtubules in *Pohlia nutans* apical protonemal cell (a), *P. nutans* tip cell after red light illumination for 2 h (b), *P. nutans* tip cell with an apical protrusion after 4 h of red light illumination (c), bar = 10 μm.

The ability of cells to grow gravitropically was lower under the higher oryzalin concentrations. It can be concluded that gravitropism gradually recovered when intracellular physiology stabilized and repolymerization of cytoplasmic MTs took place. We found that cytochalasin B insignificantly influenced the growth rate of the protonema, but it completely blocked protonema gravitropism at concentrations of 1.0–10.0 μM. This is consistent with the conclusion that cytochalasin B selectively inhibits protonema gravitropism but can not be connected with disorganization of apical cell growth. It seems that the MT-system is not involved in the phytochrome-induced MFs arrangement, but rather is responsible for keeping this process located in the apical dome. In cases where unilateral red light was applied simultaneously with anti-MT drugs, the compact arrangement of the MFs was restored within the apex, and the extent of their light-induced reorientation was pronounced. Both effects in combination might explain why

cells treated in this way grow at right angles to a gravitational vector, directly towards to the light source, remaining tubular. In cases where irradiation was performed after a preincubation with anti-MT drugs, the reorientation proceeded in an extended area and, therefore, could not restore the collar-like structure. Meske and Hartmann[44] conclude that MTs anchor the sites in the central apex that regulate the polarity of the tip and thereby the arrangement of the apical MFs.

2.2.2. *Gravity-dependent morphogenesis of protonemata cell*

It was at first shown that the apical cells that develop in darkness are able to transform into gametophore buds under the influence of low irradiance white light[47] (i.e., 0.37 μmol m^{-2} s^{-1}). In *Pottia intermedia*, gametophore buds develop on shortened side branches in the centre of a moss mat. In dark-grown gravitropic protonemata, buds are laid down on the periphery of the mats, often from the apical cells of the main stolons. When the apical cells of such buds were transferred in darkness, they differentiated back into protonemata that grew negatively gravitropically. The morphogenetic influence of light on such processes was investigated under the conditions of clinorotation. Experiments were conducted with *Pohlia nutans.* In this species, buds formed predominantly on negatively gravitropic protonemata that subsequently were irradiated with red light. Bopp[48] demonstrated that those cells that do not reach 80 μm in length have an ability to initiate bud formation. Red light seemed to inhibit growth of apical cells and to promote bud formation. Red light might act as a trigger for morphogenetic processes in dark-grown cells. Mats of *P. nutans* protonemata without buds were gathered in a tangle and then transferred into vertically oriented dishes at 1 g on the clinostat. Protonemata grown under such conditions for 7–9 days were illuminated continuously with 660 nm red light of intensity 6 μmol m^{-2} s^{-1} for 48 h. It was shown that apical cells transformed into buds considerably more often at 1 g (Table 1).

TABLE 1. Effects of microgravity and clinorotation on bud formation in red light for 48 h, λ_{max} = 660 nm, intensity 6 μmol m^{-2} s^{-1}.

Growth conditions	Number of filaments	Number of buds (%)
Pottia intermedia		
Control, 1 g	245	19.3 ± 0.9
Microgravity	964	0
Clinorotation	489	4.2 ± 0.4
Pohlia nutans		
Control, 1 g	393	8.7 ± 0.2
Clinorotation	247	3.9 ± 0..3

In the experiment on board the space shuttle Columbia (STC-87), none of 964 *P. intermedia* protonemal filaments gave rise to gametophore buds under red light. However, under clinorotation in the gravitational field (the ground experiments), some protonemal cells (4.2 ± 0.4 %) were transformed into buds. We obtained similar results with *Pohlia nutans*. Protonemata of *P. nutans* showed a weaker morphogenetic reaction to red light in comparison with *P. intermedia*. Alternating red light for 30 min with far red for 5 min considerably modified the red light effect. Results indicate that the morphogenetic action of red light is accomplished by means of the phytochrome signal transduction system. [49]

Thus, the protonemata apical cell seems to be a specialized structure that perceives exogenous signals and transforms them into photo- or gravitropic growth as well as tends them to develop the gametophore bud. Alteration of cell shape, under the influence of light and gravity, precedes the differentiation of a bud by an apical cell.

In the *Pohlia* apical cells, MFs formed a delicate network. Bright fluorescence of MFs was observed in the apical dome, which suggests an association between MFs and tip growth (Figure 3a). At about 20–30 μm behind the cell tip, MFs are arranged in thin bundles, predominantly longitudinal to the cell axis. Among thin strands, a thick longitudinally oriented strand was visualized. Bright fluorescence of MFs was at first noted in the apical dome, sometimes at the some distance from the cell tip, during early bud differentiation (Figure 3b). During the next step of bud differentiation, a local patch of MFs was observed in bud cells (Figure 3c, d). Bud cells and tip-growing protonema cells might possess a local site of growth established by the spatial organization of actin MFs. The displacement of amyloplasts during gravity-induced sedimentation might be regulated by MFs.

Figure 3. Microfilaments in dark-grown protonemata (a), microfilaments at the primary stage of bud differentiation (b) and later (c, d), bar 10 μm.

It seems likely that the whole membrane-cytoskeleton complex of a cell is involved in the responses to vectoral stimuli that determine the morphological and physiological polarities of the organism.

3. Cytoskeleton in gravisensing

In most cell types not specialized for the perception of gravity, the primary sensors are not clearly defined. The idea of positional homeostasis proposed by Nace,[50] was the first to focus on the role of the cytoskeleton in cell graviresponse. It postulates that the fixed stable position and optimal cell orientation in the gravitational field is a state of mechanical stress for the cytoskeletal elements that maintain cell membrane integrity. A significant role in stability of cell spatial-temporal organization in the gravitational field is suggested for the cytoskeleton from studies on cell gravisensing: static stimulation,[6] passive gravistimulation,[51] protoplast pressure,[52] putative tensegrity,[53] and restrained gravisensing.[11] The cytoskeleton is known to participate in cytoplasmic streaming and organelle motion, mitotic division, cytokinesis, and endo- and exocytosis, as well as in intracellular transport of substances. All these activities are potentially gravity sensitive through the cytoskeleton.[54] The intracellular cytoskeleton, the extracellular matrix, and the cytoplasmic membranes are assumed to represent compartments of sufficient macromolecular organization to be sensitive to gravity-induced phenomena.[2] The cytoskeleton and extracellular matrix are indispensable for cellular and developmental processes that are directly or indirectly linked through the cytoplasmic membrane as an intermediary between them.

In addition, recently it has been found that actin cytoskeleton could be involved in plant cell gravisensing through mechanism other than amyloplast sedimentation. There is an investigation that suggests, besides the root cap, roots may have another gravity sensor, probably located in the zone of elongation.[55]

It has been shown in the experiments with tip-growing single cells like root hairs, that they react to changes of their position with respect to a gravity vector through MF rearrangement. Thus, as with the results from clinorotation, the orientation of root hair in relation to the root axis changed from 90° to approximately 45° in the earliest stages of root hair development.[56] With further growth, the root hair restores its plagiotropic 90° orientation. It is proposed that reorientation becomes possible due to a shift of the exocytosis site, occurring as a result of altered gravity signal perception. MFs in tip region of hairs are responsible for localized exocytosis and tip growth, their rearrangement in this area can shift the exocytosis site to the new place in the apical cytoplasmic membrane thus, creating the new site of vesicle localized delivery and consequently establishing new direction of growth.[56]

Based on the data on a disappearance of two actin isoforms in wheat mesophyll protoplasts after 6 min of the microgravity action from four revealed before the experiment, the presence of specific actin isoforms responsible for certain functions related to gravisensing is suggested.[57] A dependence of MT self-organization *in vitro* on the gravitational force was recently revealed.[58] Flight results also suggest that polymerization of actin and tubulin into MFs and MTs, known to be regulated by Ca^{2+} ions,[59] may be altered in microgravity.[60]

Experiments conducted under clinorotation, where direct affect of the gravity vector is excluded, showed that the MT cytoskeleton is also sensitive to altered gravity. Thus clinorotation caused to some extent random MT organization in epidermis and cortex cells of *Beta vulgaris* seedling roots.[61] In clinorotated samples, patterns of MTs looked more variable. Even new, longitudinally-oriented MT arrays were noted in the meristem. The majority of MT bundles did not span the entire cell (Figure 4). In the postmitotic transition region MTs seemed to fan out from one point and some of them deviated from the transverse orientation (observed in control) approximately up to 45°.

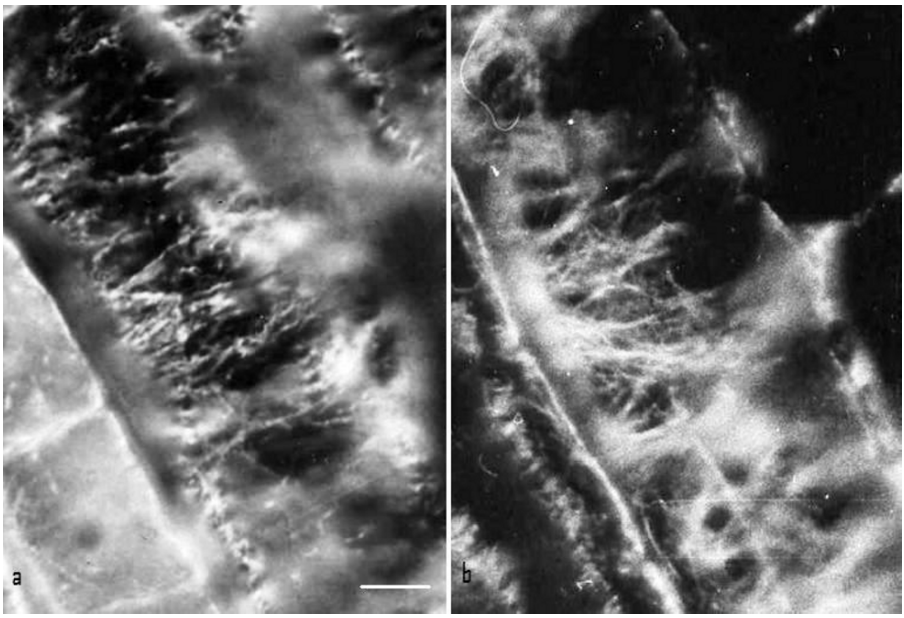

Figure 4. Patterns of cortical microtubules in epidermal and cortex cell files in the *Beta vulgaris* root elongation zone in the control (a) and under clinorotation (b), bar 10 μm.

Experiments on *Brassica rapa* (Figure 5) and *Arabidopsis thaliana* seedlings. carried out under clinorotation confirmed that the MT cytoskeleton is sensitive to altered gravity.[62,63] In the stationary conditions, cortical MTs

in root apex cells demonstrate certain changes in their pattern during cell growth and differentiation. In the meristem, cortical MTs are plentiful and have predominantly a transverse orientation relative to the long axis of the root. In the distal elongation zone, MTs are not such numerous and transversely aligned in the parallel arrays.

At the end of the central elongation zone, cortical MTs gradually shifted from transverse to an oblique orientation. Recent studies indicated that the dynamic status of the cortical MT array depends upon the developmental state of the cells, i.e. their level of differentiation.[64] Thus, the cortical microtubules within recently subcultured tobacco cells are highly dynamic (as inferred by their cold-sensitivity) but relatively stable as the culture begins to reach stationary phase.[65]

In clinorotated *B. vulgaris* seedlings, the patterns of MTs appeared more variable. Even new longitudinally-oriented MT arrays were noted in the meristem. In the distal elongation zone, MTs seemed to fan out from one point and some of them deviated from the transverse orientation up to approximately 45°.[61] An appearance of shorter, variably oriented cortical MTs was also observed in the distal elongation zone of *B. rapa* and *A. thaliana* (GFP-MAP4) seedling roots under clinorotation.[62,63]

Cortical MT array patterns in the distal elongation zone is presumed to be influenced by altered gravity due to special physiological properties of cells in this zone. Cells of the distal elongation zone, located between the apical meristem and the distal part of rapid cell elongation region, accomplish a developmental transition from cytoplasmically driven expansion to vacuole-driven elongation.[66,67] Cells of this zone are characterized by specific sensitivity to different external and internal stimuli such as auxin, ethylene, extracellular calcium, aluminium, mechanical pressure, and gravity,[68,69] and, probably, they contain highly dynamic cortical MT arrays. Simultaneously, a significant decrease in a cell length in the central elongation zone was revealed (Figure 6). The decline of anisotropic growth, that is typical for these cells, is suggested to be connected with cortical MT disorientation under clinorotation.

Observation on the cell wall showed that they were wrinkled more often than in control. Other investigations in the field of root gravisensing revealed that under clinorotation the thickness of longitudinal cell walls of peripheral root tissues is decreased compared with control roots.[3] This may result from the altered MT arrangement. Thus, MTs are presumed to be connected *via* MAPs with the cellulose-synthesizing complexes, which move within the plasma membrane and generate the microfibrils of the cell wall.[70] Changes in MT orientation may impact the movement of the cellulose-synthesizing complexes and processes of cell wall construction. In

turn, the loss of cell wall thickness may lead to alterations in growth rate and or direction.[61]

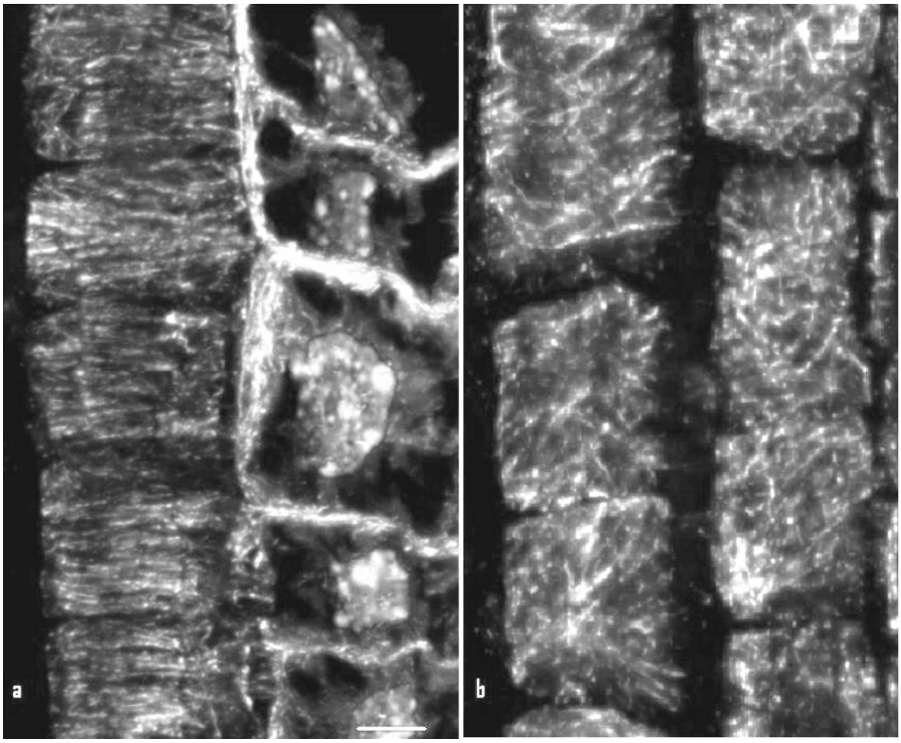

Figure 5. Microtubules in epidermal and cortex cells of the distal elongation zone (DEZ) in *Brassica rapa* roots in the control (a) and under clinorotation (b), bar 10 μm.

Microtubules are growing at each end by the polymerization of tubulin dimers, and are shrinking at each end by the release of tubulin dimers.[71] This dynamic instability makes them sensitive to MT disrupting agents. To monitor total changes in MT dynamics, the sensitivity of MTs to oryzalin was used as an indicator. We determined how oryzalin affects MTs in the control and after clinorotation, and showed that cortical MTs reveal a different level of disruption depending on the time of treatment and the root growth zone. MTs were the most sensitive to oryzalin in cells of the distal elongation zone and least sensitive in the central elongation and mature zones. In clinorotated seedlings, oryzalin action was similar to that in the meristem and the central elongation zone of control seedlings, excluding the distal elongation zone. Unlike control, 15 min of oryzalin treatment caused the rapid disappearance of shorter disoriented cortical MTs in the distal elongation zone under clinorotation (Figure 7).

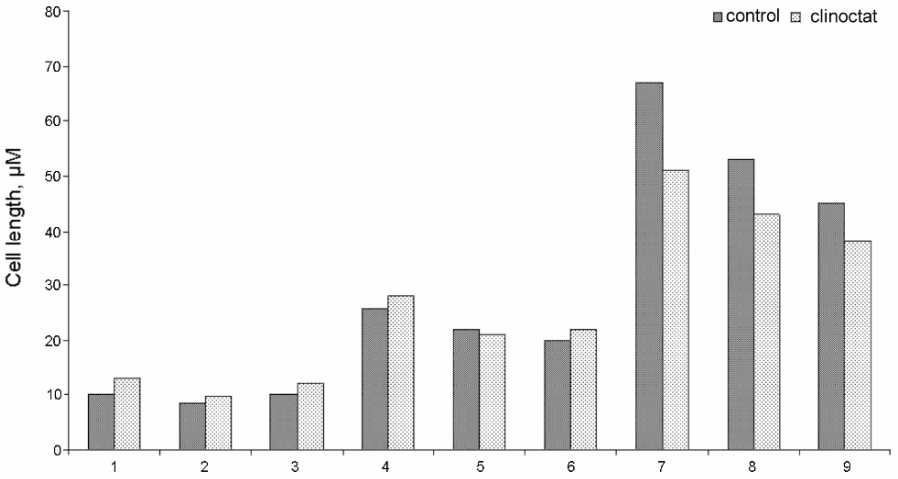

Figure 6. Cell length in the different growth zones of main root of *B. rapa* seedlings. 1, 2, 3 – meristem; 4, 5, 6 – distal elongation zone; 7, 8, 9 – central elongation zone; 1,4,7 – epidermis; 2, 5, 8 – subepidermal layer of cortex; 3, 6, 9 – cortex.

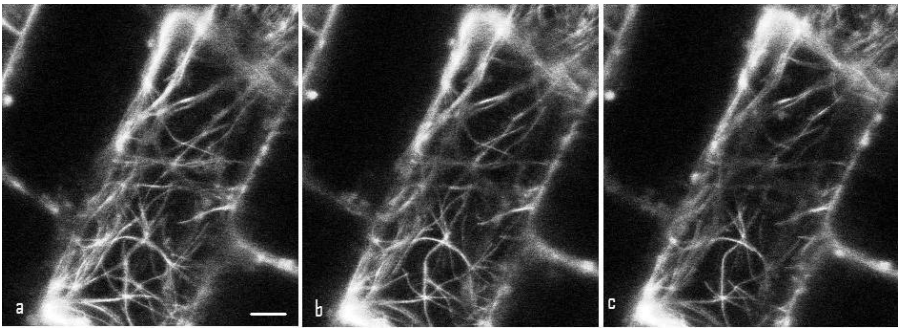

Figure 7. Rapid depolymerization of disoriented microtubules in clinorotated seedling of *A. thaliana* -GFP–MAP4 (5 μM oryzalin treatment during 5, 10 and 15 min), bar 10 μm.

Hypersensitivity to tubulin-targeted drug is an indirect consequence of altered polymer status in appeared MTs. As far as oryzalin blocks the addition of tubulin dimers to the growing end of a MT, a dynamic MT with high turnover will be more sensitive to such drugs as compared to a stable microtubule with a low rate of dimer exchange. MAPs are regulators of tubulin turnover rate in cortical MTs, which stability is generally believed to depend on the activity of structural MAPs that decrease the frequency of MT catastrophe.[72,73] The *Arabidopsis* MAP MOR1 could be one of the proteins involved in this process, as mor1 mutants have abnormally short

microtubules in interphase cells, probably as a result of altered dynamic instability.[74–76] It was shown that interphase MTs originate *de novo* along the cell cortex,[77] and they exhibit rapid growth and shrinkage at their plus ends and slow intermittent depolymerization at their minus ends. This 'hybrid treadmilling' drives MT repositioning and the reorganization of MT arrays.[77] So, an increase in the rate of MT dynamic turnover may underlie the rearrangement of MT cortical arrays in cells of the distal elongation zone under clinorotation.

4. Interrelations between cytoskeleton elements during gravisensing

Investigation of an *Arabidopsis*-GFP-MAP4 line have shown that CD treatment resulted in distortion of MT arrays and formation of bright spots randomly distributed through the meristem and the DEZ (Distal Elongation Zone; Figure 8). In comparison with the meristem, spots in the DEZ were less numerous and no spots were observed in the CEZ (Central Elongation Zone; Figure 8b, e). In clinorotated roots localization of spots was similar to the control, but their number in the meristem and DEZ was even lower (Figure 8c, f).

The same bright spots appeared in *Arabidopsis* roots following oryzalin- or amiprophosmethyl-induced MT depolimerization.[64] Additionally, measurements of cell parameters have shown that CD significantly affects the length and width of DEZ cells in the correctly-oriented control roots (Table 2). In contrast, CD applied under clinorotation does not change cell size to a great extent (Table 2).

Because MAP4 decorates microtubules and provides polymerization and stability of MT network,[78] formation of MAP4 clusters leads to the distortion of MT arrays. The observed random spots are composed of clusters of disrupted microtubules. Appearance of disrupted MTs after CD treatment serves as the basis for the conclusion that MFs contribute to the stabilization of MT arrays during cell differential growth in the meristem and the DEZ. This is in agreement with the observations of others.[79] The fact that spots were observed primarily in cells growing isotropically, and disappeared in cells growing anisotropically, supports the difference in interrelation between MTs and MFs performed under different growth types. It is likely that cooperation between MTs and MFs is important for isotropic growth in the meristem and less important in the DEZ where it starts to change for anisotropic, and probably not so essential in the CEZ, where growth is strictly anisotropic. The interrelation between MTs and MFs also might depend upon the type of tubulin cytoskeleton.

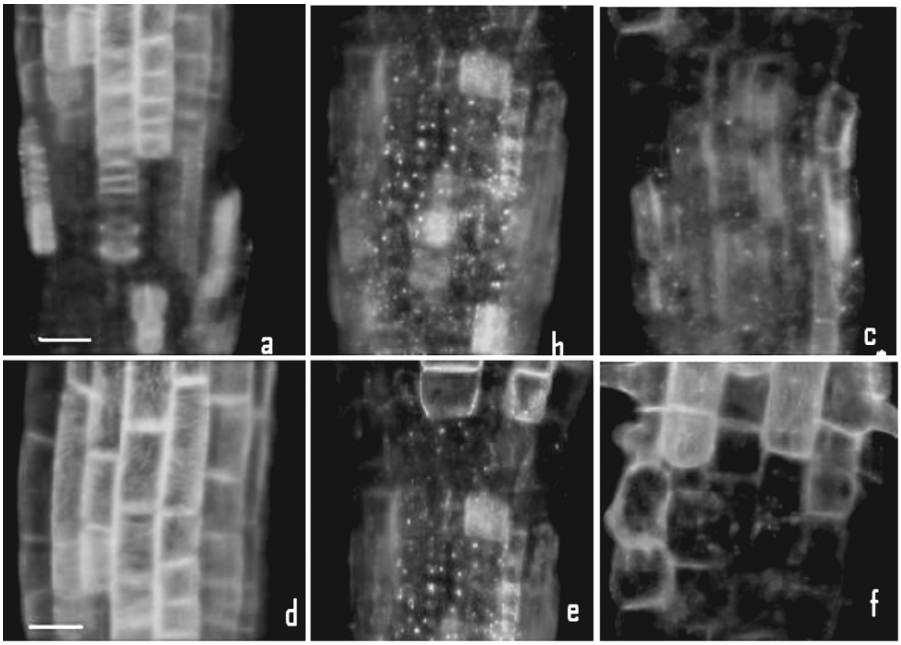

Figure 8. Distal (DEZ) and central (CEZ) elongation zones of *Arabidopsis*-GFP-MAP4 roots in the untreated cells (a, d) and in cells after cytochalasin D treatment: control (b, e) and clinorotation (c, f), bar 20 μm.

TABLE 2. Cell parameters in the distal elongation zone (DEZ) of *A. thaliana* roots.

Cell parameters (μm)	Untreated	
	Control	Clinorotation
Length	14.37 ± 0.51	14.06 ± 0.40
Width	14.42 ± 0.31	14.11 ± 0.30
	Cytochalasin D treatment	
	Control	Clinorotation
Length	17.33 ± 0.86	15.04 ± 0.72*
Width	17.34 ± 0.94	14.6 ± 0.53*

*Statistically significant difference at $p \leq 0.05$.

Thus, numerous spots in the meristem and the DEZ means that intact MFs are of great importance for functioning of endoplasmic MTs which predominate in these root sites. Progressive decrease of spot number through the DEZ and their absence in the CEZ coincides with the appearance of prominent cortical MTs; implying that once cortical MTs are stabilized, they become less dependent upon the microfilaments. Damage to MT organization after MF disruption, as well as the increase of cell parameters,

allows one to conclude that the actin cytoskeleton is a key player in the process of growth restriction[80] and regulates it via affecting MT alignment. Surprisingly, under clinorotation the number of spots was significantly lower in all root tissues, and CD treatment does not result in a great effect on cell size. Decrease in the number of spots, in our opinion, proves the existence of complex interrelations between MTs and MFs under the above condition. Thus, based upon data obtained, two main conclusions can be made. One is that MTs and MFs exhibit different organizations at the different stages of cell growth in the DEZ of *Arabidopsis* roots. Cross-talk between MTs and MFs is important for isotropic growth and less important for anisotropic growth. Stabilization of anisotropic growth, probably, is driven by MTs and that is derived from observations that long-term absence of cortical MTs in root cells prevents any anisotropic growth and hence morphogenesis.[80] The second conclusion, or rather a hypothesis, is that because growth stability has been evolutionary established on earth under constitutive g, MTs and MFs can be a part of the default mechanism of growth coordination, which is probably unmasked in altered gravity.

Also, we observed visual enhancement of the actin cytoskeleton in the DEZ of *Beta vulgaris* roots.[81] At the same time, actin expression under clinorotation was stable and there were no changes in the total protein spectrum, the actin quantity, nor the level or localization of actin gene expression (i.e., mRNA transcription rate).[82] The visual F-actin cytoskeleton enhancement in cortex cells of the DEZ occurred under clinorotation at the same level of total actin content as in the stationary conditions, and this is suggested to be caused by transformation of G-actin into F-actin.

5. Conclusions

Summarizing all known aspects of plant cell gravisensitivity it is worth noting that the cytoskeleton is definitely a key player in signaling events caused by gravity. But, in spite of much discussion on cytoskeleton function in a gravitropic reaction (graviperception and graviresponse)[10,11,21] there is very little experimental data to support that conclusion. Moreover, general acceptance of a role for MTs and MFs in graviresponsive signal transduction is still absent.

Mutually dependent functioning of cytoskeleton elements is revealed only under clinorotation. Such compensation is hypothesized to provide the cell with growth stability in altered gravity and may be considered an adaptive feature. Therefore, elucidation of cytoskeleton involvement in cell graviperception, gravisensing and gravity signal transduction is still a great challenge for molecular and cell biology.

The following questions are of a special interest:

1. Whether microgravity affects the cytoskeleton directly or not
2. Through what signaling pathway does altered gravity affect both the molecular organization and dynamics of actin MFs and tubulin MTs and expression of cytoskeletal protein genes
3. What associated proteins support cytoskeletal reorganization during gravitropic reaction
4. What future spaceflight and clinostat experiments will be necessary to define the cytoskeleton's role in plant cell gravisensing and graviperception
5. What objects are the most convenient for such experiments; and
6. What cytological, biochemical and molecular-biological methods should be employed to obtain complete information about the cytoskeleton's involvement in plant cell gravisensitivity

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PART 4

GENOMICS OF THE CYTOSKELETON

THE IMPORTANCE OF BEING AN INTRON, BY WILD....TYPE TUBULIN GENES

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Abstract. Introns of plant tubulin genes are important regulatory elements for supporting gene expression. Introns can either increase the level of expression through intron-mediated enhancement (IME), or they can change the actual site of gene expression. The molecular basis of both IME and intron dependent spatial expression (IDSE) remains largely unknown. IME and IDSE seem to be regulatory traits common to many cytoskeletal genes. Introns of tubulin genes are also useful tools for genotyping plant species and varieties, for parental assessment and for assisting breeding programs. This can be done with Tubulin-Based Polymorphism (TBP), a specific intron length polymorphism-based method that exploits both length variation and positional conservation of the introns of the plant beta-tubulin gene family.

Keywords: Tubulin genes, spliceosomal introns, IDSE (intron-mediated enhancement), IME (Intron Mediated Enhancement of gene expression), ILP (Intron Length Polymorphism), genotyping, TBP (Tubulin Based Polymorphism)

1. Introduction

Genomic studies have begun to shed light on the importance of non coding sequences such as repetitive elements, pseudogenes, antisense sequences and introns. Spliceosomal introns, confined to the nuclear genome of all eukaryotes, occupy a significant proportion of the whole genome. The range of intron densities in annotated eukaryotic genomes spans more than three

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orders of magnitude from about 140,000 introns in the human genome to a total of only 15 introns in some microsporidia.[1] In humans, introns account for approximately 95–97% of the average protein-coding gene which means that although protein-coding sequences occupy only about 1.5% of the human genome, at least one-third of the genome must actually be transcribed. Hence, we are dealing with a system where overall RNA transcription is overwhelming RNA translation, indicating the importance of an RNA-based regulatory system. The discovery of microRNAs and the involvement of RNA interference (RNAi) in developmental pathways further support this prediction.[2–4]

Non-coding RNA (ncRNA) genes increasingly are being identified and many of them are associated with the development of human diseases (Mattick[5] and references therein). They may originate from a vast amount of transcripts that do not contain any significant open reading frame. Figures are indeed impressive: out of 40,000 human genes, each containing an average of 8.4 introns, there are about 8,000 (20%) associated antisense transcripts and around 20,000 pseudogenes. The transcriptionally active, non-coding part of the genome is therefore an impressive task force.

1.1. SPLICEOSOMAL INTRONS: EVOLUTIONARY GENOMICS ASPECTS

Intron-dense and intron-sparse species are interspersed throughout the eukaryotic tree of life.[1] Regardless of the species of reference, spliceosomal introns have quasi-random nucleotide sequences and generally lacks ORFs. Their lengths vary widely between species, from just ten bases in some protists to hundred of kilobases in mammals.[6] In plants, there are two to four introns per gene with an average length of 200 bases.[7,8] Data available from the whole genomic sequence of *Arabidopsis thaliana* and of *Oryza sativa* reveal that the former has 4.38 introns per gene while the latter has 3.[9] This further confirms that also in plants, the sizes and complexity of the genome is not linearly related to the number of introns, because the rice genome is about four times bigger than *A. thaliana*'s.

Yet, the analysis of 684 KOGs (Eukaryotic clusters of orthologous genes) for six different species (Humans, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Arabidopsis thaliana*) comprising a total of 21,434 introns, shows a statistically significant proportion of introns observed in the same positions.[10]

The finding that *A. thaliana* shares more intron positions with humans than with the other four species, and the related evolutionary parsimony analysis, suggest that ancestral introns (possibly present in the Last Universal Common Ancestor) have been conserved to a large extent in plants and vertebrates while eliminated in fungi, nematodes and

arthropods.[10,11] These observations show that intron locations are not suitable markers for phylogenetic analysis that extends over long evolutionary distances. This is certainly true for the alpha- and beta-tubulin genes where intron position is not the same between plants, animals and fungi. In fact tubulin intron position is conserved among different plant species but varies when plants are contrasted with vertebrates, invertebrates, algae, fungi and protists.[12] Within the alpha- and beta-tubulin genes of theses different eukaryotic lineages, 19 and 33 are the overall number of conserved sites of intron positions, respectively. There are few exceptions to this pattern of substantial conservation, the more remarkable being the hypervariable and highly divergent intron-exon organization found in the chordate *Oikopleura dioica*.[13] On the contrary, in plants intron position is fairly well retained within the alpha- and the beta-tubulin genes, to such an extent that the introns of the latter are used as target sequences for the detection of species- and variety-specific intron length polymorphisms in the combinatorial tubulin-based polymorphism method (see below).

Recent genomic studies show that the rates of IGL (intron gain or loss) differ significantly between eukaryotic genomes. Analyses of data concerning paralogous gene families indicate that intron gain has largely prevailed over intron loss.[14] Evolution of introns in eukaryotic genes did not follow the species tree. Yet, in multi-cellular organisms the proportion of protein-coding sequences declines as a function of complexity and is only a small proportion of the genomic programme of complex organisms.[5] This is the reason why those programmes that aim to construct artificial chromosomes from chemically synthesized sequences are bound to failure when approaching organisms more complex than a bacterium. In fact, prokaryotes contain less than 25% of non coding DNA, chiefly promoters and regulatory sequences. In contrast, simple eukaryotes have between 25% and 50% non-coding DNA (*S. cerevisiae*) and more complex fungi, plants and animals have more than 50%. This percentage of non-coding DNA rises to approximately 98.5% in humans.[5] The tubulin gene family across all the eukaryotic lineages represents an interesting case for studying IGL. Tubulin intron position is somewhat in the middle between the 24% of human introns that are at the exact same positions as intron in the orthologous gene of *A. thaliana* and the 20–68% of introns that are specific for a given species.[10] This nonlinear intron dispersion is an important component of genome adaptation.

1.2. SPLICEOSOMAL INTRONS: REGULATORY ASPECTS

Introns fulfil the three essential conditions reported for a system capable of connecting, in variable combinations, elements of a common network for a

multitask purpose. These are: (1) multiple output in parallel with gene expression; (2) large numbers, many choices; and (3) potential for specific targeted interaction that may involve regulatory molecules as short as 20–30 nucleotides, capable of sufficient specificity for homology-dependent or structure-specific interactions.[7]

Once thought of as non-functional sequences, interesting only for molecular evolutionary studies, introns have now gained increasing interest for the regulatory aspects in which they are shown to be involved and for the significance of non-coding nucleotide variation.[1]

Besides the well known involvement in alternative splicing, that comprise no less than 40% of plant genes and 60% of human's,[9,15] new roles for introns at both the RNA and DNA level continue to be discovered in what is a very active field of investigation.[9] They may influence protein variability and many aspects of mRNA metabolism, including intronic transcripts, the function of which is still to be thoroughly investigated.

Mirtrons, precursors of microRNA molecules (pre-miRNAs/introns), are a fact. They provide an alternative pathway for miRNA biogenesis. Mirtron production is independent upon Drosha (an RNase III dsRNA-specific endonuclease) cleavage because it is based on the debranching of the mirtron lariat and subsequent folding of the processed RNA into a pre-miRNA.[16] Their number and sequence conservation among species have suggested that they have been the progenitors of the Drosha-dependent miRNA mechanism of production. Mirtrons are precursors of miRNAs that are involved in regulatory events, chiefly silencing. Mirtrons can direct miRNA-like gene repression.[16] They may originate from the outer edges of an intron via formation of miRNA:miRNA duplex. Intronic miRNAs can results from an intron-based transcriptional unit independent from their host protein coding gene and can be part of a negative regulatory feed-back mechanism controlling the same cognate coding sequence.[17] The majority of human miRNAs (22 nt) are located within intronic regions and are transcribed by RNA pol II as part of their hosting transcription unit.[18] miRNA have also been discovered in plants. Their processing could similarly bypass one of the RNase III cleavages[19,20].

So, the RNA regulatory system, to which introns give a substantial contribution, cannot go unnoticed and neglected anymore. On the contrary, it must be regarded as one of the major sources of evolutionary diversity of complex organisms, even more so when key steps such as the cytoskeleton structures were formed. In view of all this, it is certainly true that nowadays introns can no longer be regarded as "junk" DNA, as they were at the time dominated by the "central dogma". Rather, introns are increasingly considered as functional elements. Recent discoveries about the patterns

and causes of intron evolution make this one of the most hotly debated and exciting topics in molecular evolutionary biology today.[1]

Intron length may also be a factor with respect to gene regulatory aspects (shown for nematodes and flies[16]). In fact intron length may vary remarkably in different organisms and it influences the probability of new mirtrons arising during evolution. Intron length variation represents also a convenient tool to identify DNA polymorphisms in comparative molecular biology. Intron length polymorphisms can actually be exploited as genetic markers once the limit of the long evolutionary distances, previously reported, is respected. Actually, they have been successfully utilized in population genetics surveys and gene mapping. We will provide several examples below when describing the tubulin-based polymorphism (TBP) method.

2. Introns in cytoskeletal genes regulate gene expression

As already mentioned, introns may have different ways to influence eukaryotic gene expression. They can enhance transcription, can contain promoters and transcription start sites, and can favor the production of miRNAs. In addition they may influence different steps of the cascade that leads to protein production including mRNA differential expression, stability, transport and translation efficiency.[21]

2.1. INTRON-MEDIATED ENHANCEMENT OF GENE EXPRESSION: IME

Besides alternative splicing, the most well known regulatory event that involves introns is Intron-Mediated Enhancement (IME) of gene expression.[22,23] To this regard, the figures can be impressive: i.e. in the yeast *S. cerevisiae* only 3.8% of the genes contain introns but these genes account for 27% of the total mRNA.[24] This is also in accordance with the finding that IME correlates with increased steady state level mRNA.[25,26] As a consequence, an intron capable of IME (i.e. maize ubiquitin1 and ADH1) is often used in plant transformation to achieve high transgene expression. In fact, vectors are now commonly designed with a regulatory intron placed in close proximity of the promoter and other additional 5' regulatory motifs. Despite its frequent occurrence, the mechanism by which IME works remains elusive. Few are the certainties: in IME, introns work in close proximity of the promoters, that is that they do not act as true transcriptional enhancers. An intron's influence on the level of gene expression cannot be directly associated with any specific features of the nucleotide sequences; that is, no specific signature can be defined besides a generic AU enrichment that may be as high as 65–67%. However, different introns

placed in the same position show different enhancing effects. The importance of splicing and of the nearby gene sequence context are additional issues that have not found any conclusive evidence yet. So, IME remains an important issue to be further and more deeply investigated by molecular biologists.

All this being said, it remains an interesting observation that in plants introns are often reported to be involved in the enhancement of the expression of cytoskeletal genes or to be essential for supporting their strong and constitutive expression (Table 1). This has been the case for rice alpha- and beta-tubulin genes,[27–30] for maize actin intron 3,[31] for petunia actin depolymerising factor1,[32] for *Arabidopsis* profilin[33] and for *Arabidopsis* actin genes.[34] This indicates that introns of cytoskeletal genes may have co-evolved by maintaining a common regulatory function that, nevertheless, seems to be exerted in a somewhat different way.

The work on Arabidopsis actin shows that the leader intron of the ACT1 gene is required to sustain high level expression in pollen, and that this function could not be replaced by the leader intron of ACT2. This suggests a differential role of introns in actin gene expression.[34]

The work on rice alpha-tubulin shows that the first intron present in the coding region of each of the three isotypes (*OsTua*1, *OsTua*2, *OsTua*3) is capable of IME in transient transformation assays.[27,29] Introns 2 and 3 of *OsTua*1 are also capable of sustaining increased levels of expression but to a lower extent, compared to intron 1.[27] In addition, transgenic rice plants expressing GUS under the control of the *OsTua*1 promoter exhibited a strong and constitutive expression in vegetative tissues when intron 1 was present.[27] In transient transformation assays, intron 1 of *OsTua*1 can increase the strength of the heterologous 35S-CaMV promoter[27] and its activity can be replaced by any intron 1 from the other two rice alpha-tubulin isotypes.[29] All these experiments demonstrate IME for the first introns of all three rice alpha-tubulin genes.

2.2. INTRON DEPENDENT SPATIAL EXPRESSION: IDSE

IME can also be sustained by the leader intron of rice isotype beta-tubulin16 (now *OsTub*4 according to the recent nomenclature of Yoshikawa[35]) and rice isotype beta-tubulin 6.[30] The leader intron of *OsTub*4 is of special interest because it contains the promoter and the transcriptional start sites for two low abundance mRNAs of as yet unknown functional significance.[28] This suggests that one of the possible molecular mechanisms by which an intron can influence gene expression is to promote the synthesis of alternative transcripts. Some clues to this have been recently found monitoring GUS expression in transgenic rice plants transformed

with the upstream regulatory region of *OsTub*4 depleted of the leader intron (D. Breviario, 2007). Compared to plants that carried the whole regulatory region, including the leader intron, GUS expression was altered, but not necessarily lower, and, more importantly, displaced. This means that GUS expression was redirected in different organs and tissues (D. Breviario, 2007).

Recent works on the first intron of members of the *Arabidopsis* profilin gene family[33] and the petunia actin-depolymerizing factor[32] further address the issue of intron-dependent spatial expression. In fact, replacing the first intron of the reproductive profilin PRF5 gene with the first intron of the vegetative PRF2 profilin, GUS expression in transgenic *Arabidopsis* plants changed correspondently, indicating a distinct functionality for these two introns.[33] Also, not all of the first introns of the five profilin genes were capable of IME, reinforcing the concept for distinct functions.

Similar findings (i.e. intron-dependent spatial expression) were obtained by the same authors by producing transgenic *Arabidopsis* where the first intron of the PRF5 gene, known to be expressed in reproductive tissues, was replaced with the first intron of the petunia actin-depolymerizing factor 1 gene (*PhADF1*). In these transgenic plants, GUS expression was redirected to vegetative tissues.[32] It was suggested that this alteration of the expression pattern may result from mechanisms acting at post-transcriptional level. Altogether, these experiments show that regulatory introns may influence not only the level, but also the site of gene expression. So, we propose the term of IDSE (Intron-Dependent-Spatial-Expression) for the former of these two possibilities.

2.3. PREDICTABLE FUNCTIONAL VERSATILITY OF THE INTRONS OF CYTOSKELETAL GENES

From the literature, we should have suspected the great versatility of action that introns of cytoskeletal genes can display. In 1990 in the yeast *S. cerevisiae*, it was shown that the intron of the actin gene (ACT1) contained a promoter and transcription start site for producing an 840 nucleotide antisense RNA (ASR1) complementary to the 5' leader and the first ten nucleotides of the coding sequence of ACT1. The presence of the ASR1 transcript confers growth rate advantage to the yeast strains.[36] This was the first clue for the presence of naturally occurring antisense RNAs in cytoskeletal genes, and indicated the possibility that introns can be transcriptionally active, and therefore can directly contribute to gene regulation. This is reminiscent of what we have just described for the leader intron of rice *OsTub*4.[28]

TABLE 1. Cytoskeleton-related genes for which IME or IDSE effects have been reported.

Gene	Effect	Experimental system	Reference number
Maize actin	IME	Transient assays, cultured cells	31
Arabidopsis actin[a] ACT1	IME	Transgenic plants, reproductive tissues	34
Rice α-tubulin1	IME	Transient assays,	29
OsTua1	IME	vegetative tissues	27
Rice α-tubulin2	IME	Transient assays	29
OsTua2	IDSE	Transgenic plants, vegetative tissues	D. Breviario (2007)
Rice α-tubulin3	IME	Transient assays	29
OsTua3	IME	Transgenic plants, reproductive tissues	D. Breviario (2007)
Rice β-tubulin4[a]	IME	Transient assays	28
OsTub16	IDSE	Transgenic plants	D. Breviario (2007)
Rice β-tubulin 6[a] Ostub128	IME	Transient assays	30
Arabidopsis profilin1 PRF1	IME	Transient assays	33
Arabidopsis profilin2	IME	Transient assays	33
PRF2	IDSE	Transgenic plants, vegetative tissues	
Petunia ADF1	IDSE	Transgenic plants, vegetative tissues	32

[a]Leader intron

2.4. ADDITIONAL PREDICTABLE REGULATORY FEATURES?

In view of these data and recent findings about mirtrons, analysis of the untranslated sequences of some plant tubulin genes should be reconsidered. In fact, analysis of the 5'-untranslated region (5'UTR) present in the three rice alpha-tubulin genes shows the occurrence of a biased nucleotide sequence composition together with complex arrays of repeats.

For example, the 5' UTR of the *OsTua1* gene is highly enriched in C residues that account for 48.28% of the total nucleotides. This unusual nucleotide composition results in the presence of several direct repeats: CCTCC (three times), CTCTCT (twice) and CCGCCG (twice). In addition, a stem and loop structure can originate from the interaction of the AGAGAAA and the TTTCCTCCT sequences. Cytosine is also the predominant nucleotide in the 5'untranslated sequence of *OsTua2* at

53.57%. This sequence is similarly enriched for different di- and tri-nucleotide motifs. Worthy of mention is the presence of six CCGCC direct repeats. G and A are the nucleotides most commonly present in the 5'UTR of *OsTua3*. Together they account for 81.25% of the total nucleotides. In this 5'UTR region the tetranucleotide GAGG is repeated 12 times, the direct repeat GAGAGG(A/C)GAG three times and twice for the GGAGGAG repeat. To this regard, the preferential complementary enrichment in CT and GA dinucleotides of the 5'UTR of *OsTua1* and *OsTua3*, respectively, determines a 60% match pairing of the two sequences (42 nucleotides out of 73), a finding that deserve further study.

Besides their uniqueness, an interesting point concerning these motifs is that no corresponding features are found in the upstream region of the rice beta-tubulin genes, three of which show instead the presence of a leader intron with a potential regulatory role in gene expression, ascertained for at least two of them (*OsTub4* and *OsTub6*). This calls for a different organization of the alpha- and beta-tubulin sequences that is likely to influence cognate gene expression through different mechanisms. This is in accordance with the well known modes of regulation that distinguish alpha from beta-tubulin gene expression.

3. Introns in tubulin genes: a rationale for the cTBP method

All living eukaryotes are likely to share a nuclear intron-bearing ancestor. This is supported by the observation of shared intron positions between paralogues from duplication events dated before the ramification of the eukaryotic tree of life, as well as between ancient orthologues compared across distantly related eukaryotes.[1,10] Then, molecular evolution proceeded with different and often opposite trends from massive loss of introns to extensive gains. This different trend is also related to factors that may constrain the site of intron insertion. In fact, there is growing evidence that the differences in intron gain and/or intron loss (more than three orders of magnitude across the eukaryotic lineages) are subject to selection acting on introns, depending on the biology of the organism and the gene involved.[9] Intron dynamics differ between particular types of genes[9] depending on the pattern of expression, on the possibility of encoding transcripts such as small nucleolar RNAs (snoRNAs) in ribosomal genes, and depends on coupling to transcription-splicing-mRNA transport processes.

3.1. INTRON LENGTH POLYMORPHISM: ILP

Conservation of intron site insertions provides the basis for the use of introns as phylogenetic markers. Intron length polymorphism (ILP) can actually be

a very rapid and useful tool for genotyping species and varieties.[37] Compared with more popular methods, substantially based on the amplification of anonymous DNA sequences, ILP has several advantages. ILP is specific because it is a sequence-tagged marker, and it is functional because it directly detects variation occurring within genes. Therefore, it also offers a convenient method for the rapid isolation of the gene/sequence of interest. ILP provides codominant markers such that one can obtain more-complete information on the genotypes and can identify parental lines. An ILP marker is often transferable from one plant species to another because of the conservation of the sites of intron insertion. In turn, this provides an additional advantage such that ILP can often be performed on unexplored genomes because it doesn't require preliminary information on the target sequences. ILP is easy to visualize, it is convenient (just a PCR reaction), neutral (no interference from phenotypes), reliable, fast and inexpensive.

3.2. COMBINATORIAL TUBULIN-BASED POLYMORPHISM: CTBP

Combinatorial TBP (cTBP) is an ILP method worked out for plant tubulin genes that possesses all the advantages just mentioned.[38] Success is based upon a very simple fact that strong conservation of intron positions is observed in members of the plant beta-tubulin gene family. With very limited exceptions, that should be a matter for evolutionary studies, introns of plant beta-tubulin genes are always located at position +396 and +672 from the ATG initiation codon.[39,12] This is a clear case of a functional constraint acting to conserve intron position, dictated by a specific gene context. Tubulin is not an inconstant and whimsical regulatory protein. On the contrary, tubulin is a structural protein that carries out fundamentally important jobs where 'distractions' are deleterious or lethal. For example, tubulin in its polymerized form, the microtubule, is intimately involved in chromosome attachment and separation with the responsibility of performing well in cell division. This and many more similar functions in plants, require a tremendous conservation of specific protein domains. This amino acid sequence and interactive domain conservation is reflected in a correspondingly conserved DNA sequence. This helps explains why intron position is well conserved in beta-tubulin genes, because in the course of speciation positions where introns could be allocated without provoking negative effect on the function of the genes have been selected.

We can exploit this by designing oligonucleotide primers to the conserved coding sequence at the boundaries of the tubulin introns to be amplified. Because introns do not suffer the same constrains as their neighbouring coding sequences, because we are dealing with members of a gene-family and because one simultaneously recovers the information from both introns,

the chances of finding a polymorphic marker are very high as our experience with cTBP and the results shown in the next section can prove.

Ultimately the cTBP studies performed on many different plants should help to establish the intron evolution flow within species with appropriately similar biologies to be confronted with similar investigations carried out on distantly related taxa.

4. cTBP method: some applications

Up to now, we have applied the TBP method (based solely on the first intron of the beta-tubulin genes)[40] or the cTBP method (combinatorial: based on both first and second intron) to no less than thirty different plant species and many varieties within them. We have always detected variability among plant species. Each species shows a specific pattern of amplification that is simple and easy to score. cTBP represents one of the most intelligible ways to show the occurrence of a differential rate of evolution between coding and spliceosomal intron sequences. On the other hand, TBP and/or cTBP success is not always guaranteed when analyzing varieties within a species. It depends very much on the degree to which a specific variety has been inbred, as is the case for some commercially valuable crops. Otherwise, cTBP can successfully characterize wild relatives of highly inbred varieties, landraces, ecotypes, and more so if we are dealing with allogamous plants. Through an extensive application of the method we found that cTBP is useful for classification at lower taxonomic levels, either as the sole experimental method or as a first-order method. We have often compared the outputs of the cTBP analysis with those of more detailed, but also more laborious and expensive methods, such AFLP or SSR, to find out that the information we gathered with cTBP was always consistent with that obtained with the other methods. We have also cloned several of the cTBP-amplified products to find out that they were indeed tubulin gene sequences at the exon-intron boundaries. Sometime these sequences have been a convenient handle to easily fish out novel, sometime suspected but not previously isolated, tubulin isotypes. Here to follow we provide some experimental evidences for the different possible applications of the cTBP method.

4.1. GENOTYPING PLANT SPECIES

Figure 1 shows the TBP pattern, originated from the amplification of only the first intron, obtained by analyzing 12 different species, mainly grasses or cereals, that are commonly used in different combinations to feed livestock (one such mixture is shown in lane 13). It can be immediately

appreciated that each of the different species is easily distinguishable from all the others for its own specific, simple pattern of amplification. This has obvious practical consequences but, beyond that, a clear indication of the rate of evolution of tubulin intron across the species is offered. The number of the amplified bands is also in strict relation with the ploidy of the analyzed species. For instance, common wheat (*Triticum aestivum* L) shows numerous bands as expected for an hexaploid species (lane 3 of Figure 1)

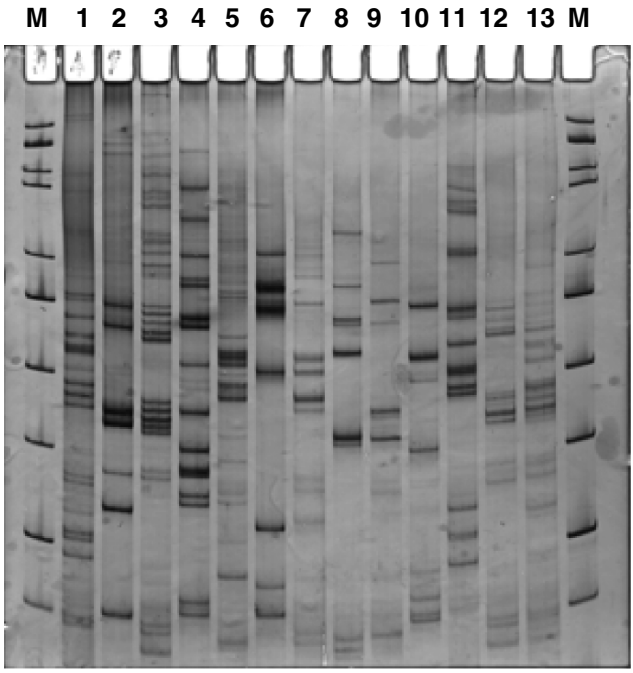

Figure 1. Results of the TBP analysis performed on different plant species. (1) *Avena sativa* L.; (2) *Phaseolus vulgaris* L.; (3) *Triticum aestivum* L; (4) *Linum usatissimum* L.; (5) *Lolium multiflorum* L.; (6) *Medicago sativa* L.; (7) *Zea mays* L.; (8) *Panicum miliaceum* L.; (9) *Hordeum vulgare* L.; (10) *Pisum sativum* L.; (11) *Glycine max* L; (12) Triticale; (13) QT fodder (a mixture of common wheat, maize and sugar beet). M stands for molecular sizes markers.

In addition, immediate correlation concerning evolutionary distances and parental origin can be drawn. Triticale, which is a hybrid obtained from crossing wheat and rye, shows a TBP pattern closely related to common wheat. Unfortunately we didn't have access to rye to further prove this point. Nevertheless, the point of easy identification of the crops present in a mix is proven by the pattern of lane 13 that corresponds to a fodder substantially made up by wheat and maize (lane 3 and lane 7 respectively).

4.2. PLANT ORIGIN RECOGNITION

cTBP can also help to identify the provenience and the origin of newly uncovered species as was the case for the *Eleusine* taxon "Calhoun".

This new weed was recently discovered in the cotton fields of Calhoun County in South Carolina, USA. From many morphological and growth habit traits it was considered to be derived from *Eleusine coracana* ssp. *africana*, and because there is no record of the introduction of the related cultivated species *E. coracana* ssp. *coracana* in the USA was ever produced. Plant and seed morphology of *E. coracana* ssp. *coracana* are also remarkably different from "Calhoun". Despite all this, cTBP clearly shows that the "Calhoun" subspecies is much more similar to *E.c.* ssp. *coracana* than *E. c.* ssp. *africana* (Figure 2).

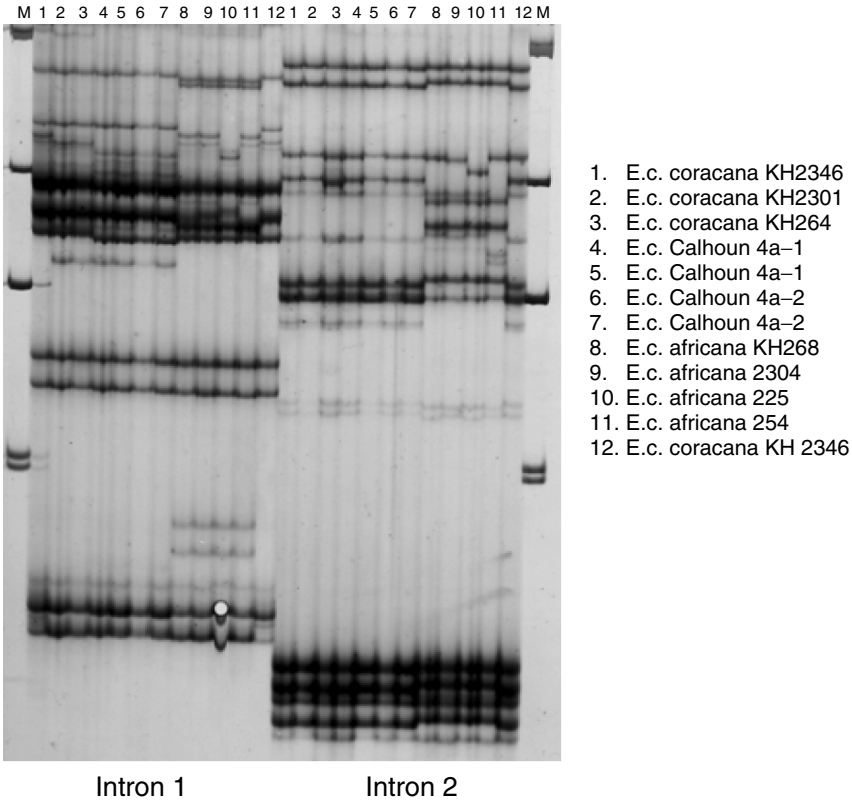

Figure 2. cTBP analysis performed on different individuals of the *Eleusine coracana* subspecies *E.c. coracana* ; *E.c. africana* and *E.c. Calhoun*. M stands for molecular size markers.

In fact, *E.c* ssp. *Calhoun* shows just a couple of specific bands that differentiates this subspecies from *E.c.* ssp. *coracana* while none of the

bands that are specific for *E.c.* ssp. *africana* are visible in the "Calhoun" subspecies for either the first or the second beta-tubulin intron. The cTBP data and related conclusions about the origin of *Eleusine* ssp. *Calhoun* have been entirely confirmed by a parallel study conducted with the use of nuclear ITS markers and plastid *trnT-trnF* sequences.[41]

4.3. GENOTYPING PLANT VARIETIES

As previously stated, and as shown in Figure 3 for *Phaseolus vulgaris*, the TBP method can be used to distinguish varieties within the same species. Here we present data on just four varieties, but we have collected data from no less than 20 varieties, among which are ecotypes grown in close proximity to one another in rural areas of southern Italy and yet are discernable from each other. For each one of the 20 bean varieties, we were able to define a specific cTBP pattern (D. Breviario, 2006).

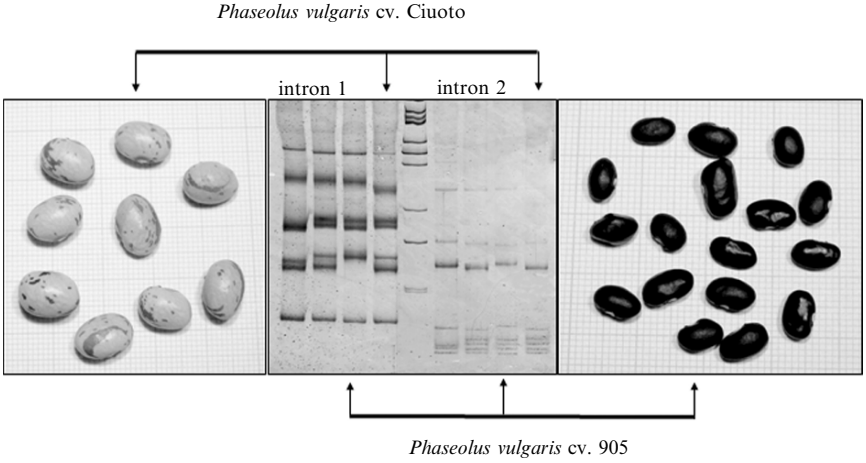

Figure 3. cTBP analysis performed on four different bean varieties. Correspondence between seeds and cTBP pattern is highlighted for two of them.

As a matter of fact, cTBP has insofar proven capable of discriminating varieties within species in the vast majority of the plants we analyzed. However, we have failed in the following two cases: either the varieties are the result of highly inbred lines such as the case of *Arachis hypogea* L. or the varieties were generated from a very narrow genetic base as is the case for tomato (*Lycopersicon esculentum* L.) varieties imported and grown in Italy. While for the former case cTBP cannot be of much help, the negative results obtained in the second case may nevertheless be informative,

particularly so when obtained from an initial screening of previously uncharacterized germplasm.

4.4. PARENTAL ASSESSMENT

Figure 4 documents another case for which the TBP method can be very useful for its ease and rapidity of analysis. In one day, with a single gel run, we were able to assess the parental lineage of the coffee species *Coffea arabica*. As can be appreciated from Figure 4, the diploid progenitors of *C. arabica* can easily be identified in the *C. canephora* and *C. eugenoides* species, each one contributing to the hybrid product.

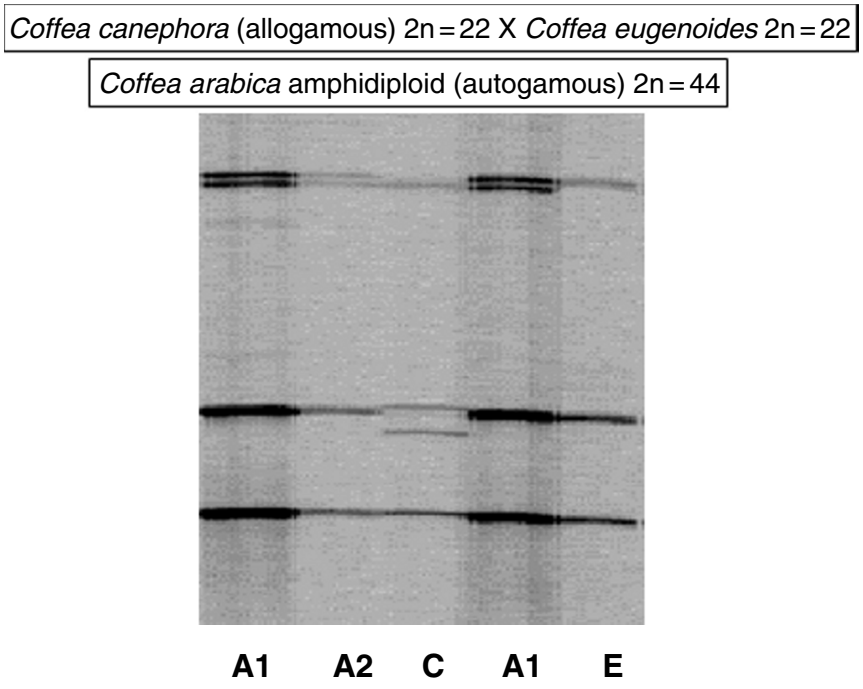

Figure 4. TBP analysis of coffee. Two different accessions of *Coffea arabica* (A1, A2) and one each of *Coffea canephora* (C) and *Coffea eugenoides* (E) are shown.

The upper band of *C. arabica* shown in the gel of Figure 4 was inherited from *C. eugenoides* and since then it has been strongly maintained in all of the *C. arabica* accessions we were able to characterize. This is true to such an extent that the upper band may well be considered a molecular signature of *C. arabica*.

In the case just reported for coffee, parental assessment performed with TBP has elegantly confirmed data already known, though in a much simpler

and faster way. There are cases though in which the parental lineage of a hybrid is not known. We have run into this problem at least two times and solved it using the cTBP method. The first time cTBP helped to clarify the sequence of genetic crosses that eventually led to the achievement of a new bean variety with a low phytate content, due to a single gene mutation originally produced by EMS mutagenesis in one experimental variety and then introgressed into a more valuable genetic background. The second time was when we had to identify the parental lines of some *Jatropha* interspecies hybrids (D. Breviario, 2006).

4.5. IN VITRO GENOMIC INSTABILITY

We applied the TBP method to identify DNA rearrangements during the *in vitro* development of oil palms (*Elaeis guineensis*) ramets, derived from ortets we characterized previously. *In vitro* development of oil palm ramets is a common method to produce a vast amount of plantlets in a reasonably short time with the guarantee of the absence of infections.

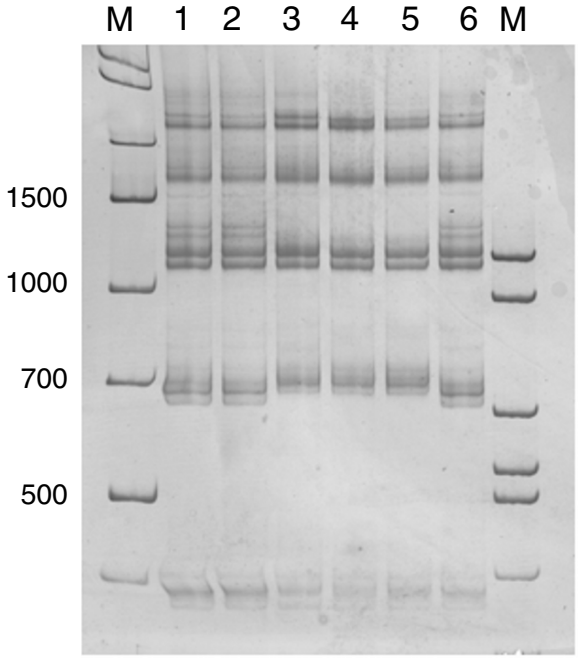

Figure 5. TBP analysis on two oil palm Ortets and *in vitro* derived ramets. Ramets 1 and 2 were derived from ortet 3; Ramets 5 and 6 from ortet 4. Numbers on the left refer to molecular size markers (in basepairs).

The *in vitro* procedure though, is known to influence the average content of DNA that generally decreases. This reduction in the DNA content often correlates, in some yet uncharacterized way, with the frequent development of abnormal flowers, and hence lower seed production in mature plants. As shown in Figure 5, TBP can be a marker for these rearrangements because we found a large percentage of oil palm ramets with polymorphisms occurring at the beta-tubulin loci, presumably introduced during the *in vitro* practices of reproduction. We have actually sequenced those polymorphic bands and found that the sequence changes occurr within a short region of the beta-tubulin intron. This result was unexpected and, although difficult to explain, it provides evidence for an unforeseen tendency toward modifications of the beta-tubulin intron genomic loci.

5. Conclusions

We think we have provided data and clues to prove the concept that introns are a gold mine for studies on eukaryotic gene expression, molecular evolution and comparative biology. Investigation into these different aspects is expected to proceed with mutual benefit. Comparative biology studies have a practical output that helps in the easy identification of plant species, plant varieties, landraces and the progeny of crosses, even recognition of DNA rearrangements. At the same time, because TPB has the intrinsic advantage of providing immediate sequence-specific information, the possibility of getting a deeper understanding of eukaryotic molecular evolution through changes in tubulin introns, is very tempting. Although it may sound like an over simplification, due to the difficulty in identifying a suitable DNA barcode for plants (that could perform as well as the *CO1* mitochondrial gene in animals), our data and experience lead us to propose that introns of the beta-tubulin genes should be considered among the valid candidates.

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We are in debt, for the data on the cTBP method we have reported, with numerous colleagues and friends who have provided us with genomic DNAs or plant material from which we could extract it.

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THE TRANSCRIPTOME OF THE TUBULIN GENE FAMILY IN PLANTS

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Abstract. Microtubules are dynamic multi-protein structures involved in many basic cellular functions and are comprised of an α-tubulin (TUA) and a β-tubulin (TUB) subunit. In plants, both subunits are encoded by small gene families. *TUA* gene family consists of four *TUAs* in Arabidopsis to at least thirteen *TUAs* in cotton, while *TUB* gene family number varies from eight members in barley, rice and maize to twenty in aspen. Expression of almost all tubulin genes is spatially and temporally controlled during growth, development and in response to specific signals. Vegetative plant organs and developing seeds are characterized by redundant expression of several *TUA* and *TUB* genes. Specific tubulin isoforms were described to be expressed in pollen. The present review describes the distribution and possible functional significance of the various α- and β-tubulin genes in plants based on their expression patterns in different tissues, organs as well as during distinct developmental stages and in response to different internal and external factors. Possible mechanisms of regulation of tubulin gene expression are also discussed.

Keywords: α-Tubulin, β-tubulin, gene expression, microtubules, development

1. Introduction

Microtubules are highly dynamic cellular structures that are involved in many important physiological processes. Apart from their conserved role in the process of chromosome separation during cell division[1] and cell expansion,[2] plant microtubules are involved in the development of the plant cell wall and, thus, in determining cell shape and polarity.[3,4] They play an

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important role in generating localized cell wall ingrowths in cells which have ceased expanding, as, for example, during the formation of vascular tissues[5] and transfer cells,[6,7] and for organelle positioning.[8] The basic component of microtubules is the heterodimeric protein tubulin formed by a non-covalent interaction between an α-tubulin (TUA) and a β-tubulin (TUB) subunit, which are replenished by both *de novo* biosynthesis and by depolymerization.[9] Tubulins occur in all eukaryotes being presumably evolved at the time eukaryotic cells became established. Plant tubulin subunits are encoded by multigene families, and the stability and assembly of microtubules are both transcriptionally and post-transcriptionally regulated.[10,11]

It is not surprisingly, that microtubules and their components have been intensively studied and is further summarized by a number of excellent reviews[11–15] and in several books[16,17] over the last decade. However, most of them are concentrated on the cellular roles of microtubules in plants largely avoiding the fact that the tubulins, the major constitutive of microtubules, are encoded by multiple families of genes which can be differentially expressed and regulated in specific plant tissues and organs. The main aim of the present review is to describe the tubulin gene families and the expression of distinct tubulin genes in relation to different developmental processes in plants. Newly developed tools like sequencing of a number of plant genomes, extensive EST collections for most agronomically important species, coupled with gene expression profiling by micro- or macro-arrays has allowed for the analyses of all the members of the tubulin gene families and the simultaneous studies on their relative expression levels in different plant tissues and/or over developmental course.

2. Genes and gene families for tubulins

TUA and TUB proteins are very conservative among different taxa usually sharing more than 88% amino acid sequence similarity among animal, plant and fungal genes.[11]

In lower eukaryotic organisms, including lower algae (e.g., *Chlamydomonas reinhardtii*), both tubulin subunits are usually encoded by one or two almost identical genes. In higher eukaryotes, including all flowering plants, tubulins are encoded by small gene families. Mammalian and bird species often have equal numbers of genes for α- and β-tubulins (e.g. seven genes for each in both human and mouse genomes[18]). In higher plants, however, the family sizes vary from four *TUAs* and eight *TUBs* in rice expanding to eight *TUAs* and 20 *TUBs* in the newly described aspen genome.[19] Also, the growing number of different cell types and specialized cell functions during evolution is accompanied by expanding number of both α- and β-isotypes. Plant tubulin sequences have been highly conserved throughout the plant

evolution, particularly in the middle regions of the proteins. These much conserved regions may be involved in basic tubulin functions, such as subunit polymerization and dimer interaction. Even small amino acid substitutions may cause relevant changes in the plant morphology. As an example, the exchange of serine with a phenylalanine in AtTUA4 and AtTUA6 proteins cause a left-handed helical growth and clockwise twisting in elongating organs of Arabidopsis plants.[20] In addition, missense mutations in the TUA proteins are correlated with the resistance to antimicrotubule drugs in goosegrass.[21,22] More divergent N- and, especially, C-termini are more vulnerable to sustainable mutations and may contribute to protein isotypic specificities. The sequence and domain structure of tubulins in relation to their functions and reactivity with diverse antimicrotubule drugs was reviewed early.[12,23]

The *TUA* gene family consists of six genes in Arabidopsis[24] and maize,[25] four expressed *TUA* isotypes in rice,[26] whereas the barley genome contains five *TUA* genes.[27,28] Four to five *TUA* genes may be expressed in goosegrass.[22] A complex wheat genome contains 15 different expressed *TUA* genes, which are clustered into five homeologous groups of three genes comprising three ancestral genomes of hexaploid wheat.[29] Surprisingly, aspen (*Populus tremuloides*) *TUA* gene family is largely expanded compared to their herbaceous counterparts and contains eight expressed genes and two most likely pseudogenes.[19] Twelve *TUA* genes were found in *Miscanthus*, an herbaceous plant species that is also characterized by very tall growth.[30] The cotton genome contains at least 13 expressed *TUA* genes.[31] As shown from recent results, the TUA gene family in some plant species can be expanded possibly due to specification and diversification of microtubule functions involved in the development of specific tissues like secondary wood or fibers (see below).

Two Arabidopsis members, *AtTUA3* and *AtTUA5*, are located as tandem repeats on the chromosome 5 and encode identical proteins, implying a very recent genome duplication event. *AtTUA2* and *AtTUA4* are also located very closely on the chromosome 1 and encode identical proteins, which appear to have duplicated as an independent event. A high sequence similarity exists also between orthlogous *TUA* genes of wheat and barley than between paralogs within the wheat gene family, suggesting that the *TUA* gene family originated before the divergence in the *Triticea* subfamily.[29] The aspen TUA protein sequences are more diverse than in any other species reported so far, especially in the hypervariable C terminus. All plant TUA proteins identified so far contain a C-terminal Tyr residue (referred to as Y-type), which has been implicated in the control of posttranslational modifications of tubulin stability in animal models.[32] Interestingly, only three (PtTUA1, PtTUA6 and PtTUA8) of the eight aspen TUA proteins are Y-type, while

the others terminate with either Met (M-type: PtTUA3, PtTUA5 and PtTUA7) or Glu/Gln (E/Q-type: PtTUA2 and PtTUA4).[19]

Concerning the amino acid sequence similarity, all TUAs can be divided into two subclasses[19] (Figure 1) reflecting early evolutional diversion.[25]

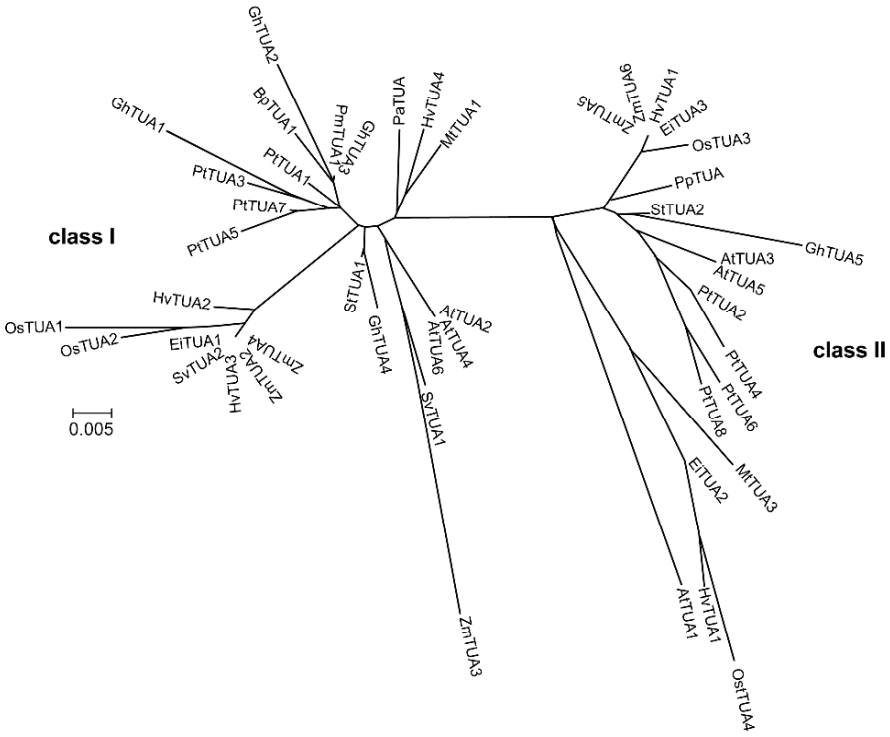

Figure 1. Minimum evolution tree of representative full-length TUA proteins. GenBank accession numbers of the sequences are as follow: *Arabidopsis thaliana*, AtTUA1 (At1g64740), AtTUA2 (At1g50010), AtTUA3 (At5g19770), AtTUA4 (At1g04820), AtTUA5 (At5g19770), AtTUA6 (At4g14960), *Betula pendula*, BtTUA (CAB66336), *Chlamydomonas reinhardtii*, CrTUA (AAN87017), *Eleusine indica*, EiTUA1 (CAA06618), EiTUA2 (O22348), EiTUA3 (O22349), *Gossipium hirsutum*, GhTUA1 (AAQ92661), GhTUA2 (AAQ92662), GhTUA2 (AAD50627), GhTUA4 (AAQ92663), GhTUA5 (AAD50629), *Hordeum vulgare*, HvTUA1 (CAA67942), HvTUA2 (CAA69724), HvTUA3 (CAA10663), HvTUA4 (CAM58977), HvTUA5 (CAM58978), *Medicago truncatula*, MtTUA1 (ABN08980), MtTUA2 (ABE86411), *Oryza sativa*, OsTUA1 (Os03g072610), OsTUA2 (Os11g024730), OsTUA3 (Os07g057480), OsTUA4 (Os03g021930), *Pseudotsuga menziesii*, PmTUA (AAV92379), *Populus tremulus*, PtTUA1 (AY229881), PtTUA2 (AY229882), PtTUA3 (EF583813), PtTUA4 (EF584828), PtTUA5 (EF583814), PtTUA6 (EF583816), PtTUA7 (EF584829), PtTUA8 (EF583815), *Setaria viridis*, SvTUA1 (CAE52514), SvTUA2 (CAE52515), *Solanum tuberosum*, StTUA1 (ABB02631), StTUA2 (ABB16994), *Zea mays*, ZmTUA2 (CAA33733), ZmTUA3 (CAA44861), ZmTUA5 (CAA44862), ZmTUA6 (CAA44863).

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Subclass I contains α-tubulins with very similar sequences and often overlapping expression profiles in vegetative tissues. More divergent sequences often with specific expression profiles belong to subclass II. Moreover, these classes differ both in number and position of introns. Class II of *TUA* genes contains four introns and shows a conserved exon-intron structure as in monocot (maize, rice) as well in eudicot (Arabidopsis, *Populus*) plants. The class I of *TUAs* contains three introns, however, their positions are less conserved. The most unusual α-tubulin with unique expression pattern was cloned from the sunflower.[33] The gene could be family-specific, since similar sequences were found only in Asteraceae plants. All of this data suggests that the TUA genes diverged into two classes before separation of monocot and dicot plants.

The Arabidopsis genome contains nine *TUB* genes,[34] while four *TUB* sequences (presumably not all) were identified in *Eleusine indica*.[35] Maize,[36] rice[37] and barley[28] all encode eight *TUB* genes. Twenty expressed *TUB* genes and two likely pseudogenes were identified in aspen.[19] *Populus TUB* family has undergone expansion giving rise to ten pairs of highly homologous genes.[19] The predicted amino acid sequences of all TUBs are highly conserved (89–98% identity overall), except for the C-termini. These highly variable tubulin C-terminals were successfully used for the production of antibodies against specific tubulin protein isoforms[38,39] in consequent studies which involved of individual isotypes in microtubule arrays.

Phylogenetic analysis revealed at least five distinct classes of plant *TUB* genes (Figure 2). These classes are comprised of *TUBs* from different monocot and eudicot plants supposing that the expansion of the *TUB* gene family took place very early in evolution, before splitting into two major plant classes. Further gene multiplication in some species might have occurred by gene duplication as shown in *Populus* and Arabidopsis genomes. There is also no strong correlation concerning the similarity of *TUB* genes from different species and their mode of expression in these plants. Most of plant *TUBs*, for which genomic structure is available, has two introns located at conserved positions. The exceptions are maize *ZmTUB1* and rice *OsTUB2*, both of which contain only the first intron, while other closely related *TUB* genes have two introns. This suggests that the second intron was lost independently in two different species. Some genes, such *AtTUB6*, *ZmTUB2*, *ZmTUB5*, *OsTUB4* and *PtTUB13* to *PtTUB18*, contain an additional intron within the 5' untranslated region. For rice, it was shown that this intron plays an important role in the regulation of the respective gene expression.[40]

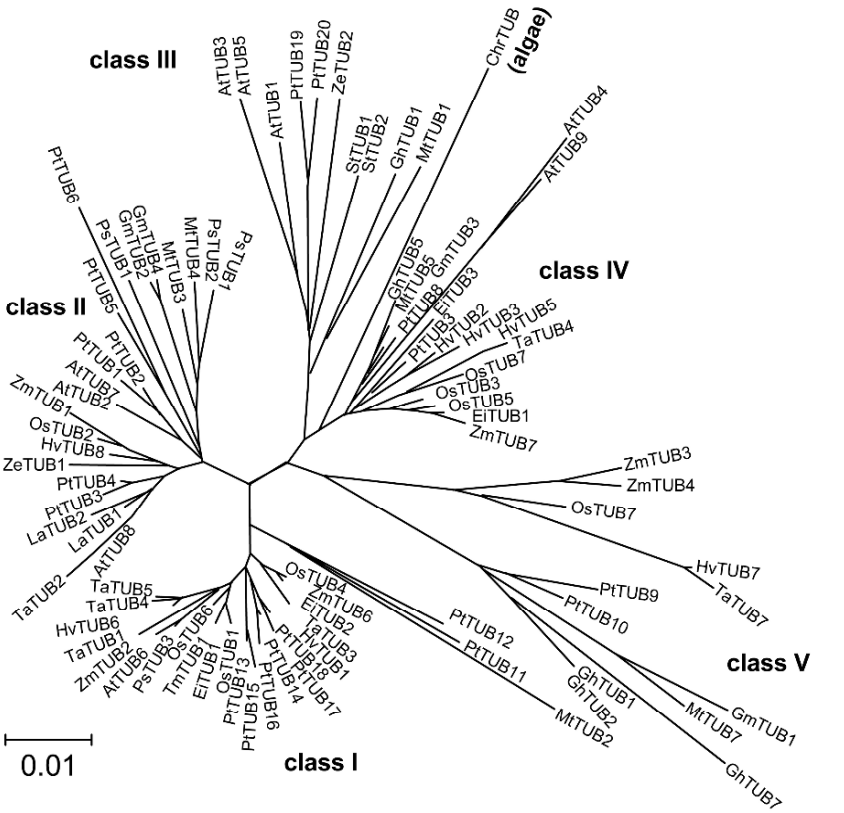

Figure 2. Minimum evolution tree of full-length TUB proteins of selected plant species. The predicted amino acid sequences were aligned using CLUSTALW, and the unrooted dendrogram was obtained using MEGA 4. GenBank accession numbers of used sequences are as follow: *Arabidopsis thaliana*, AtTUB1 (At1g75780), AtTUB2 (At5g62690), AtTUB3 (At5g62700), AtTUB4 (At5g44340), AtTUB5 (At1g20010), AtTUB6 (At5g12250), AtTUB7 (At2g29550), AtTUB8 (At5g23860), AtTUB9 (At4g20890); *Eleusine indica*, EiTUB1 (AAD20178), EiTUB2 (AAD20179), EiTUB3 (AAD20180), EiTUB4 (AAD20181), *Gossipium hirsutum*, GhTUB1 (AAL91118), GhTUB2 (AAN32988), GhTUB5 (AAQ92665), GhTUB6 (AAQ92666), GhTUB7 (AAQ92667), *Hordeum vulgaris*, HvTUB1 (CAM58979), HvTUB2 (CAM58980), HvTUB3 (CAM58981), HvTUB4 (CAM58982), HvTUB5 (CAM58983), HvTUB6 (CAM58984), HvTUB7 (CAM58985), HvTUB8 (CAM58986), *Medicago truncatula*, MtTUB1 (ABE81376), MtTUB2 (ABE81426), MtTUB3 (ABE81854), MtTUB4 (ABE83857), MtTUB5 (ABE90057), MtTUB6 (ABE92128), *Oryza sativa*, OsTUB1 (Os01g18050), OsTUB2 (Os03g01530), OsTUB3 (Os06g0671900), OsTUB4 (Os01g59150), OsTUB5 (Os02g0167300), OsTUB6 (Os05g0413200), OsTUB7 (Os03g566810) OsTUB8 (Os03g45920); *Populus trichocarpa*, PtTUB1 (EF584830), PtTUB2 (EF584831), PtTUB3 (EF584832), PtTUB4 (EF584833), PtTUB5 (EF584834), PtTUB6 (EF584835); PtTUB7 (EF584836),PtTUB8 (EF584837), PtTUB9 (EF584838), PtTUB10 (EF584839), PtTUB11 (EF584840), PtTUB12 (EF584841), PtTUB13 (EF584842), PtTUB14 (EF584843), PtTUB15 (EF584844), PtTUB16 (EF584845), PtTUB17 (EF584846), PtTUB18 (EF584847), PtTUB19 (EF584848), PtTUB20 (EF584849), *Triticum aestivum*, TaTUB1 (AAD10487), TaTUB2 (AAD10488), TaTUB3 (AAD10489), TaTUB4 (AAD10490), TaTUB5 (AAD10492), TaTUB6 (AAD10493), *Zinnia elegans*, ZeTUB1 (BAA82637), ZeTUB2 (BAA82638), *Zea mays*, ZmTUB1 (P18025), ZmTUB2 (P18026), ZmTUB3 (CAA52718), ZmTUB4 (CAA52719), ZmTUB5 (CAA52720), ZmTUB6 (AAA20186), ZmTUB7 (AAA19708), ZmTUB8 (AAA19709).

In plant cells, each genetic isotype may give rise to several biochemical isotypes because tubulins are able to be modified posttranslationally, thereby adding to the complexity of tubulin isoforms in a cell.

3. Expression of tubulin genes

Tubulins are often expressed at high levels in most tissues because the cytoskeleton requires large amount of microtubules. However, as shown in many studies, expression of almost all tubulin genes is spatially and temporally controlled during growth, development and in response to specific signals. The different patterns of tubulin expression suggest that tubulin isoforms may play specific roles during plant development. Distinct gene isotypes often exhibit partially overlapped expression patterns and the different isoforms are believed to co-assemble in microtubule arrays. So, as shown by immunofluorescence microscopy of antibodies specific to individual tubulin isoforms, these tubulin isoforms appear to be incorporated into microtubule arrays in at least some cell types in maize plants.[38] Although the developmentally and environmentally dependent tubulin gene expression was estimated for years, it still remains unknown whether and how different tubulin isotypes contribute to the functional specificity of plant microtubules. As will be shown below, both functional redundancy and specificity of individual types are likely possible.

3.1. TUBULIN GENES ARE EXPRESSED HIGHLY REDUNDANTLY IN VEGETATIVE TISSUES

Five of six Arabidopsis *TUAs* are expressed in vegetative tissues,[24,41] albeit at different levels (Figure 3). *AtTUA2* is expressed in most tissues of mature Arabidopsis plants, with the highest expression levels found in actively dividing and elongating cells.[42] Similarly, all five *TUA* genes in barley are expressed in leaves, although at different levels and with slightly different expression profiles, generally correlating with changes in the arrangements of the microtubules in the developing mesophyll.[27] The *HvTUA1* and *HvTUA5* genes express almost exclusively in a very restricted leaf zone during elongation. Transcripts of *HvTUA2* and *HvTUA4* are most abundant in the meristematic leaf tissue while *HvTUA3* shows nearly constitutive level of expression during leaf growth.[27]

Sequence comparisons of *AtTUB1* and *AtTUB5* suggest that these genes have evolved from a common progenitor.[34] They, however, exhibit very different expression patterns. While *AtTUB1* is expressed predominantly in the cortical cells of roots[34,43] (Figure 3), the *AtTUB5* transcripts accumulate

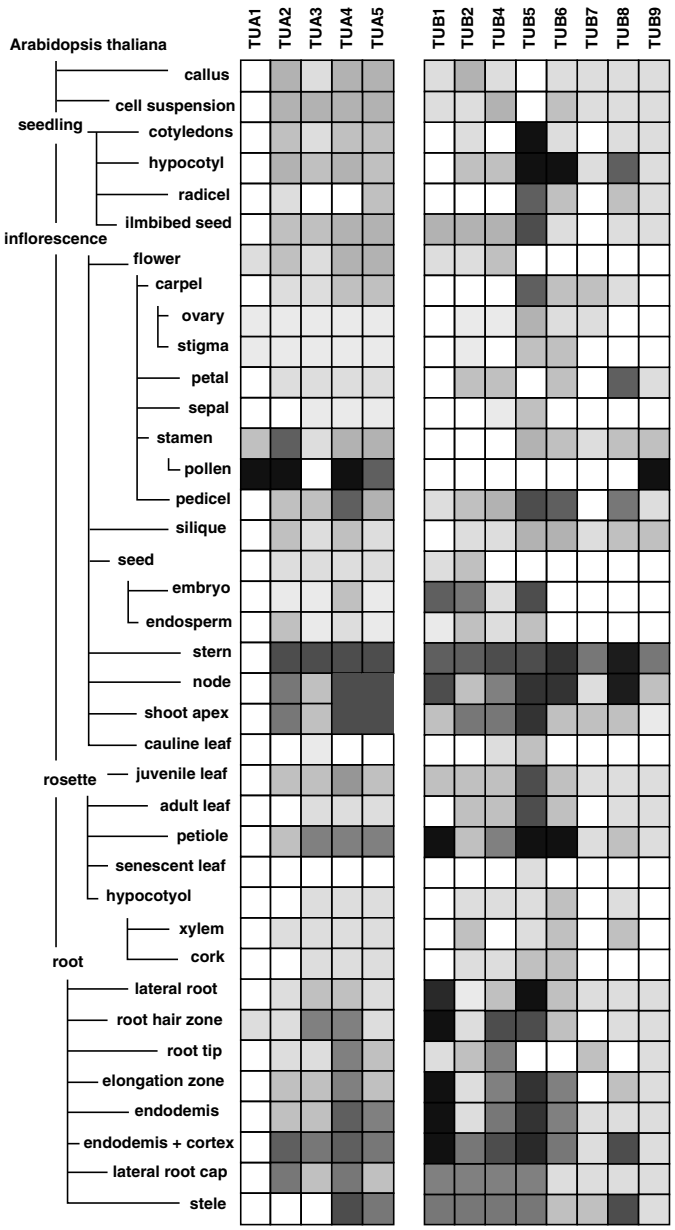

Figure 3. Atlas of relative expression of α- and β-tubulin genes in different tissues and organs of Arabidopsis plants as revealed by microarray expression analysis and visualized by Genevestigator tool.[97] Darker grey color corresponds to higher expression of the corresponding gene.

primarily in leaves, seedlings and stems.[43] *AtTUB1* also exhibits high mRNA abundance in leaf petioles, nodes and pollen (Figure 3). The results from dot blot hybridization experiments showed that all of the *ZmTUB* genes were expressed in most tissues examined, although each gene showed a unique pattern of differential transcript accumulation.[36] The *ZmTUB1* gene showed a high level of transcript accumulation in meristematic tissues and almost no accumulation in the late stages of anther development and in pollen. Transcripts of *ZmTUB4*, *ZmTUB6*, *ZmTUB7* and *ZmTUB8* genes were predominant in the shoot.[36] Seven of eight rice *TUBs* show high expression in leaves including apical meristem. *OsTUB1* and *OsTUB4* were specifically expressed in dividing and elongating parts of the leaf sheath.[37] It is likely that the development and differentiation of the complex structure of a leaf requires different isotypes of tubulins for microtubule formations. It is still a question whether all of these tubulins genes are expressed in a single cell or if some cell/tissue specificity exists, as was shown for such specialized cells as cotton fiber and tracheary cells.

Specific tubulin transcripts are strictly associated with cotton fiber development[31,44–46] and *Zinnia* tracheary element differentiation,[47] supporting the view that microtubules are involved into cell elongation and microfibril deposition during secondary wall synthesis. Seven of 13 cotton *TUAs* are predominantly expressed in elongating fibers. Among them, *GhTUA1*, *GhTUA5* and *GhTUA9* display the highest and specific to fiber level of expression around 5–10 days post anthesis, coinciding with the elongation stage and the primary cell wall synthesis of fiber.[31,44] However, only the *GhTUA2*, *GhTUA3* and *GhTUA4* transcripts remain abundant following the onset of secondary cell wall synthesis.[44] Furthermore, *GhTUB1* gene is also preferentially expressed in the early stages of fiber development.[45] These TUAs and TUB of cotton may be involved in formation of specific microtubule arrays essential for rapid fiber elongation. Experimental results indicate, that over-expression of *GhTUA9* on one of *GhTUBs* in fission yeast (*Schizosaccharomyces pombe*) can promote atypical longitudinal growth of the host cells, supporting the view that these tubulins are specifically involved in cell elongation during cotton fiber formation.[31,46]

In *Zinnia elegans*, two genes, *ZeTUB1* and *ZeTUB3*, are expressed in differentiating xylem cells, as well as in the ground meristem and the procambium of seedlings.[47] The expression of these genes also increases during trans-differentiation of mesophyll cells into tracheary elements, contrasting with the constitutive *ZmTUB2* expression during the whole cell culture period.[47] The Arabidopsis *AtTUB8* gene also appears to be expressed almost exclusively in vascular tissues[43] possibly fulfilling the same function in tracheary element formation as corresponding genes in *Zinnia*. More recently, cortical microtubules have been shown to influence

secondary cell-wall development in cell suspension-induced tracheary elements of *A. thaliana*.[48] Furthermore, over-expression of the green flourescent protein-tagged AtTUA6 in transgenic Arabidopsis plants causes disruption of cortical microtubules leading to a marked reduction of cell wall biosynthesis.[49]

Populus PtTUA1 and *Eucalyptus grandis EgTUB1* were also identified among the most abundant transcripts in xylem, as detected by microarray and EST analyses.[19,50,51] PtTUA1 protein possesses the highest homology to the three aforementioned fiber-specific cotton TUAs at the amino acid level (Figure 1), consistent with the supposition of its involvement in secondary cell wall development. Immunolocalization of the PtTUA1 protein confirmed its localization in newly formed xylem fibers and in phloem fibers with extensive secondary cell wall development.[19] The aspen *PtTUB9* and *PtTUB15* are also predominantly expressed in developing xylem, followed by *PtTUB16* and *PtTUB13*.[19] The use of polyclonal antibodies against TUB proteins revealed similar distribution patterns for PtTUBs as was found for PtTUA1 in the developing wood. It has been recently shown, that the *Eucalyptus grandis EgTUB1* gene is involved in determining the orientation of cellulose microfibrils in plant secondary fiber cell walls. Cellulose microfibril angle is correlated with *EgTUB1* expression, and has been significantly altered in woody pieces *in vivo*, where the expression of the *EgrTUB1* was moderately down-regulated by homology-dependent gene silencing.[51] The authors provide functional evidence that β-tubulin is directly involved in cellulose microfibril orientation during xylogenesis.[51] Contrary to the results from perennial woody plants, *TUA* and *TUB* gene expression has not been associated with secondary growth in Arabidopsis.[52,53] This discrepancy in tubulin regulation appears to coincide with differential evolution of the secondary cell wall-associated tubulin isoforms, where the increased tubulin isoform diversity, along with elevated transcript abundance, characterizes wood and fiber formation in woody and fiber plants. How the microtubules exert an influence over microfibril deposition remains unclear, but likely involves multiple factors, including microtubule-associated proteins and diverse regulators. As shown recently by time-lapse visualization, functional associations exist between cellulose synthase complexes and microtubules providing further evidence for the direct guidance of cellulose deposition by the cytoskeleton.[54] Moreover, Oakley et al.[19] found that xylem-abundant *PtTUA1*, *PtTUA5*, *PtTUB9*, *PtTUB15* and *PtTUB16*, together with less abundant *PtTUA4*, *PtTUA8*, *PtTUB3*, *PtTUB4*, *PtTUB7* and *PtTUB19*, were all up-regulated twofold to fourfold in tension wood, which is characterized by increased abundance of microtubules, in parallel with cellulose microfibrils.[55] Their up-regulation was correlated with expression of three cell-wall associated cellulose

synthase genes and Korrigan endoglucanase gene.[56] Thus, a number of tubulin genes are co-ordinately regulated with cellulose biosynthesis machinery. A similar picture was shown for developing seeds (see below).

Microtubules play an important role in the location and number, as well as growth, of root tips and root hairs.[57,58] Studies with drugs that depolymerize or stabilize microtubules, as well as mutants with altered microtubule organizations support the notion of microtubule involvement in the control of root hair growth.[59] Some tubulin genes are specifically up-regulated during these processes in roots. *PtTUA5* from *Populus* is highly expressed in root tips, while *PtTUA3* is primarily detected in xylem and shoot apices and *PtTUA7* in apices and young leaves.[19] *PtTUB15* transcripts were also detected in roots and pollen grains.[19] High expression levels of *OsTUB3*, *OsTUB5*, *OsTUB6* and *OsTUB7* were observed in rice roots.[37] At least three *TUA* genes were expressed in maize roots, each showing different temporal and spatial patterns of distribution. *ZmTUA1* expression appears to be higher in the cortex-producing cells, whereas *ZmTUA3* is associated with the cells that differentiate to vascular bundles. Expression of *ZmTUA3* is limited to the root apex epidermis.[60] It is likely that specific tubulin isoforms are synthesized in cells with different morphological features. Reduced *AtTUA6* expression in transgenic Arabidopsis plants has been shown to affect the patterning of root hairs.[61]

As seen from the examples described above, the tissue specific expression of plant tubulin genes is clearly established; however, whether any plant tubulin isoform has a unique function remains to be investigated. In general, the smaller TUA family appears to exhibit broader functionality, whereas the large TUB family shows gene redundancy and functional specialization.

3.2. POLLEN-SPECIFIC TUBULIN GENES

Although most *TUAs* and *TUBs* genes are redundantly expressed in distinct plant organs and tissues, some tubulin genes show pollen-specific expression patterns.[62,63] For instance, three (*AtTUA1*, *AtTUA2* and *AtTUA4*) of six Arabidopsis *TUA* genes are highly active in pollen (Figure 3); however, only *AtTUA1* is expressed almost exclusively in pollen and is specifically up-regulated during pollen tube growth,[62] concomitant with microtubule assembly. Aspen pollen grains express *PtTUA1*, *PtTUA5* and *PtTUA7* genes at high levels, as well as, most notably, *PtTUA6* and *PtTUA8*, that are negligibly expressed elsewhere.[19] The most divergent and likely family-specific plant α-tubulin isoform has been described in sunflower (*Helianthus annuus*) pollen[33] with restricted expression in male gametophyte before germination. The authors concluded that the isoform

could play a role in the gametophytic regulation system by preventing microtubule assembly prior to pollen germination. However, besides the putative three-dimensional model of the protein, no further confirmation for this function has been presented.

One of nine Arabidopsis *TUBs*, *AtTUB9*, also shows preferential pollen expression pattern[34] (Figure 3). Cheng et al.[64] established the activity of this gene in pollen, elongating pollen tubes and ovules, using 800 bp of the 5'-flanking ("promoter") region of *AtTUB9* gene fused to the β-glucuronidase (GUS) reporter gene coupled with the *in situ* investigation of the *AtTUB9* mRNA localization. The expression of the reporter gene in ovules ceased at the time of fertilization. The similar patterns of *AtTUA1* and *AtTUB9* gene expression in male gametophytic tissues suggest that these tubulins may be incorporated into microtubules with unique functions during pollen tube growth.

Aspen pollen expressed *PtTUB7*, *PtTUB8*, *PtTUB19* and *PtTUB20* transcripts at high levels,[19] showing expression patterns similar to other pollen-specific β-tubulins, including *AtTUB9* from Arabidopsis, *OsTUB8* from rice[37] and *ZmTUB2* and *ZmTUB4* from maize.[36] The accumulation of ZmTUB2 protein in pollen was also confirmed by using isotype-specific antibodies.[38] High mRNA levels for two rice genes, *OsTUB6* and *OsTUB8*, were detected in pollen and the vascular bundle of the anther filament, whereas *OsTUB8* was not expressed in any other tissue.[37,65] Transgenic rice plants, expressing the antisense sequence of *OsTUB8*, driven by its own promoter, were viable but showed 20–60% reduced growth. Interestingly, only *OsTUB8* expression was down-regulated while *OsTUB6* expression was not affected, despite the very close similarity of their sequences. Moreover, *OsTUB6* could not complement the shortage of *OsTUB8* transcripts.[65] During the reproductive stage, antisense transgenic plants produced normal panicles and anthers. However, the seed set was drastically reduced, experimentally confirming that the microtubule cytoskeleton is essential for reproductive growth of the pollen tube.[65] This is the first direct confirmation about functional specificity of tubulin isoforms in plants so far.

Pollen-specific tubulin isoforms are phylogenetically conserved, although the role of microtubules in pollen development remains elusive. In the case of *Drosophila melanogaster*, the substitution of the β3-tubulin isoform for the β2-isoform results in the disruption of microtubule-mediated events during spermatogenesis.[66] In plants, no microtubules are detected in the vegetative cell of the mature pollen grain, but actin filaments are present.[67] However, in the generative cell, microtubules assemble in the cytoplasm and control cell shape. The mature pollen grain contains a stock of pre-synthesized mRNAs, including those of tubulins, which are

translated later, during pollen tube growth.[68] Later, up-regulation of pollen-specific tubulins coincides with pollen tube growth, directing, possibly together with actin filaments, vesicle and organelle transport in the elongating pollen tubes.[8] This is supported by the existence of pollen specific kinesin-related motor proteins[8] and microtubule-associated protein.[69] Microtubules may also play a role in the direction and deposition of the pollen cell wall compounds, including callose and cellulose, and in the maintenance of the shape of the growing pollen tube.[70]

3.3. ROLE OF THE MICROTUBULES DURING SEED DEVELOPMENT

Contrary to the vegetative organs, the developing seed consists of three genetically distinct tissues. Covering tissues, mainly consisting of the seed coat in dicotyledonous plants and of pericarp in monocots, are derived from the mother plant and cover newly developing filial embryo and endosperm. The embryo arises from the fusion of an egg cell with a sperm nucleus, while the endosperm is a product of double fertilization of two central cell nuclei with one sperm nucleus, thereby resulting in a triploid genome.

The role of microtubules in seed development is the most comprehensively studied in barley. During early grain development in barley, the pericarp represents the bulk of the seed, and this continues to grow in coincidence with the development of filial tissues until the onset of starch and protein accumulation in the endosperm. Afterwards, the pericarp loses its significance and degenerates rapidly.[71] High up-regulation of three *TUAs* (*HvTUA2-HvTUA4*) and all eight *HvTUBs* between 2 and 10 days after flowering (DAF) with rapid declining of their mRNA abundance thereafter perfectly correlated with the pericarp growth[72] and may reflect the involvement of microtubules in cell divisions.

Two expression peaks for tubulins were detected in the developing endosperm.[72] Shortly after fertilization, the endosperm nucleus begins to divide without cell wall formation or cytokinesis, thereby leading to the endosperm coenocyte[73] or, in other words, to the large endosperm cell with thousands of nuclei. These nuclear divisions require high amounts of microtubules, which is reflected by the very strong up-regulation of *HvTUA2-HvTUA,* all *HvTUBs* and some kinesin genes around two DAF.[72] Proteome analysis of maize kernels also revealed a strong accumulation of TUA and TUB proteins during early endosperm development.[74] The mitotic microtubule spindles serve to separate the two daughter sets of chromosomes, and are involved in the formation of the phragmoplast separating the daughter nuclei. In the early developing endosperm, unlike phragmoplasts in meristematic cells, no cell plate is formed between the two separating nuclei.[75] Cellularization of the endosperm coenocyte begins

around four to six DAF and is mediated through a tightly orchestrated interplay between cell cycle machinery and the microtubular cytoskeleton.[73] In the barley syncytial endosperm, microtubule rearrangements prepare for the cellularization of the coenocyte by forming a radial microtubular system, marking out a nucleocytoplasmic area for each nucleus,[76] which coincides with the formation of the adventitious phragmoplast.[75] Synchronous mitoses and periclinal cell divisions continue until the complete filling of the central vacuole marks the end of the cellularization phase (till 10–12 DAF). With the onset and completion of cellularization, the requirements for tubulins go down, which is reflected in the gradual down-regulation of all tubulin genes till 12 DAF.[72] The endosperm cells differentiate into starchy endosperm, transfer cells, aleurone and the embryo-surrounding region.[73] The establishment of aleurone cells is visible from around 8 days after flowering, and is marked by a full complement of cytoskeletal arrays in the meristematic cells. In contrast to the aleurone cells, the starchy endosperm cells develop cortical microtubules, but lack any pre-prophase bands,[75] which subsequently leads to a random orientation in cell division.[73] The second, less pronounced and more extended, expression peak included only *HvTUA3*, *HvTUA5* and *HvTUB1* genes and might be associated with the cell wall organization in aleurone and starchy endosperm. This is supported by the co-expression of the cellulose synthase *HvCesA2* gene, and the *KORRIGAN* and *COBRA* (*HvCOB2*) genes.[72] The *COBRA* genes play a role in the diverse types of cell expansion and cell wall biosynthesis,[77] while the *KORRIGAN* gene encodes for an endo-1,4-β-D-glucanase and is essential for cytokinesis.[56,78]

The zygote enters a developmental pattern typical of plant meristems, giving rise to the embryo.[79] The microtubule cycle of the embryo differs from that occurring in the co-developing endosperm by the absence of hoop-like cortical MTs in the interphase. Instead, cytoplasmic organization and cell wall formation is a function of nuclear-based radial MT systems.[79] During embryo development, some tubulin genes (*HvTUA2* and *HvTUB1*) are expressed at almost constant levels, while others are up-regulated (*HvTUA2*, *HvTUA5* and *HvTUB1*) or down-regulated (*HvTUA3*, *HvTUB2*, *HvTUB3*, *HvTUB5* and *HvTUB6*). These changes in mRNA accumulation of particular genes may reflect their involvement in the development of the leaf or root primordia, but this speculation requires further experimental confirmation.[72] Interestingly, *HvTUA5* is highly expressed specifically during embryo development but not in endosperm and pericarp. Besides the developing and germinating embryo, this gene is also particularly active in leaves, stems and internodes.[72]

3.4. MODIFICATION OF TUBULIN GENE EXPRESSION IN RESPONSE TO DIFFERENT FACTORS

Since microtubules are involved in many very important cellular processes, it is not surprising, that the expression of tubulin genes can be affected by internal and external stimuli like phytohormones and stress factors.

Phytohormones can influence tubulin gene expression by enhancing (giberellic acid (GA), brassinolides) and repressing (ABA) mRNA accumulation, or it may also show no effect (cytokonins). Oat *TUA* and *TUB* transcripts were up-regulated by GA.[80] In rice, the expression of *OsTUB5*, *OsTUB6* and *OsTUB7* genes was also elevated by the GA.[37] Promoter analysis revealed two elements of the GA response complex upstream of the *OsTUB5* coding region.[37] Moreover, all rice *TUB* genes were strongly repressed by external ABA, a known antagonist of GA; however, this was shown to occur only at the mRNA level,[37] while amounts of the TUA and TUB proteins were likely unaltered.[81] Brassinolides enhance transcripts of rice *OsTUB5* and *OsTUB6* genes,[37] as well as Arabidopsis *AtTUB1* gene.[82] No significant effect of auxins and cytokinins on the tubulin gene expression was reported.[37,83]

Specific β-tubulin genes were shown to be regulated by light[84] and temperature.[85] The role of the microtubule has been especially established in the cold stress response and in adaptation to low temperatures.[16] Microtubules undergo transient disorganization after exposure to low temperatures during cold acclimation.[86] Enhanced freezing tolerance can even be artificially achieved by the short disassembly of microtubules with herbicide pronamide. It is supposed that a single amino acid exchange in the conservative region of the TUA sequence can increase cold resistance.[87] As was shown for tubulin genes in wheat, the expression of all 15 TUA genes was affected by cold treatment and was generally down-regulated after 1 day of cold treatment with partial recovery of transcript abundance afterwards.[29] However, some genes are differentially expressed during cold acclimation and low-temperature tolerance stages. Moreover, it was shown that the wheat protein kinase PK-F29, which is induced during cold acclimation, interacts specifically with the α-tubulin TaTUA2-3 isoform[88] pointing out that the changes in TUA gene expression may be associated with the changing composition of microtubules. This can subsequently influence specific protein interactions on the surface of microtubules leading to an enhanced cold tolerance.[29]

Moreover, activity of some tubulin genes is environmentally regulated. Indeed, the expression of the *AtTUB1* gene is down-regulated by light in the hypocotyl, but not in the root.[84] The *AtTUB8* expression is down-regulated in response to low temperature[85] whereas the expression of *AtTUB9*, a gene

from a different TUB subfamily, is up-regulated.[64] Thus, tubulin gene expression is regulated by both developmental and environmental signals.

4. Regulation of the expression of tubulin genes

Tubulins play a crucial role in plant development and, therefore, their expression and post-translational modification are tightly regulated. As shown above, the number of *TUA* genes is generally lower that of *TUB* genes for a single plant genome, and their overall expression can greatly vary in the same tissue. This raises the question as to how the cell maintains a balance between the levels of the various TUA and TUB isotypes. Both subunits are incorporated into an MT in an equimolar ratio, and any excess in free TUB is lethal in yeast cells.[89] Co-transformation of *TUA* and *TUB* was an essential requirement for recovery of transgenic plants, as over-expression of only a single gene was lethal in maize[21] and tobacco,[90] as well as in other plants[91] (see also a chapter in this book). Up-regulation of *Eucalyptus* β-tubulin in poplar was also shown to be cytotoxic.[51] Therefore, plant cells are unable to tolerate large imbalances in the ratio of α- to β-tubulins when constitutive over-expression of only one subunit occurs, presumably because large cytoplasmic pools of free β-tubulin are cytotoxic. Using an antisense approach, full Arabidopsis plants were able to regenerate, where only the α-tubulin subunit (*AtTUA6*) was targeted, however, only small decreases in α-tubulin were detected in regenerated plants.[61] Despite only a moderate reduction in *TUA* expression, root development was severely affected. Root tip cells contained aberrant microtubular structures and were unable to undergo regular cell division.[61] A decrease in the regeneration efficiency was also noted in this study, and the authors suggested that in the cells, in which the silencing effect was high, this imbalance was likely to have caused cell death, and, therefore, reduced transformation and regeneration efficiency.[61]

The 5'-flanking regions of tubulin genes were often used to study the patterns of tubulin gene expression using the GUS reporter system.[31,60,62,64,65] The level and distribution of GUS expression was mostly consistent with the patterns of mRNA expression for the corresponding gene. However, the systematic mutational analysis of the tubulin promoter sequences was not done so far, although it was also shown that a sequence of only 97 bp 5'-upstream of the ATG was sufficient to confer a specific pattern of expression of the Arabidopsis *AtTUA1* gene in developing anthers and pollen.[62] The tissue-specific GUS expression was largely dependent on the length of the maize *ZmTUA1*[92] and *ZmTUA3*[60] 5'-upsteram regions. Recently, it has been also shown that introns are essential and specific elements for the regulation of the *TUA* genes at least in rice[40] (also in this

book). The first intron position (between 31/32 codons) in Arabidopsis *AtTUA2*, *AtTUA4*, *AtTUA6* and cotton *GhTUA6*, *GhTUA9* and *GhTUA13* genome sequences is exactly identical for all these genes, while other introns placed in different positions or are absent. This implies that the first but not the other introns in *TUA* genome sequences may be essential for regulation of the respective gene expression.

Early, Breviario and Nick[12] proposed that the expression of multigene β-tubulin family fulfills regulatory functions rather than resembles the functional specificities of distinct subunits. Altogether, the transcriptional regulation of tubulin gene expression may be an even more important aspect of MT function in plants than previously anticipated.

Tubulin folding cofactors TFC B and Arl2 have been characterized in Arabidopsis as *PILZ* group mutants and are generally embryo lethal, because they fail to form specific microtubule arrays in a cell cycle-dependent manner.[93] Moreover, in the absence of microtubules, both nuclear division and the cellularization of the syncytial endosperm were blocked.[93] The tubulin-folding co-factor A (TFC A), a microtubule chaperone protein, is not essential for proper seed development in *A. thaliana*, but the gene is involved in maintaining a correct balance of α- and β-tubulins by binding and consequently reducing the toxic effects of free β-tubulins.[94] The expression of the barley orthologs *HvTFC A*, *HvTFC B*, along with that of the associated small G-protein *HvArl2*, is perfectly correlated with those of *TUA* and *TUB* genes during barley seed development.[72] We have shown that *HvTFC A*, *HvTFC B* and *HvArl2* are co-expressed with the tubulin genes, and have concluded that the tubulin folding cofactors, together with the *HvArl2*, must participate in MT formation during the development of the barley grain. Furthermore, we suggest that the *PILZ*-like genes are involved in the control of polymerization of TUA/TUB heterodimers.[72]

Besides transcriptional regulation, plant tubulins undergo diverse post-translational changes, including polyglycylation, tyrosination, detyrosination, acetylation and polyglutamination.[95] Certain types of posttranslational regulation of α-tubulins preferentially occur in a tissue-specific way.[96] On the other hand, as discussed by Mayer and Jürgens,[9] a number of microtubule-associated proteins are involved in the organization of tubulin subunits and, therefore, additional factors may specify the recognition of particular TUA/TUB isotypes.

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ARRAY TECHNOLOGY FOR STUDYING MAIZE TUBULIN

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Abstract. Microarrays can quantitatively measure the expression of thousands of genes simultaneously but they are also powerful tools to examine the expression of specific genes in different tissues and cell types. They complement other methods used to detect gene expression at the transcript level, including Northern blotting and RT-PCR. This study uses oligonucleotide microarray expression profiling to compare maize β-tubulin (*tub*) gene expression at the transcript level in different tissues and during various stages of development and adds to what is known about maize β-tubulin expression. In maize, at least nine β-tubulin genes are expressed. The presence of multiple *tub* genes suggests that some could have evolved to provide fine control of expression or even have distinct biological or physiological properties in the plant. This is supported by the differential expression at the transcript level of the maize β-tubulins in different tissues during development. The following work has added evidence of maize β-tubulin gene differential expression and regulation. Based on microarray data, some β-tubulins exhibit statistically significant differences in transcript abundance in 11-day old seedling shoots, immature ears, 19-DAP embryos, 13-DAP endosperm and 19-DAP endosperm. There are also different expression patterns for many of the tubulin genes within the tissues studied. As the plant develops it may use β-tubulins in different amounts in specific tissues, and these tubulins may even interact differently with other proteins present at these developmental stages. In immature ears, *tub1* and *tub2* have higher transcript abundances. Comparisons of expression patterns indicate that *tub3* and *tub4* have very similar expression patterns, which may be a result of similar transcriptional regulation. The highest transcript abundance of these two genes occurs in developing seedling shoots. *tub3* and *tub4* transcripts have also been detected for the first time in maize endosperm. This chapter describes a variety of methods

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for array analysis of tubulin gene expression as well as important considerations before taking on this type of work.

Keywords: GCRMA, GeneChip, hybridize, maize, MAS 5.0 (Affymetrix Microsuite 5.0), microarray, RMA, Significance Analysis of Microarrays (SAM), *tub* (tubulin gene), cDNA

1. Introduction

The major components of microtubules are heterodimers of α- and β-tubulins. In maize (*Zea mays* (L.)), there are at least eight α-tubulin genes but the expression of only six has been detected.[1] There are at least nine expressed β-tubulin (*tub*) genes.[2,3] While some or all may be functionally redundant, some could have slightly different biochemical properties or distinct roles in the plant, for example, during development or in response to different stimuli. Determining if there are significant differences in the properties and physiological roles of the maize α- and β-tubulins is an ultimate goal, but the focus of this research is the β-tubulins. Studies using Northern blots, dot blot hybridizations, RNAse protection assays, 2D electrophoresis, and antibodies to the β-tubulin family provide evidence of differentially expressed β-tubulins within maize tissues.[2–7] Additional evidence is provided here through the use of oligonucleotide microarray expression profiling of maize β-tubulins.

A DNA microarray is also known as a DNA chip, gene array, genome chip, or GeneChip®, a registered trademark of Affymetrix, Inc. (Santa Clara, CA), referring to its high density, oligonucleotide-based DNA arrays. Affymetrix GeneChips® have become the industry standard but other companies do manufacture oligonucleotide based chips.

Briefly, on a DNA microarray, DNA is covalently attached to suitable, solid matrices as microscopic spots at known, fixed locations. These probes are high density; each spot contains a large number of identical DNA molecules or fragments. These probes range from 20 to 100s of nucleotides long. Targets are hybridized under highly stringent conditions and hybridization is usually determined by fluorophore-based detection.

Microarrays are used as a means for expression profiling at the mRNA level. If a gene is active, many mRNA molecules are produced and the RNA levels are used to extrapolate gene expression. Some benefits of working with RNA rather than proteins are that RNA is more easily produced, fewer precautions are needed with its handling, and RNA will

naturally hybridize with complementary sequence given the proper environment.

Thousands of publications exist on the subject of microarrays. This is not meant to be a complete review of everything that is available but a review of some of the more relevant tools available for analyzing the cytoskeleton.

2. Array platforms

The three major types of microarray platforms are cDNA arrays, short oligonucleotide arrays, and long oligonucleotide arrays (reviewed, for example, in Gabrielson et al.[8]; Barrett and Kawasaki[9]). The cDNA arrays are constructed using long, highly variable length cDNA clones from cDNA libraries, which consist of plasmids maintained in bacteria. The cDNA inserts are PCR amplified using universal primers that correspond to the vector sequence on each side of the gene sequence. The amplified PCR products are analyzed, quantitated, printed onto slides, and fixed according to one of numerous methods.[9] The cDNA on the array are hybridized to samples that were generally radio-nucleotide or fluorescence-labeled.

Oligonucleotide array[10] construction avoids the need for cDNA libraries. Synthesized oligonucleotides are either short (20–25 base) or long (50–80 base), with the short synthesized oligonucleotide arrays being the most widely used. Long oligonucleotide arrays are produced by making oligonucleotides that are the same size, but have little sequence homology. They can be produced by *in situ* (on-chip) synthesis and printing onto a suitable surface. The longer the oligonucleotides the more dependent the process is on using algorithms to keep the oligonucleotides at a similar G-C content, limit homology, and avoid repetitive or hairpin sequences.[9]

Short (25-mer or 30-mer) oligonucleotides are arrayed *in situ* using, for example, a photolithographic oligonucleotide deposition process; methods vary by manufacturer. High-density maize oligonucleotide microarrays produced by Affymetrix use EST contig sequences or chromosome segments to produce the synthetic oligonucleotides. A typical short oligonucleotide array (GeneChip® array) format is a 1.28 x 1.28 cm chip with up to 500,000 different oligonucleotide sequences.[9] Probes are grouped into sets containing 11–20 distinct probes that may have overlapping sequence.

Published reports on the effects of the array technology chosen for expression analyses are conflicting. Notably, among the plethora of reports are those of Kane et al.,[11] Yuen et al.,[12] and Yauk et al.[13] Yuen et al.[12] compared the accuracies of the short oligonucleotide array (Affymetrix GeneChip®) and a custom printed cDNA array. They found that both

performed identically, each time underestimating changes in mRNA expression. Comparing the bias in the two, the cDNA array was more predictable than the oligonucleotide array and therefore lends itself more to calibrations to compensate for the bias. Kane et al.[11] compared cDNA and long (50 mer) oligonucleotides on the same glass microarray and showed no difference in sensitivity, each having a sensitivity that could detect ~10 mRNA copies per cell (reviewed in Barrett and Kawasaki[9]). Yauk et al.[13] performed a comprehensive evaluation of different platforms, labeling techniques, and hybridization protocols to compare gene expression between two samples. They compared spotted cDNA sequence or PCR product, short oligonucleotide and long oligonucleotide platforms, various methods of probe spotting, labeling techniques from fluorescent labeling with cyanine dyes to secondary labeling with biotin-/streptavidin-conjugation, and various protocols for running the analysis. Their findings were that, when using high-quality systems and proper normalization, biological factors, not technological factors caused the variances in data between systems.

Many aspects of this type of research, including the choice of array, data analysis methods, and validation methods (reviewed in Barrett and Kawasaki[9]; Schadt et al.[14]; van Hal et al.[15]; Schulze and Downward[16]; Šášik et al.[17]) should be considered based on specific needs. The requirements of each experiment will dictate some of the specific tools that are necessary for analysis, but when possible, a conservative approach, using multiple forms of analysis, should be employed.

2.1. SINGLE-CHANNEL AND TWO-COLOR MICROARRAY ANALYSES

2.1.1. *Two-color spotted microarray (also known as two-dye design, ratio-based, or two-channel microarrays)*

Two-color array probes are spotted onto the microarray surface in a matrix pattern, by one of many fabrication/production methods. These probes can be oligonucleotides, cDNA, or small PCR product fragments; usually 80–100 nucleotides long.

These arrays are used for comparison of two samples from different biological sources. Labeled cDNAs, or targets, are produced by reverse transcriptase labeling of mRNA isolated from each of the two samples. Each of the two sets of cDNAs is labeled with one of two different fluorophores[18] then the two labeled sets are combined in equal amounts and hybridized with the probes on the array. The fluorophores used differ by the wavelength at which they emit their fluorescence. Examples of common fluorescent dyes used are Cy3, with a maximum absorption wavelength of ~550 nm and fluorescence emission wavelength of 570 nm

(green), and Cy5, with a maximum absorption wavelength of ~649 nm and fluorescence emission wavelength of 670 nm (red). When the combined targets are washed over the microarray, they competitively hybridize to their complementary sequences, with the proportion of hybridization corresponding to the amount of target. Laser beams, set to defined wavelengths corresponding to the absorption wavelengths of the dyes, are used to excite the fluorophores (Figure 1a). Visualization and measurements are done with a microarray scanner.

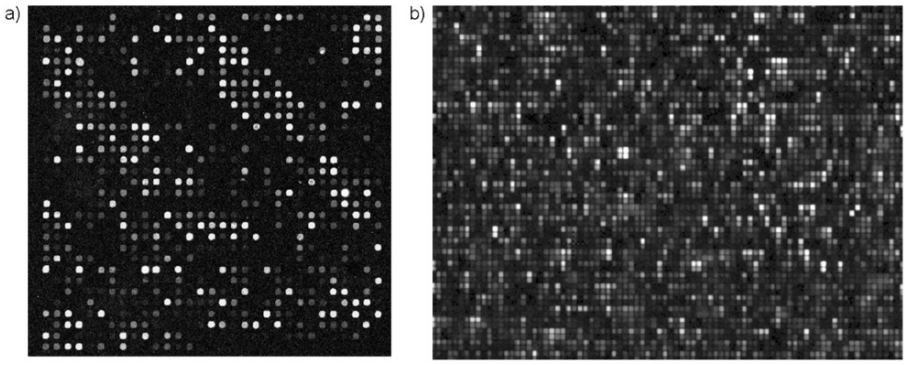

Figure 1. Two-color and single-channel microarrays. This example of a two-color microarray (a) is a maize 70-mer spotted oligonucleotide microarray (www.maizearray.org). The maize Affymetrix microarray (b) is representative of a popular single channel microarray (http://www.affymetrix.com/products/arrays/specific/maize.affx).

The ratios of the two samples are determined based on the level of fluorescence emitted. Using the common fluorescent dyes, Cy3 and Cy5, as examples, the RNA from the Cy3 labeled sample will appear as green spots if it is abundant and the RNA from the Cy5 labeled sample will be red. When they are equal in abundance, the spots will be yellow. A black spot is the lack of fluorescence indicating no expression of the gene corresponding to that spot. While this method cannot determine absolute levels of gene expression, it can determine relative expression levels.

2.1.2. *Single-channel microarray (also known as one-color and single-dye oligonucleotide microarrays)*

Compared to ratio-based arrays, the one-color analysis has considerable advantages. With the GeneChip®, for example, a one-color intensity-based system using immuno-fluorescent detection of biotinylated nucleic acids, comparisons can be made between samples that were not hybridized to the same array, and it does not rely on signal comparisons to a common reference or to other samples of that series.[19]

Sample preparation starts with total RNA extraction from cells/tissues of interest. This is followed by reverse transcription, synthesis of Biotin-labeled (complimentary) cRNA through transcription of the cDNA, fragmentation of cRNA, and then hybridization of the fragmented, Biotin-labeled cRNA to the array. Unhybridized cRNA are then washed off, and the array is stained. The chip (Figure 1b) is then scanned with a confocal laser scanner to determine fluorescent intensity at each probe cell, and the resulting images undergo signal intensity-based analysis by computer.

The more mRNA that is present, the brighter the fluorescent area. Fluorescence is directly proportional to the amount of mRNA in the pool. Unlike the two-channel microarrays that require comparison, these can give an estimate of absolute value of gene expression and avoid the need for competitive hybridization. A sample is labeled with a single fluorophore and hybridized to the array. The resulting fluorescence is proportional to the number of molecules that hybridized to each spot. This allows for the quantitative measurement of the expression of thousands of genes simultaneously by measuring fluorescence of gene-specific targets or probes.

2.1.3. *Affymetrix maize GeneChip®*

The Affymetrix maize GeneChip® (reviewed in Schadt et al.[14]; Meldrum[20]) contains probe sets consisting of 15 short (25-mer) oligonucleotides designed to detect a particular transcript. The probes are designed using EST contig sequences from multiple genotypes to account for possible sequence differences. The fluorescence intensity measured is related to absolute mRNA concentration.[19,21] Because the Affymetrix probes can distinguish most of the maize β-tubulins, these microarrays are a useful way of examining maize tubulin expression at the transcript level.

In this study, the abundance of transcripts for various β-tubulins is compared between inbred line B73 11-day old seedling shoots, immature ears, 19-DAP embryos, 13-DAP endosperm and 19-DAP endosperm. The very similar sequence of the tubulin genes and the correspondingly similar sequences and physical characteristics of the proteins they encode cause problems with their analysis. These similarities make it difficult to separate individual isoforms, create probes to distinguish each, and interpret data.

The maize GeneChip® contains approximately 17,555 probe sets that are designed to detect transcripts of 13,339 genes. Each probe set on the glass surface of the array includes 15 short oligonucleotides designed for a particular transcript and relies on redundancy to overcome a lack of specificity. There is not a one probe set to one gene ratio; instead there are multiple probe sets for some genes to detect sense and anti-sense expression or alternate transcript expression. There are two types of GeneChip probes

that make up a probe pair: perfect match (PM), which is identical to the templates found in databases, and a corresponding single mismatch (MM), which has a substitution of a single base near the middle (18th) position. Each PM probe and the corresponding MM probe contains thousands of short (25-mer) sequences (ideally identical) that are taken from the transcribed sequence of the gene represented by that probe set. Hybridization of labeled mRNA molecules to the GeneChip® follows the Langmuir adsorption (formula) model in which absolute mRNA concentration is related to fluorescence intensity.[21] When hybridized with a fluorescent-dye-labeled RNA, each probe in the probe set captures a certain amount of RNA, whose fluorescent intensity, based on the amount of RNA bound, is subsequently measured. MM probes are used as controls because they are expected to detect any non-specific hybridization. By determining the difference of the match versus mismatch (PM – MM) signal, the true signal is extrapolated; the true signal is proportional to the mRNA concentration of the target. One complication is that the sequences in each probe set have an unknown hybridizing efficiency unless each is analyzed.

Other methods used to detect transcript levels include Northern blotting and RT-PCR, but microarray analysis has clear advantages. With microarrays being automated, one can analyze expression of a greater number of genes in a fraction of the time that it would take with the other methods. Microarrays also allow for the processing of many of these genes in parallel thus avoiding setbacks inherent with sequential processing. Because the Affymetrix probes can distinguish the maize β-tubulin mRNAs, these microarrays are a useful way of examining maize tubulin expression at the transcript level.

There are several steps to the analysis of these high-density oligonucleotide arrays, and the different options available have different effectiveness. The key steps are (1) using the estimate of the nonspecific signal to adjust the perfect match probe signal (2) normalization at the probe set level to center the plot and (3) choosing the best test statistic.[22]

3. Analysis software packages and their properties

There are many software packages that calculate gene expression levels from fluorescence intensities, each having characteristics that may be desirable or limiting depending on individual experimental needs.[21–29] The model-based method of Li and Wong[23] depends on a large number of microarrays in an experiment, assumes probe-specific background measured by mismatch probes and cannot be used for an isolated microarray. Šášik et al.[26] proposed a multiplicative, not additive noise model that assumes the PM probe intensity, after uniform background

adjustment derived from both MM and PM intensities, is directly proportional to the concentration of the transcript. A method by Naef et al.[24] is designed for paired microarrays, such as resulting from an experiment in which each "treatment" sample has a corresponding "control" sample.[26]

The Affymetrix Microsuite 5.0 (MAS 5.0) and Bioconductor's log scale robust multi-array analysis (RMA) utilize algorithms of a purely statistical or empirical nature. MAS 5.0 is based on taking the anti-log of a robust average (Tukey biweight) of values derived from log(PMij – CTij) where i = probe pair and j = array (see next section for definition of CT), assumes probe-specific background measured by mismatch probes and is tailored for analysis of a single microarray.[28] Therefore, MAS 5.0 can be used with any experimental design. RMA employs a log-additive robust multi-array analysis. GCRMA is based on physical properties of the hybridization process. It uses sequence information to perform background adjustments.

Both RMA and GCRMA make theoretical predictions to subtract background estimates before fitting the expression measures. GCRMA is fairly insensitive to low concentrations (less than 1 pM) while MAS 5.0 can potentially detect changes in concentration as low as 0.1 pM. RMA has even better precision for lower expression values than MAS 5.0.[27] Both GCRMA and MAS 5.0 become less sensitive in the high concentration range, with MAS 5.0 showing evidence of saturation at concentrations above 100 pM.[28] Both have a tendency to overestimate concentration changes.[29]

When using microarray-based technology, there are potential non-biologically relevant variations to consider. A variety of software programs correct for them, by means of background correction, between-slide normalization, or perfect match correction to eliminate non-specific hybridization.

This work uses the Bioconductor Affy Package (http://www.bioconductor.org) within the R statistical language (http://www.rproject.org/) and processes the data using the Robust Multi-chip Average (RMA) algorithm. To determine which *tub* transcripts were present in each tissue, MAS 5.0 was used to compare the PM to the MM intensity and then label the transcript to which it correlates as Absent, Marginal or Present.

4. Considerations when designing a microarray experiment

There are limitations to the ability of microarray experiments to detect small differences.

Choe et al.[22] assessed the performance of the most popular methods (at the time) for analysis using control datasets. They found, through

comparison studies, that the performance of the normalization methods was similar, but the method chosen to adjust the PM can influence accuracy. When attempting to perform these analyses using different combinations of each step of the available methods, Choe et al.[22] found that, in the "best-case scenario," approximately 95% of truly differentially expressed genes with changes greater than twofold could be detected. Less than 30% of differentially expressed genes with changes below 1.7-fold could be accurately detected. They concluded that even with a "best-route" combination of analysis methods, only approximately 70% of true positives were detected before reaching a 10% false-discovery rate. This shows that there is a definite need for improved analysis methods for Affymetrix microarrays.

Two major factors that affect raw data of microarrays are sequence-dependent probe hybridization and chemical saturation resulting from a surface adsorption process. The affinity between probe and target is dependent on both GC content and length of sequence, and some of the probes do not perform as well as others. Without proper planning before manufacturing of the arrays, probes on the same array may have different affinities for their targets. In other words, they do not hybridize to the target as well as others, and this may confuse the results of the expression analysis.[17]

Work is underway to correct for these effects, but with what is currently known of GeneChip® analysis, it is incorrect to assume that there is a linear relationship between measured intensity and concentration.[21] If GeneChip® hybridization follows the Langmuir adsorption model, it is possible to predict how the calibration curves saturate; this induces a "marked compressive bias in differential expression estimates, most severely at high concentrations".[21] Hekstra et al.[21] discuss a simple model of surface adsorption and how, with knowledge of the sequence composition of the probes, the GeneChips® can be calibrated. With this model, based on GeneChip® data following Langmuir isotherms and fitting three of the model's parameters to the probe sequence composition, estimators can be produced for both absolute concentrations and expression ratios.

Currently still under scrutiny is the process of removing non-specific background. A common method is the use of the MM process to estimate the background signal. This method assumes that the majority of the MM signal is non-specific. However, there is evidence to show that the MM signal is a result of both specific and random non-specific signal components. The MMs are not considered an independent reference since the MM sequence only differs from the PM sequence by a single-nucleotide and may bind the targets of the PM probe.[17,25] Although many of the standard forms of analysis rely on the removal of the MM signal from the

PM signal, making a direct subtraction will unlikely be effective. MAS 5.0, in particular, allows the expression index or average difference to be negative when the MM signal is larger than the PM signal; if negative, the transcript is still considered present. When the expression index is a negative value, an imputed value for the contrast value (CT), which takes the place of the MM value (PMi – CTi, where i = probe pair), is used.[28] If, within a probe set, there are a few probe pairs that result in a negative value when subtracting PM – MM then the CT value is created by using the average ratio of the complete probe set's PMi/MMi values.[28] Beyond difficulties in choosing the appropriate method to subtract non-specific signal, some studies have found that including MMs in the analysis, either by subtracting them from the PM or pooling them with the PMs, results in increased noise levels.[21,29] Hekstra et al.[21] suggest that the MM probes should be replaced with additional PMs with non-redundant sequences and Irizarry et al.[27] suggest just ignoring these values is preferable. However, no change in methodology has been adopted by Affymetrix. Multiple methods should be tried. Bolstad et al.[30] proposes the use any of three probe intensity normalization methods that use data from all of the arrays used in an experiment. These are available as part of the R package (from the Bioconductor project http://www.bioconductor.org).

Hybridizing temperature is also a factor. The experimental protocol calls for a 42°C hybridizing temperature, but the ideal setting would be that at which the PM probes bind to their corresponding RNA and the MM probes do not. Temperatures below the ideal may result in stable, unspecific binding, and result in the MM probes binding the transcript. Because the G-C content of the individual probes may vary, their optimum hybridizing temperature would vary. MAS 5.0, for example, is designed with the assumption that it is possible to compare concentrations of the same transcripts from different experiments but not different transcripts, and it does not compute sequence specificity during analysis.[21,23] Depending on the extent of G-C variation between probes, the inclusion of GCRMA may be advantageous.

In this study, Affymetrix oligonucleotide microarrays for maize gene analysis are used to study the expression at the transcript level of maize β-tubulin genes. The expression of each β-tubulin is compared across all of the tissues studied, and a comparative study of each tissue is performed to determine difference in expression of all of the β-tubulin. RMA was chosen as the main software package to analyze the data obtained from the GeneChip study. RMA is used in conjunction with SAM (Significant Analysis of Microarrays) analysis[31] to control the parameters that indicate statistically significant changes in expression. MAS 5.0 was used to confirm that the *tub* transcripts were present in each tissue.

5. Materials and methods

Maize Affymetrix GeneChips® were used to profile the expression patterns of the known β-tubulins in maize inbred lines B73. Microarray hybridizations were performed with RNA isolated from 11-day old seedling shoots, a stage at which there is rapid cell division at the shoot apex and in leaf basal meristems, and cell elongation everywhere else, immature (3.7–5.4 mm long) ear containing meiotic cells, developing embryo tissue collected 19-DAP, and tissue from 13-DAP and 19-DAP endosperm, developmental stages when cell division is still occurring in the outer region of the endosperm.[32]

Dr. Nathan Springer (Department of Plant Biology, University of Minnesota) contributed raw GeneChip® data from microarray analyses.[33] All subsequent analyses were performed using that data. The materials and methods section is split into two parts; the first presents materials and methods used by the Springer laboratory for collection and processing of materials and microarray hybridizations. The second includes the methods used to select maize tubulins, analysis by MAS 5.0 and RMA, and statistical analysis of significance by SAM performed using .cel files received from the Springer laboratory.

5.1. PLANT GROWTH AND TISSUE COLLECTION

Details of the growing and collection methods of the seedling shoots, ear, and embryo can be found in Stupar and Springer.[33]

11-day old seedling shoots: After growing for 11 days in a green house, the plants were cut to include all above-ground tissues and meristems. All except embryos were flash frozen in liquid nitrogen and stored at -80°C prior to RNA isolation. *Immature Ears*: A range of immature ears from 3.7–5.4 mm in length were collected from field-grown maize plants. *Embryos*: Embryos were collected from kernels of field-grown maize plants at the 19 days after pollination (DAP) stage, frozen in a dry-ice ethanol bath and stored at -80°C. *Endosperm*: Endosperm was collected from field-grown maize plants 13 days after pollination and 19 days after pollination.

5.2. RNA ISOLATION AND MICROARRAY HYBRIDIZATIONS

Complete details of RNA isolation and microarray hybridizations can be found in Stupar and Springer.[33] The biological replicates of each of the tissues were ground in liquid nitrogen. RNAs were extracted, DNAse treated, and extracted with phenol:chloroform. Resuspended RNAs were further purified, quantified, and qualified. Probe sets chosen had closest identity to *tub* transcripts (data not shown).

5.3. PRESENT-ABSENT GENE ANALYSIS

Details of the present-absent gene analysis can be found in Stupar and Springer.[33] Due to the difficulty of concluding whether a transcript was absent, present or marginally represented each data point was given a score. Absent was given a score of 1, Marginal a score of 2 and Present a score of 3.

5.4. STATISTICAL ANALYSIS USING MAS 5.0 AND RMA

Raw data of the spot intensities from the Affymetrix microarray analysis were analyzed using the Bioconductor Affy Package (http://www.bioconductor.org) within the R statistical language (http://www.r-project.org/), and Robust Multi-chip Average (RMA) algorithm. RMA was used in conjunction with SAM (Significant Analysis of Microarrays) analysis to control the parameters that indicate expression changes of twofold and greater. MAS 5.0 was used to confirm that the *tub* transcripts are present in each tissue. All necessary analyses, standard deviation calculations, and t-test statistical analyses (two-tailed distribution, assuming equal variance, false discovery rate (FDR) of $P = 0.05$) were then performed.

5.5. DETERMINATION OF SIGNIFICANCE

Significance Analysis of Microarrays (SAM) software (© Trustees of Leland Stanford Junior University) was performed to determine if each of the tubulin genes was at least twofold more or less expressed between the tissue types. The data, in logged (base 2) scale, were analyzed by SAM software programmed to calculate using two class unpaired response type, T-statistic, 100 permutations, automatic estimate of sO factor for the denominator. The imputation engine was set at K-nearest neighbors imputer with the number of neighbors set at 10, and the random number seed was generated randomly for each pair analysis. Following initial SAM analysis using these parameters, the data output was limited to a twofold change and the delta table was calculated. The delta value was manually determined by limiting the number of median false positives to less than 1.0. SAM analysis was run again using the twofold change cutoff and the delta value correlating to less than 1.0 median false positives. A list of significant genes was then compiled showing all genes with at least a twofold increase or decrease in expression. From this data, using the Affymetrix GeneID for each β-tubulin gene, the *tub* genes that were shown to be significantly expressed were manually determined.

6. Results

6.1. PRESENCE OF TRANSCRIPTS

The data from the MAS 5.0 presence-absence calls (Table 1) indicate that the β-tubulins 1, 3, 4, 5, 6, 7 and 8 were called "present" in all biological replicates of all tissues and all genotypes. β-tubulin 2 was called present in all replicates of seedling shoot, ear, embryo, and 13-DAP endosperm but was marginal in 19-DAP endosperm.

TABLE 1. Assignments of absent, present and marginally present scores to determine presence of transcripts of each maize β-tubulin. Scores of 1, 2 and 3 indicate absence, marginal presence, and presence, respectively. A score of 2.5 is the lowest acceptable score for confidence in presence of the transcripts.

β-Tubulin	Probe set	Seedling shoot	Ear	Embryo	13 DAP endosperm	19 DAP endosperm
tub1	Zm.2458.1.S1_at	3	3	3	3	3
tub2	Zm.79.1.S1_at	3	3	3	3	2.25
tub3	Zm.7172.1.A1_at	3	3	3	3	3
tub4	Zm.3257.1.S1_at	3	3	3	3	3
tub5,8	Zm.4718.1.S1_at	3	3	3	3	3
tub6	Zm.7750.1.S1_at	3	3	3	3	3
tub7	Zm.16988.1.A1_at	3	3	3	3	3

Probe sets have been named based on which β-tubulin gene they are specific to, and correspond to the following AffyID's: *tub1*(Zm.2458.1.S1_at), *tub2*(Zm.79.1.S1_at), *tub3*(Zm.7172.1.A1_at), *tub4*(Zm.3257.1.S1_at), *tub5,8*(Zm.4718.1.S1_at), *tub6*(Zm.7750.1.S1_at), *tub7*(Zm.16988.1.A1_at).

6.2. EXPRESSION PATTERNS OF EACH β-TUB GENE BETWEEN B73 TISSUES

B73 is chosen as the representative genotype for *tub* expression studies. B73 is a commonly studied inbred line and it was used in previous maize β-tubulin transcript studies by Villemur et al.[3]

Figure 2 shows the normalized tissue-specific *tub* gene expression patterns in B73. There are clear differences in the *tub* expression levels between the tissues studied. *tub3* and *tub4* have the same pattern of expression within the tissues studied but have different patterns than the rest of the *tub* genes, indicating a commonality in how these two genes are regulated. *tub2* also has a very unique pattern of expression compared to the

other *tub* genes. The remaining probe sets also show patterns that differ from the others, with *tub1* having very definitive expression differences between the ear, embryo and 19-DAP endosperm, and probe sets for *tub6* and *tub7* showing less dramatic changes of these two tubulins between the tissues studied. Probe sets *tub1*, *tub2*, *tub5*,*8* and *tub6* also show that these tubulins have significantly less expression in 19-DAP endosperm. The pattern changes that exist between the *tub* genes (Figure 2) suggest a difference in their transcriptional regulation within tissues.

6.3. COMPARISON OF EXPRESSION OF EACH β-TUB GENE ACROSS B73 TISSUES

The data suggests that there are many cases of differences in particular *tub* gene expression between the tissues studied. There are also some more striking transcript abundance differences that are greater than twofold (Table 2). *tub2* shows at least a sixfold increase in expression in the young, 3.7–5.4 mm long ear compared to all other tissues studied, and *tub3* is least expressed in the embryo. *tub4* expression is least expressed in the embryo and more expressed in the seedling when compared to all other tissues.

TABLE 2. tub intensity scores in the B73 tissues studied with the locations of over twofold difference indicated. Microarray intensity scores for the probe sets specific to *tub* genes in B73 are presented with standard deviations indicated within parentheses. Arrows indicate over a twofold *tub* intensity difference in a tissue compared to the other tissues studied.

	Seedling	Ear	Embryo	13-DAP endosperm	19-DAP endosperm
tub1	3,679 (±87)	9,754 (±432)	7,891 (±178)	4,374 (±563)	1,865 (±41)
tub2	249 (±48)	1,373 (±69) ↑	198 (±33)	225 (±43)	119 (±12)
tub3	2,232 (±67)	1,565 (±244)	202 (±8) ↓	466 (±14)	581 (±32)
tub4	4,879 (±495) ↑	770 (±151)	128 (±9) ↓	446 (±46)	555 (±22)
tub5,8	5,976 (±341)	7,784 (±125)	6,660 (±188)	3,399 (±463)	995 (±75) ↓
tub6	2,691 (±202)	2,502 (±179)	3,065 (±110)	2,397 (±75)	1,835 (±100)
tub7	7,546 (±183)	9,353 (±199)	6,416 (±176)	7,583 (±123)	7,697 (±281)

Due to the available probe sets and the similarity in sequence, *tub5* and *tub8* were studied together. The expression data obtained from the probes in probe set *tub5, 8* may be the levels of expression for the combination of both *tub5* and *tub8* or the expression of only one of these if the other *tub* is not expressed. While this limits the conclusions that can be drawn, the data can still be used to detect if patterns exist in reduced expression levels. The data indicate that *tub5* and/or *tub8* are less expressed in 19-DAP endosperm than all other tissues.

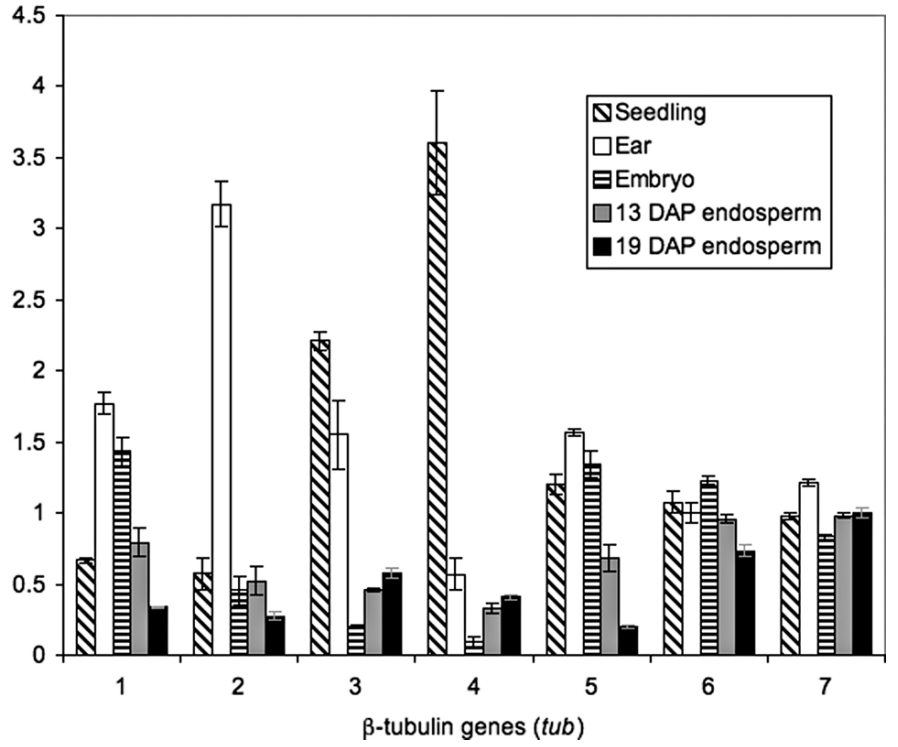

Figure 2. Relative tub gene expression in B73 tissues. Samples from B73 11-day old seedling shoots, immature ear, 19 DAP embryo, and 13 DAP and 19 DAP endosperm tissues were analyzed. Relative expression levels were determined for each *tub* gene represented by one or more microarray probe sets. There are similarities in expression patterns between the *tubs* but *tub2*, and *tub3* and *tub4* show more unique expression patterns in comparison to the remaining *tub* probe sets.

7. Discussion

In this study, the GeneChip® data was analyzed by GCRMA (not shown), MAS 5.0, and RMA followed by SAM (Statistical Analysis of Microarrays),[31] to determine significance of expression. A conservative approach to analyze expression was chosen here based on what is commonly used in microarray analyses. One's individual experimental needs as well as changing industry standards will dictate which analytical methods are appropriate, as presented in the introduction.

7.1. DETERMINING SIGNIFICANCE OF EXPRESSION LEVELS

SAM parameters were adjusted to control the criteria that would indicate significant differences in expression; the main criterion is that it must have a minimum expression level fold change of two. Using SAM in traditional expression studies, a twofold difference is commonly utilized to determine, among the thousands of genes being studied, that there are genes with significant differences in expression, and that they are in need of further analysis. This is not necessary for biologically significant expression differences when looking at a manageable number of genes. The twofold difference requirement may hide smaller but still significant changes, especially those expected between genes as similar as the maize β-tubulin genes. Table 2 shows, by the inclusion of arrows, the *tub* genes that show a twofold expression level difference when compared to the other *tub* genes (as determined by SAM). While these are indicative of a difference in expression, Figure 2 and expression scores in Table 2 show statistically significant differences in expression at a less stringent, more inclusive level, and gives a clearer picture of how the expression of each *tub* compares in all of the tissues studied.

7.2. CHOOSING REPLICATION STRATEGY WHEN DESIGNING A MICROARRAY STUDY

As with any biological analyses, the number and type of replications must be considered. First, the hybridizations should be repeated with RNA from multiple samples rather than by repeating the hybridizations with RNA from the same sample. This approach fulfills the need to account for biological variability. A minimum of three replicates is needed for more accurate statistical analysis. These considerations are commonly accepted and have been adopted in this research. Although this is common in array studies there may soon be a movement to increase the number of replicates. Pavlidis et al.[34] focused on determining the number of replicates that would

be necessary for array experimentation, and concluded that at least five biological replicates are needed for stable results.

Microarray analysis used independently is sufficient to analyze transcript abundance; although sufficient, it is still commonly expected that, to ensure validity of the microarray analysis, results from additional quantitative gene expression techniques accompany the microarray results.[17] Based on the direction of the field, there will be continuing refinement of the technique, like increasing the number of replicates that are commonly used, to establish greater confidence in the resulting array data.

7.3. DETERMINATION OF PRESENCE OF TRANSCRIPT CORRELATED TO INTENSITY OF GENECHIP® SIGNAL

Transcripts of all the *tub* genes, except *tub2*, were found in all of the tissues studied (Table 1). *tub2* may not be in 19-DAP endosperm of B73, but it does show "presence" and "marginal presence" in the 19-DAP endosperm of Mo17 and F_1 hybrids of B73 and Mo17, respectively (data not shown).

Users of the Affymetrix maize GeneChip® can attempt a rough estimate of the copy number (from 1:50,000 to 1:6,667 copy number ratio) of transcripts of interest using *B. subtilis* exogenous, poly-A RNA positive controls at known final concentrations. The Affymetrix GeneChip® contains control probes corresponding to these *B. subtilis* genes, which should not hybridize to transcripts found in the maize sample. The protocol includes adding control oligonucleotides at a known concentration into each sample that is analyzed. With many different, proven technologies available to measure the amount of transcript in samples, this methodology was not employed in this study.

7.4. COMPARISON OF GENE CHIP ANALYSES AND PREVIOUS STUDIES ON *TUB* TRANSCRIPTS

A variety of experiments to examine maize β-tubulin expression, including this study, have looked at similar maize tissues, but this study also looked at additional developmental stages. The data from these GeneChip® analyses are significant additions to the collective information about *tub* expression patterns and have indicated presence of *tub3* and *tub4* transcripts for the first time in endosperm. New insights into the control of expression of the β-tubulins and new questions to be investigated are a direct result of this work.

7.5. DIFFERENTIAL EXPRESSION OF THE *TUB* GENES

At the transcript level, evidence that the *tub* genes are differentially expressed has been supported using GeneChip® analysis. In this study, *tub* gene transcript abundances varied in many of the tissues studied, suggesting that differential expression exists in different tissues and at different stages of development of the same tissue (Figure 2, Table 2). 19-DAP endosperm shows reduced *tub1*, *tub2*, *tub5,8* transcripts when compared to other tissues (Table 2). This is at a developmental stage when there are few cells undergoing mitosis or cytokinesis and no cell expansion, and thus possibly not much need for tubulin. While the level of expression of each of the *tub* genes varied significantly between some of the tissues studied, some have produced particularly interesting results.

7.5.1. *β-tubulin 1*

Past studies by Villemur et al.[3] included dot blot hybridization and RNAse protection assays to detect *tub* transcripts in tissues from inbred lines B73 and A188. They determined the relative transcript abundances of *tub1*, *tub4*, *tub6*, *tub7* and *tub8* in a number of tissues, some similar to those analyzed in this study. Among the tissues analyzed, Villemur et al.[3] looked at 6 day old seedling shoot (including first internode and coleoptile), 15 DAP embryos and endosperm, and immature cobs (1–2 cm). For *tub1*, I saw a similar pattern of transcript abundance but with slight differences. Villemur's findings showed that the immature cob and embryo had the highest transcript abundance but the distinction of which was highest could not be made with confidence. This microarray study indicates that *tub1* was expressed most highly in immature ear, at a developmental stage where there are cells going through meiosis, followed by the embryo (Figure 2, Table 2). The comparison between the abundance of seedling shoot and 15-DAP endosperm in Villemur et al.[3] showed that there was lower transcript abundance in the endosperm. This can be extrapolated by the expected range that would be found between the scores for 13-DAP and 19-DAP endosperm in this study. This is likely due to reduced levels of mitosis and cytokinesis at these later stages of endosperm development. This microarray data confirms what was found previously and has allowed for further distinction.

7.5.2. *β-tubulin 2*

β-tubulin 2 protein has previously been detected in meiotically dividing, pre-emergent anthers[6,7] and transcripts encoding *tub2* have been found at various stages of development.[3] This GeneChip® data shows that, among the tissues studied, *tub2* transcript is most abundant in the immature ear

(Figure 2, Table 2). The increase in abundance in the immature ear, selected at a developmental stage when its cells are meiotically dividing, combined with previous knowledge about β-2 tubulin in meiotic spindles, suggests that *tub2* undergoes greater expression to increase the pool of tubulin available for the microtubules of meiotically dividing cells during sexual reproduction. Although there is an increase in abundance of the *tub2* transcript, β-tubulin 2 is still a very minor tubulin relative to others in meiotically dividing tissue.[7]

7.5.3. *β-tubulin 4*

The general pattern of *tub4* transcript abundance is similar between this work and that of Villemur et al.[3] Of the tissues studied, the *tub4* transcript is statistically more abundant in the seedling shoot. This work shows a more definitive difference between the abundance in the ear and endosperm than that found in Villemur's work, and the data from this study contradicts that found in Villemur et al.[3] with respect to the transcript abundance in the embryo. Villemur's findings suggest that statistically there may not be much difference between the amount of transcript in the embryo, immature cob and 13-DAP endosperm. Data from this microarray analysis (Figure 2, Table 2) suggests that there is a (statistically significant) lesser abundance of *tub4* transcript in the embryo compared to those tissues.

Earlier studies showed the *tub4* transcript was more abundant in shoot than root but still less than the abundance in pollen, and led to the postulation that β-tubulin 4 is involved in pollen tube growth.[3,5] The current work corroborates what had been reported earlier, showing that the transcript is most abundant in the seedling shoot compared to the other tissues studied. The seedling shoot at this stage is predominantly going through cell elongation.

This GeneChip® study does not agree with a previous northern blot analysis of *tub4* transcripts in maize endosperm by Rogers et al.,[5] who found that there was no detectable transcript in the endosperm. As indicated in Figure 2 and Table 2, *tub4* transcript is present but at a lower abundance.

tub4 transcript is present in all of the tissues studied but is highest in pollen, as found in previous studies, and, as this study indicates, there is also a high amount in the seedling. This data suggests that there is a shared requirement for β-tubulin 4 during seedling development at a stage consisting of extensive cell elongation and the stages of pollen tube growth. Further study is imperative to examine the β-tubulin 4 protein during these cellular events.

7.5.4. *β-tubulin 3*

Rogers et al.[5] detected no *tub3* transcript in 12-DAP maize endosperm using Northern blots, but this GeneChip® study detected the presence of the transcript in 13-DAP and 19-DAP endosperm and in a greater abundance than found in the embryo, the site of least transcript abundance.

Comparing the relationship between *tub4* and *tub3* transcript abundances in the tissues studied, the same pattern of expression between the tissues (Figure 2), and their similar sequence,[5] it is likely that they have similar biochemical roles or at the least be involved in the same cellular events.

8. Conclusions

This work supports differential expression of maize β-tubulins, and there are cases of over twofold abundance differences in different tissues. Most interesting, the *tub3* and *tub4* transcripts were found in maize endosperm where previously they were not detected.[5]

With the findings from this study, new directions for research have been presented. The key to these is the addition of protein analyses to determine what extent each of the maize β-tubulins is expressed in different tissues at different stages of development, and where they are localized in cells. RNA is not a direct indication of protein levels. Multiple factors, including the rates of translation and RNA degradation, add to the complexity of expression studies like these. Also, RNA alone is not sufficient for determining how the cells of different tissues incorporate the individual β-tubulins during development. This data will need to be paired with detection of the proteins occurring in each tissue and to where they are located to get a more complete picture of the roles of the maize β-tubulins.

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PART 5

CYTOSKELETON PROTEOMICS

THE MICROTUBULE PROTEOME: A ROLE IN REGULATING PROTEIN SYNTHESIS AND IMPORT INTO ORGANELLES?

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Abstract. Microtubules (MTs) are dynamic components of plant cells, and are organized into four major arrays. The growth and organization of MTs in these arrays is regulated by a group of structural proteins called the microtubule-associated proteins (MAPs). A number of MAPs have been identified in plants, some of which are plant-specific. Another group of MT-binding proteins that are well represented in plants are the kinesin-related motor proteins. A third and more loosely defined group of proteins that bind to MTs are the MT-interacting proteins. Binding of these proteins to MTs can serve to concentrate the protein, to regulate the activity of the protein, or to serve other functions. Numerous putative MT-interacting proteins were identified in two large-scaled studies. A group of proteins that were represented in one of these studies were peroxisomal matrix proteins. The MT and RNA binding activity of these peroxisomal proteins has led to a model that links MTs to protein synthesis and targeting of these proteins to peroxisomes.

Keywords: Microtubule, proteome, translational control, microtubule-interacting protein, peroxisome

1. Introduction

Microtubules (MTs) function in a wide range of cellular processes, from cell division to cell signalling. All eukaryotic MTs are structurally similar,

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and are composed of α/β-tubulin dimers that are assembled at the plus end of MTs. Plant MTs, however, have several unique traits related to their organization, dynamics and function, and assemble into four distinct arrays during the cell cycle[1–3] Three of these arrays – the pre-prophase band, the phragmoplast, and the interphase cortical array – lack a counterpart in animal cells. The fourth array, the metaphase spindle, is common to both plant and animal cells and serves a conserved role in the segregation of chromosomes. The pre-prophase band appears prior to mitosis and predicts the position and plane of cell division, while the phragmoplast assists in the construction of a new cell wall during cytokinesis. The interphase cortical array consists of a dynamic arrangement of MTs that lie on the inner face of the plasma membrane and may function in several processes, including a role in regulating cellulose microfibril synthesis.[4,5] In animal cells, MTs originate from a centriolar located MT-organizing center. In contrast, plant MT nucleation sites are located at the nuclear surface and scattered over the cell cortex. Plant MTs demonstrate a dynamic instability at both the plus and minus end, with the plus end exhibiting stochastic switching between growth and shrinkage.[6–8] This dynamic nature allows for rapid reorganization of MTs in the different phases of the cell cycle and in response to environmental signals.[9]

Many of the novel aspects of plant MT organization, dynamics and function can be understood by examining the proteins that bind to MTs. These MT-associated proteins (MAPs) are often conserved among eukaryotic organisms, however, several plant-specific MAPs have been identified. Proteins that bind to MTs can be classified into three groups.[10,11] The classic structural MAPs are proteins that function in regulating MT growth dynamics and organization, and can be co-purified with MTs through cycles of MT disassembly and assembly. The motor MAPs organize directional movements of cargo, or move MTs themselves. In addition to these two groups, other "MT-interacting proteins" have been identified that do not function in MT dynamics and organization.[10,12] These proteins might use MTs as a solid-state surface to regulate their own activity, to serve as a mechanism to concentrate the protein, or to perform other functions that are associated with the protein.[13] The term "MT-binding protein" will be used here when collectively referring to a classic MAP, MT motor, and MT-interacting protein.

In this chapter, we discuss MT-binding proteins and some of their unique roles in plants. An inventory of plant MAPs, MT motors, and MT-interacting proteins is not presented here, as these have been covered extensively elsewhere.[9,10,14] Rather, we discuss a few examples of plant MT-binding proteins and the techniques that are used to purify them, and review the results of two large-scale proteomic studies aimed at identifying novel

plant MT-binding proteins. We also discuss the possible function of a group of proteins that may regulate their own translation and import into peroxisomes via binding to MTs prior to import.

2. The microtubule proteome: classical MAPs, motors, and microtubule-interacting proteins

2.1. METHODS USED TO IDENTIFY MT-BINDING PROTEINS

The identification of plant MT-binding proteins has been achieved using DNA sequence database information, mutant screens, and biochemical purification approaches.[14–18] To determine that candidate MT-binding proteins have an authentic association with MTs, evidence for their co-localization *in situ* is required. Co-immunolocalization of candidate proteins with MTs in fixed cells provides the most convincing evidence for an interaction, as this technique labels endogenous cellular protein. However, if a protein is low in abundance, or if only a small percentage of the protein in a cell binds to MTs, detection of an association with MTs can be difficult. The expression of inherently fluorescent proteins coupled to candidate MT-binding proteins has been valuable in demonstrating MT interactions, and overcomes some of the limitations encountered by immunolocalization in fixed cells. However, strong constitutive promoters are often used to drive expression of these protein fusions, leading to unusually high expression levels that can result in non-representative binding to MTs. A combination of these techniques, including biochemical interaction experiments performed *in vitro*, provides solid evidence for an authentic MT association.

2.2. CLASSICAL MAPS AND MOTORS

The availability of plant genome sequence databases provides abundant information on genes that code for plant MAP orthologs, and has been useful in identifying conserved proteins in plants.[15,16] For instance, a single CLASP gene was identified in the Arabidopsis genome, and codes for a protein with 36% amino acid identity to a mouse CLASP ortholog.[15] Expression of Arabidopsis CLASP as a fluorescent protein fusion showed enriched localization of the protein at the plus end of MTs, and along the length of MTs.[19,20] Arabidopsis CLASP is thought to have a role in promoting MT stability, with mutants showing abnormal cell division and expansion phenotypes. A limitation in using bioinformatic approaches to identify MAPs, however, is the inability to identify proteins that have

diverged significantly in primary amino acid sequence, and to identify novel plant MAPs.

Forward genetic screens have successfully identified several genes that code for general and plant-specific MAPs. One study utilized an immunofluorescence microscopy screen for Arabidopsis temperature sensitive MT mutants, and identified a mutant with disorganized and shortened MTs at restrictive temperatures.[21] Arabidopsis MOR1 belongs to the conserved XMAP215/TOB/Dis1 family of MAPs. It localizes to all plant MT arrays, and functions to regulate MT organization in these arrays. The *mor1* mutant demonstrates twisting of organs and isotropic cell expansion at restrictive temperatures. In another study, a forward genetic screen identified a mutant in one of the 61 kinesin-like genes present in the Arabidopsis genome. The *fragile fiber1* mutant was characterized by shortened stems and had disorganized cellulose microfibrils in the secondary cell wall, possibly resulting from impaired regulation of cellulose synthase complex movements.[22]

An early biochemical purification approach that was used to identify plant MAPs involved the co-purification of plant proteins with taxol-stabilized bovine brain MTs. Using this approach, MTs were incubated with plant suspension cell extracts, followed by centrifugation of MTs.[23] Taxol stabilized MTs polymerized from endogenous plant tubulin were later used as a matrix,[24] and a modification of this procedure utilized rounds of MT assembly and disassembly in order to enrich for MAPs.[25] This MT assembly/disassembly approach resulted in the purification of a major class of plant MAP called the MAP65 family of proteins.[25] Subsequent database searches have identified nine MAP65 genes from Arabidopsis, each coding for proteins that have a range of functions related to the organization of the four plant MT arrays.[26,27] More recently, a 70 kD MAP was identified by co-assembly and co-purification of plant MTs that were purified from taxol-stabilized plant MTs, and has led to the discovery of the MAP70 family of proteins.[17] One of these family members (MAP70-5) stabilizes MTs, and this stabilization has a role in maintaining axial polarity and controlling the normal extension of plant organs.[28]

MT/tubulin affinity chromatography has also been valuable in identifying MT-binding proteins.[29–35] For instance, a 90 kD Arabidopsis protein identified as phospholipase D (PLD) was purified from a membrane fraction using tubulin affinity chromatography.[31,36] PLD is involved in phospholipid signalling, and treatment with the PLD activator, *n*-butanol, caused dissociation of MTs from the plasma membrane[37] and affected plant development.[38] This suggests that PLD might mediate signalling across the plasma membrane through MTs. MT and tubulin affinity chromatography

were also utilized for the purification of EF1α,[29,35] a protein shown to conditionally associate with plant MTs *in vivo*.[39]

2.3. MT-INTERACTING PROTEINS

Fewer authentic plant MT-interacting proteins have been identified. Numerous candidate MT-interacting proteins were identified from Arabidopsis suspension cells in two large-scale studies. These studies utilized different approaches: tubulin affinity chromatography of cell suspension extracts,[13] and the co-assembly and co-purification of MT-binding proteins with endogenous taxol-stabilized MTs.[17]

The tubulin affinity chromatography study identified putative MT-binding proteins from an Arabidopsis (Columbia ecotype) hypocotyl-derived cell suspension using bovine α/β tubulin dimers bound to the chromatography matrix.[13] Previous work demonstrated that plant or bovine tubulin chromatography columns in a dimeric or polymerized form, resulted in the purification of proteins with SDS-PAGE profiles that were qualitatively similar.[29,35] The proteins purified in this study also co-sedimented efficiently with MTs,[13] as was demonstrated similarly in a previous study.[31] Proteins that were purified in this large-scale study were separated by non-equilibrium two-dimensional pH gel electrophoresis (NEPHGE) and stained with Coomassie brilliant blue. The advantage of using NEPHGE over conventional equilibrium IEF two-dimensional gel electrophoresis is that it preserved the solubility characteristics of the basic proteins in these samples.[13] Eighty-six clearly defined protein spots were excised from the gels, and the protein in each spot was analyzed by liquid chromatography, followed by tandem mass spectrometry (LC-MS/MS). A total of 122 proteins were identified with high confidence in this study and were categorized into six groups based on their known functions.[13] These included classical MAPs, and five groups of putative MT-interacting proteins: RNA-binding proteins, translation-related proteins, signalling proteins, metabolic enzymes, and proteins with other functions (Figure 1). Approximately 40% of these proteins are related to proteins that were previously shown to possess MT binding activity. Examples of MAPs fitting the classical definition were HSP70/HSP40, and a protein similar to human CLASP1. Proteins with RNA-related functions included RNA helicases, small nuclear ribonucleoproteins, and proteins containing known RNA binding domains. Translation related proteins included several initiation and elongation factors, aminoacyl-tRNA synthetases and ribosomal proteins. Among the several putative signaling proteins were protein phosphatase 2A regulatory and catalytic domain-containing protein, proteins with serine-threonine kinase domains, and a cyclin-dependent

kinase. This study also identified numerous proteins with known metabolic activities in the cell. One group of metabolic enzymes included six peroxisomal matrix enzymes with activities involved in the β-oxidation of fatty acids. Interestingly, one of these proteins, the peroxisomal multifunction protein (MFP), was previously identified in a screen for proteins with MT and RNA binding activity.[35] Another of these proteins, peroxisomal malate dehydrogenase (MDH), was also shown to possesses MT and RNA binding activity (M.C. Freeman, L.A. Vickerman, and D.G. Muench, 2006). Since peroxisomal matrix proteins are typically fully folded in the cytosol prior to import, they have the potential for cytosolic activities. Additional studies supported the MT and RNA binding activities of MFP and MDH[35,40](M.C. Freeman, L.A. Vickerman, and D.G. Muench, 2006 unpublished observations).

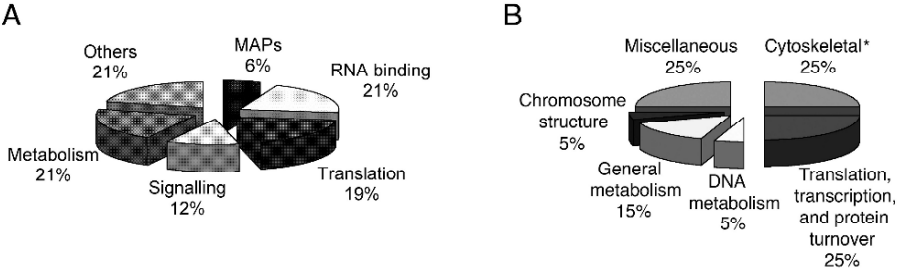

Figure 1. Classes of proteins identified in (A) a large-scale tubulin-affinity chromatography study[13] (From Chuong et al.[13] with permission from the publishers), and (B) a MT co-assembly and co-sedimentation study.[17] *The value indicated for cytoskeleton proteins in this figure was determined without including the 15 tubulin proteins that were identified in this study.

The large-scale study that used co-assembly and co-purification of MT-binding proteins with endogenous MTs identified 55 proteins from Arabidopsis (Landsberg ecotype) suspension cell protoplasts.[17] This work revealed that the cell line of choice was important, as the procedure was not successful when the more highly vacuolated Columbia ecotype cell line was used. MTs were assembled by the addition of taxol, and co-purification of MTs and associated proteins was achieved by centrifugation. Proteins were fractionated by SDS-PAGE and protein bands were excised and analyzed by MALDI-TOF mass spectrometry. This study identified several MAPs, such as MAP65 and MOR1, and as described above, a representative of the novel family of MAP70 family. Several kinesin-related proteins were also identified. Similar to the tubulin-affinity chromatography study described above,[13] numerous proteins that could be classified as putative MT-interacting

proteins were identified.[17] An RNA helicase, translation factors, ribosomal proteins, metabolic enzymes, and proteins involved in chromatin structure and DNA metabolism were among the proteins that were identified in this study (Figure 1). In an extension of this proteomic study, a novel MAP called AIR9 was identified and localized to cortical MTs as well as the pre-prophase band of MTs.[41] This protein also appears after cell division and is located the former pre-prophase band site. AIR9 might be involved in wall maturation events.

The results of these two complementary MT proteome studies indicated that the MT surface is extremely congested with proteins, and that MTs serve as a platform for the operation of numerous cellular processes. Intense competition for binding space on MTs would require that the expression levels of MT-binding proteins and their affinity for MTs is highly coordinated in a spatial and temporal manner. This type of tight control over MT binding has been observed for a MAP65 family member through protein phosphorylation events.[42]

3. Interactions between microtubules and RNA-binding proteins indicate a role for plant microtubules in localized translation and import of proteins into organelles

The cytoskeleton is involved in the localization of a broad range of transcripts to distinct regions of the cytoplasm, as well as to organelle surfaces.[43] One purpose that mRNA localization serves is to concentrate protein synthesis at a specific region of the cell where the protein carries out its function. Several RNA-binding proteins involved in mRNA localization have been identified, and these proteins bind either directly to the cytoskeleton, or indirectly through motor proteins or other adaptors. For example, *Vg1* mRNA travels to the vegetal pole of Xenopus oocytes using MTs. The RNA binding protein Vg1RBP/VERA binds to a specific sequence in the 3'UTR of the *Vg1* transcript to mediate this interaction, and the ER links this complex to MTs.[44,45] Staufen is involved in mRNA transport along MTs in dendrites. Staufen also possesses MT binding activity, implying that it has a role in directly anchoring transcripts to MTs.[46] Many cytoskeleton-bound mRNAs are components of ribonucleoprotein complexes that contain RNA-binding proteins and translational machinery, supporting a role for these mRNA-protein-cytoskeleton associations in translational regulation.[47,48]

Rice endosperm cells provide an ideal example of subcellular mRNA localization in plant cells. Here, mRNAs that code for the prolamine class of seed storage protein are enriched on a subdomain of the endoplasmic reticulum (ER) that houses the prolamine protein body (the PB-ER).[49] There

is evidence for a cytoskeletal role in the transport and anchoring of prolamine mRNAs to the PB-ER surface.[50,51] The increasing number of examples of mRNAs that are localized to the surface of organelles suggests that there exists a general process that serves to efficiently restrict translation at the surface of the target organelle. This places the protein in close proximity to the import machinery that is located in the organelle boundary membrane.[43] As discussed below, protein import into organelles may be facilitated in some instances by the localized synthesis, anchoring and concentration of proteins on the MT surface.

3.1. MT/ORGANELLE INTERACTIONS EXIST IN PLANT CELLS

MTs are functionally diverse in their roles related to organelle motility and positioning in eukaryotic cell types. In mammalian cells, organelles typically move along MTs through the action of dynein/dynactin and kinesins motors.[52] In contrast, organelle motility in plant cells is largely mediated by actin filaments and myosins.[53,54] The actin cytoskeleton is central to the rapid movement of Golgi bodies, chloroplasts, endoplasmic reticulum, mitochondria and peroxisomes in plant cells.[55–59] There are, however, exceptions to these actin-mediated movements, as several examples of MT/organelle interactions exist in plant cells.

In contrast to the involvement of actin in directional Golgi movement, MTs were found to increase the pausing of Golgi bodies in a subset of tobacco suspension cells.[58] This was revealed by a small but significant overall increase in the streaming of Golgi bodies upon MT-destabilizing drug treatment. In support of this observation, a study on trichome morphogenesis identified a kinesin, Kinesin-13A, which localized to Golgi bodies and was proposed to anchor or distribute Golgi bodies at the cell cortex.[60] Similar to Golgi bodies, movements of mitochondria in young tobacco cells and protoplasts are slowed by MTs. When MTs were destabilized, there was an increase in the number of cytoplasmic strands that contained motile mitochondria, resulting in a slight increase in overall mitochondrial motility.[55] In contrast to the movement of mitochondria in young cells and protoplasts, the cortical cytoplasm of elongating cells contained varying numbers of immobile mitochondria organized in distinct parallel alignment. Interestingly, the alignment of these immobile mitochondria is disturbed by oryzalin treatment, resulting in the positioning of mitochondria evenly throughout the cortical cytoplasm. In another example, MT inhibitor treatments caused the motility of non-green plastids to increase.[61] Taken together, these data suggest a role for MTs in slowing the movement and affecting the positioning of plant organelles.

An additional role for MTs is their role in intracellular transport of organelles. For instance, chloroplast movements in the moss *Physcomitrella patens* depends primarily on actin, but require both actin and MTs.[62] Members of the green algal group Characeae use actin and MT based mitochondrial motility and positioning in internodal cells.[63] MT-mediated interactions and motility have also been observed in higher plant cells. MTs play a critical role in cell plate formation during cytokinesis, serving as the backbone for Golgi-derived vesicle transport to the division plane.[64] In root apical meristem cells of tobacco, MTs rather than actin coordinate ER distribution during cell division.[65]

3.2. THE ACTIVITY OF MT-INTERACTING PROTEINS COULD REGULATE TRANSLATION AND FACILITATE PROTEIN IMPORT INTO ORGANELLES

A large body of literature exists on the mechanisms responsible for translocating proteins across the boundary membrane of organelles. However, little is known about the mechanisms responsible for getting the protein to the surface of organelles in order for them to interact with the import machinery. Targeting of mRNAs to the organelle surface, followed by translation, is one means of localizing proteins to organelle surfaces.[43,66] Another possibility is to move the organelle to a region or structure where the protein might be concentrated. An example of the latter mechanism might be related to the synthesis and import of proteins into the peroxisome.

Peroxisomes lack a genome, and as a result the entire complement of peroxisomal matrix proteins are synthesized in the cytosol and are imported post-translationally into the peroxisome. Unlike the synthesis and import of proteins destined to chloroplasts or mitochondria, peroxisomal matrix proteins are typically imported as fully folded and oligomerized proteins,[67] indicating that these proteins could possess cytosolic activity prior to import. As described above, peroxisomal MFP possesses both MT and RNA binding activity.[35,40] Additionally, peroxisomal malate dehydrogenase (MDH) was also shown to possess MT and RNA binding activity (M.C. Freeman, L.A. Vickerman, and D.G. Muench, 2006 unpublished observations). The identification of six peroxisomal matrix proteins in the tubulin-binding protein study described earlier[13] and one other in a different study,[68] suggests that the MT-binding activity of these proteins functions generally in their import into the peroxisome. That is, the binding of these proteins to MTs in the cytosol could function to assist in the efficient import of these proteins into peroxisomes, and could also serve as a mechanism to suppress their enzymatic activity prior to import. Real-time fluorescence imaging of onion epidermal cells with GFP-MFP revealed apparent interactions

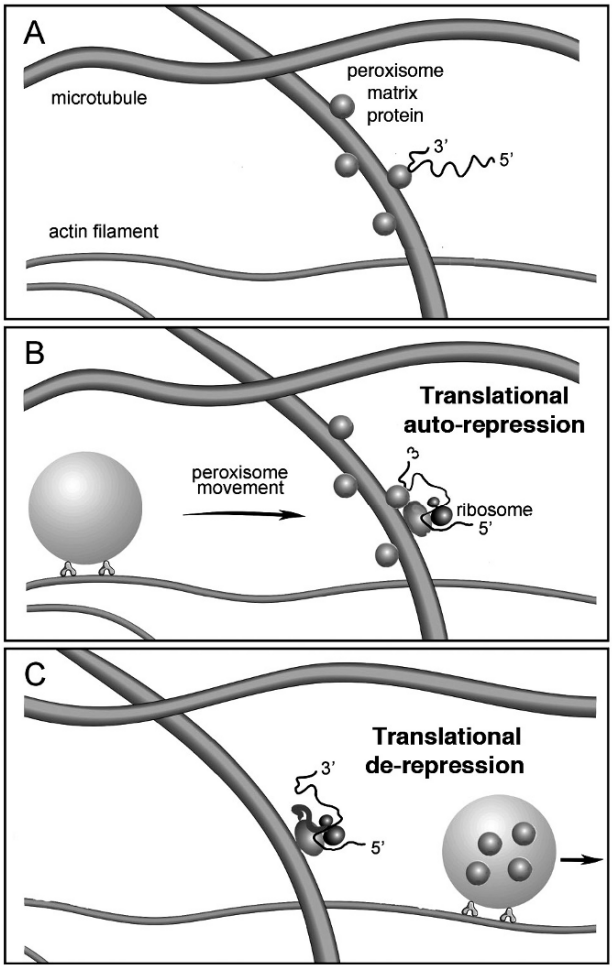

Figure 2. A working model depicting the possible role of the MT and RNA binding activities of MFP, MDH, and other peroxisomal matrix proteins on their translation and subsequent import into peroxisomes. Nascent peroxisomal matrix proteins bind simultaneously to cortical MTs and to their own mRNA (top panel). This association functions to repress translation in an autoregulatory fashion (middle panel). As peroxisomes pass by or pause on MTs, microtubule-bound peroxisomal matrix protein is imported into the peroxisome and translational repression is lifted, resulting in the synthesis of additional MFP protein (bottom panel). A pre-formed translation complex that includes ribosomes and translation factors (middle panel) ensures that the rapid initiation of translation takes place once repression is lifted. The dynamic nature of the plant cytoskeleton would provide a constant repositioning of microtubule-actin filament intersection sites, thereby assisting in the efficient import of the peroxisomal protein that is uniformly localized along the length of the microtubule. Modified from Muench and Park.[48]

between MTs and peroxisomes,[40] thereby bringing peroxisomes into close proximity to MTs. The RNA binding activity of MFP could anchor mRNAs to MTs. Other metabolic enzymes also possess RNA binding activity, some of which bind to their cognate mRNAs to suppress their own translation in an autoregulatory manner.[69,70] This data led to a model that links the MT and RNA binding activities of peroxisomal matrix proteins to translational autoregulation and import (Figure 2).[48] The model proposes that peroxisomal matrix proteins bind simultaneously to cortical MTs and their cognate mRNA thereby repressing translation in an autoregulatory manner. De-repression occurs when peroxisomes interact with MTs and import the protein. Thus, translational autoregulation would ensure that peroxisomal matrix protein synthesis is controlled by the rate of import into peroxisomes.[48]

4. Summary

The discovery of a wide range of proteins with MT binding activities has begun to change our view on the role of MTs in plant cells. Continued efforts to identify and characterize classical MAPs will improve our understanding of how the four MT arrays coordinate cell division, differentiation and function. As well, the identification and characterization of MT-interacting proteins demonstrates new roles for MTs as cellular structures that provide a matrix for coordinating a multitude of processes, including translation and protein sorting to organelles. Translational autoregulation mechanisms, such as the one described for peroxisomal proteins, could serve to assist in the controlled interaction of a wide range of proteins on the surface of MTs. The growing field of MT proteomics has great potential to expand our understanding of the mechanisms responsible for gene expression, protein activity, subcellular localization, signalling, and other complex activities that go on in plant cells.

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QUALITATIVE AND QUANTITATIVE ANALYSES OF ß-TUBULIN Cva (C-TERMINAL VARIABLE ACIDIC AMINO ACID REGION) BY MALDI-TOF MASS-SPECTROMETRY

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Abstract. The C-terminal region of tubulin is highly acidic and its amino acid sequence is divergent among the isotypes. This region is also known to have various types of post-translational modifications. Comprehensive analyses of C-terminal proteolytic fragments of tubulin by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) will be useful in understanding the role of this region in microtubule function during cell proliferation and differentiation. Lysylendopeptidase (LysC)- and endoproteinase AspN (AspN)-digested fragments were effective for predicting/identifying the isoforms in a complex mixture. It was also shown that tobacco BY2 cells were labeled efficiently by ^{15}N-containing medium. The stable isotope-labeled components were detected as separate peaks from their ^{14}N-counterparts with their masses increased as predicted by their nitrogen contents, and this method is useful for the quantitation of specific fragments.

Keywords: ß-Tubulin isoform, C-terminal region, MALDI-TOF MS, stable isotope, tobacco BY2

Abbreviations: AspN = endoproteinase AspN, Cva = C-terminal variable acidic amino acid region, DAC = diammonium citrate, LysC = lysylendopeptidase, HNM = heavy Nagata's medium, IPG = immobilized pH gradient, MALDI-TOF MS = matrix-assisted laser desorption/ionization-time of flight mass spectrometry, NM = Nagata's

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medium, PTM = post-translational modification, THAP = trihydroxyacetophenone, TTLL = tubulin tyrosine ligase-like

1. Introduction

1.1. BACKGROUND

Plant cell culture has been considered to be an excellent system for investigating the control mechanism of cell division and differentiation. Plant tissue de-differentiates into callus when it is grown on an appropriate medium containing auxin. The callus is a kind of stem cell and can differentiate into a whole plant.[1,2] Recently, receptors for plant hormones were found[3–7] and the basic mechanism of tissue development seems to be quite similar to the patterning during early development of animal embryos.[8] Plant callus cells generally grow in a completely defined medium.[9] This is a great advantage over animal cell culture, which usually grows in a medium containing uncharacterized serum, when studying cell cycle events and differentiation.

Patterning at the root differentiation accompanies with the cessation of proliferation and initiation of cell elongation. This process proceeds with close relation to the auxin gradient.[10] The translocation of signal molecules into nuclei is thought to be mediated by microtubules, but the mechanism for the process is not fully elucidated. Carrot GD2 cells were shown to change its mitotic activity and cell shape after the medium auxin was changed from 2,4-D to IAA.[11,12] This phenomenon was thought to be a model for the switching from proliferation to elongation mode at the early patterning of root tissue. In both of the process of cell division and elongation, microtubule distribution and its function change dramatically. The main microtubule protein subunits, α-, ß-tubulin, consist of multiple isoforms in multicellular organisms. Tubulin was isolated from carrot and its properties were studied.[13,14] The composition of isoforms seems to be different according to the physiological state of the cells[15,16] or differentiated tissues.[17] Carrot GD2 cells were quite useful for the biochemical studies. However, they are not suited for cell cycle study because they can not be synchronized well by aphidicolin. However, tobacco BY2 cells can be fairly well synchronized for cell cycle events.[18] In this chapter, we describe the experiments performed mainly on BY2 cells.

1.2. TUBULIN Cva (C-TERMINAL VARIABLE ACIDIC AMINO ACID REGION)

Tubulin is a highly conserved molecule. However, the C-terminal region of α- and β-tubulin is divergent among isotypes and extremely rich in acidic amino acids. In addition to the existence of isotypes, tubulin Cva has been reported to be glutamylated, phosphorylated, glycylated and detyrosylated.[19] These post-translational modifications (PTM) further increase the divergence among isoforms. The combinations of diversity in genetic isotypes with glutamylation, phosphorylation and others at specific sites in Cva may encode information defining interactions with factors such as MAPs, motor proteins or others at a definite time and/or location.[20]

In the proposed structural model of the tubulin molecule from electron-crystallographic data, the C-terminal region of tubulin was not defined because of its heterogeneity and flexible nature.[21,22] The amino acids consisting this region have a high propensity to make α-helix, but abundant negative charges likely repulse each other and keep the chain extended.[23] Therefore, at physiological pH, Cva is expected to protrude from the surface of microtubules as a flexible arm.[24] Consequently, various substances surrounding microtubules interact first with Cva, and so Cva would play the role of an antenna for microtubules. The polyvalent negatively charged chain would behave like a sticky, flexible strand that attracts substances with basic sites. Then, according to the structure of the various counterparts, more specific interactions may be made with Cva itself or with other specific sites of the tubulin molecule. Counterparts of binding elements might be MAPs, motor proteins, signal molecules, kinases, phosphatases, transcription factors and others either directly or indirectly. For the controlled binding of such diverse factors, diversity of Cva might be advantageous.

1.3. DETECTION AND DETERMINATION OF TUBULIN CVA BY MALDI-TOF MASS SPECTROMETRY

MALDI-TOF MS detects peptide fragments at small amounts on the order of femtomoles, which corresponds to several hundred million molecules [i.e. a single molecule in a several hundred milliliter culture of log phase BY2 cells (~10^6 cells/)]. In addition to high sensitivity, the advantage of this method over other techniques such as ESI-MS or Edman degradation is that, isolation of target molecules at a high level of purity is not necessarily required. Minimum purification steps reduce the risk of artifacts. In addition, mixed components in a sample can be analyzed simultaneously. Tubulin tyrosine ligase-like (TTLL) protein family enzymes recognize specific glutamate residue in the C-terminal sequences of tubulin and add tyrosine or glutamic acid.[25] As there are still some TTLL enzymes with

unknown functions in the genome, it is possible that any one of them adds any one of the other amino acids to a specific site of Cva.[26] Although the available information is restricted to mass value, we can predict the isotypes and their modifications, and the sites of the modifications simultaneously. The available resolution is around a ten thousandth, but the assignment of the peaks is not easy if amino acid sequence information is not available. Prediction of unknown sequence and unknown modifications by post-source decay (PSD) or sequential digestion with carboxypeptidase Y[27] are not always successful, especially when the signal intensity is low. Even if the sequence data exist, there are several problems to be solved. These include possible artificial change by the cell degradation, biased recovery of tubulin subtypes, incomplete digestion, co-incidence of the m/z values with other fragments or noise, difficulty of ionization, etc. Some of them can be overcome, but some are not easy.

In this article, we present our studies on elucidating the composition of tubulin isoforms by MALDI-TOF MS. This includes assignment of spots in 2-D PAGE to the products of the known isotypes and their possible modifications. An method for the quantitation of isoforms, using a stable isotope-labeled control, is also described.

2. Results

2.1. PREPARATION AND DETECTION OF CVA

It is most important that cellular activity is stopped as rapidly as possible and that the sample for mass spectrometry is preserved as it was in the cell when harvested. Harvested cells were immediately frozen in liquid nitrogen and used soon or stored at -80°C. Cells were pulverized in a mortar filled with liquid nitrogen and drops of frozen extraction buffer. The extraction buffer contains inorganic phosphate, protease inhibitors and EDTA to prevent the action of enzymes for phosphorylation, de-phosphorylation, glutamylation and proteolysis as the pulverized cells thawed. Sucrose, sodium tartrate and ammonium sulfate have been shown to be effective in stabilizing tubulin during the extraction.[28] The nitrogen gas, which was released by evaporation during grinding of the cells, prevents oxidation. Polyphenols are easily oxidized and might interact with proteins, but they were efficiently removed by including polyvinylpolypyrrolidone (PVP) in the extraction buffer.

In order to eliminate possible interactions with positively charged groups, tubulin was adsorbed to DEAE-Sephacel immediately after the separation of cell extract. The acidic protein fraction rich in tubulin was isolated as quickly as possible.

We amplified five different cDNAs from log phase BY2 cells corresponding to the C-terminal region of β-tubulin and designated NTB1~5[29] (*Nicotiana tabacum* beta-tubulin 1 through 5).

The β-tubulin Cva's are 19–21 amino acid peptides flanking the conserved region of helix 12 of the molecule. When β-tubulin is completely digested with *Achromobacter* lysylendopeptidase (LysC), the C-terminal fragments are produced by cleavage at K-392. The resulting fragments are heterogeneous in size.

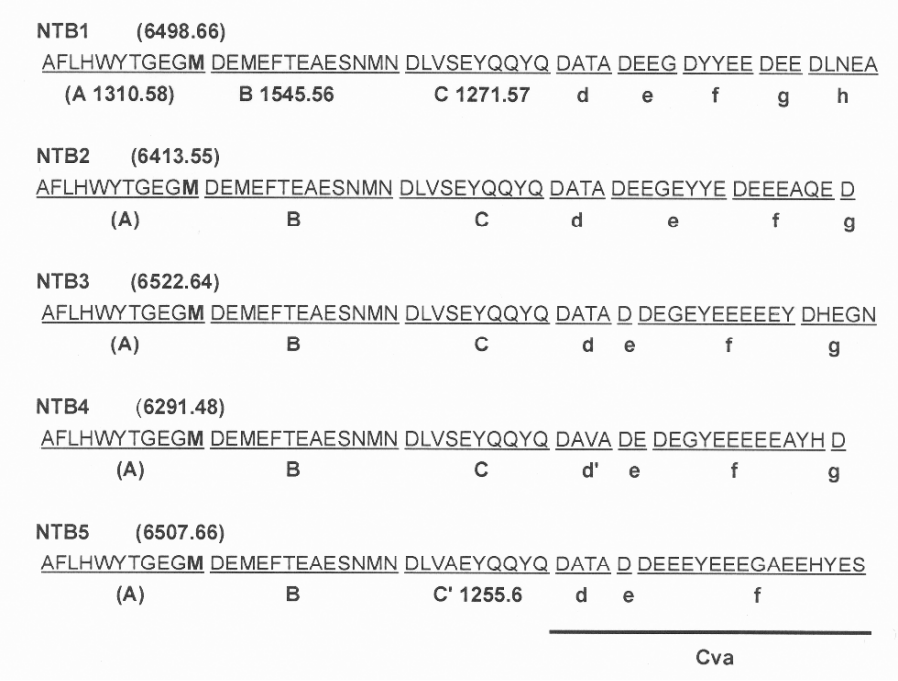

Figure 1. Sequences of C-terminal LysC and AspN-fragments of tobacco β-tubulin isotypes. The C-terminal amino acid sequences predicted from the nucleotide sequence of β-tubulin isotype cDNA[29] are shown. In the parentheses above the sequence, the average masses of C-terminal LysC-fragments cleaved at the consensus ^{392}K are shown. Expected fragments after digestion with AspN are shown under the peptide sequence. Fragments in consensus region are in capital letters and those in the Cva are shown in lowercase letters. "A" fragment is available only when the LysC-fragment is further digested by AspN. **M** shown in bold type is the site where the C-terminal of GST was fused in the experiment for data presented in Figure 2.

The expected size and sequence of the C-terminal LysC fragments are shown in Figure 1 with the cleavage sites of endoproteinase AspN (AspN) and the expected peptides.

Methionine residue seemed to be oxidized during the sample preparation. Increases of mass by 16 Da were useful for identifying the peptides containing methionine (Figure 2, common fragment B). The sizes of C-terminal

LysC-fragments are around 6,500 Da. It is too large to analyze MALDI-TOF MS with reflector mode, but can be detected with lower accuracy using the linear mode. AspN digestion gives smaller isotype-specific fragments, most of which are suitable for detection using the reflector mode, which gives more accurate mass values.

Because Cva is rich in acidic amino acids, isotype-specific fragments were not detected efficiently. In order to find the proper conditions, the end of isotypes NTB1~5, at residue M-403 (Figure 1), were fused to the carboxy-terminal end of GST and expressed in *E. coli*. The recombinant fusion proteins were isolated and digested with LysC or AspN, and different matrices and modes of measurements were tested to detect Cva-specific

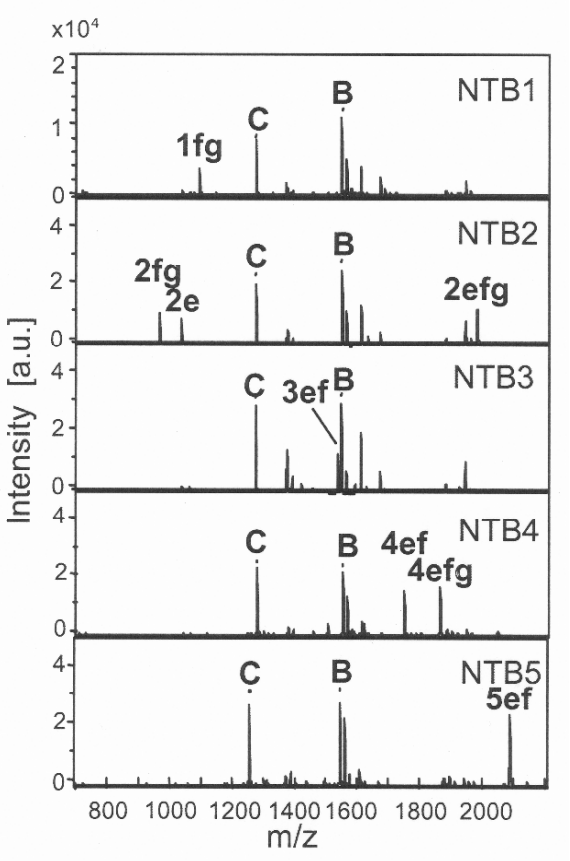

Figure 2. Identification of isotype-specific AspN-fragments from recombinant β-tubulin isotype GST-Cva fusion proteins. Complementary DNAs containing NTB1-5 Cva (from M-403 (bold type in Figure 1) were fused to the C-terminal of GST and expressed in *E. coli.* Purified proteins were digested with AspN and negative ions were detected in MALDI-TOF MS with THAP/DAC matrix. Uppercase letters at the top of the peaks are common fragments, and those in lowercase letters are isotype specific fragments shown in Figure 1.

fragments. Some of the acidic fragments were detected more efficiently by the negative mode than the positive mode. The common fragments among most of the isotypes (B and C in Figure 1) appeared as paired peaks with comparable intensities by the negative mode. The isotype-specific peptides were detected with efficiencies of 30–50% compared with common fragments B and C (Figure 2).

2.2. DETECTION OF ß-TUBULIN CVA IN LOG PHASE BY2 CELLS

The β-tubulin band was excised from the PVDF blot of an SDS-polyacrylamide gel. It was treated with the reagents for denaturation, reduction and alkylation, and enzymatically digested with LysC. Digestion products were analyzed by MALDI-TOF MS with linear mode.

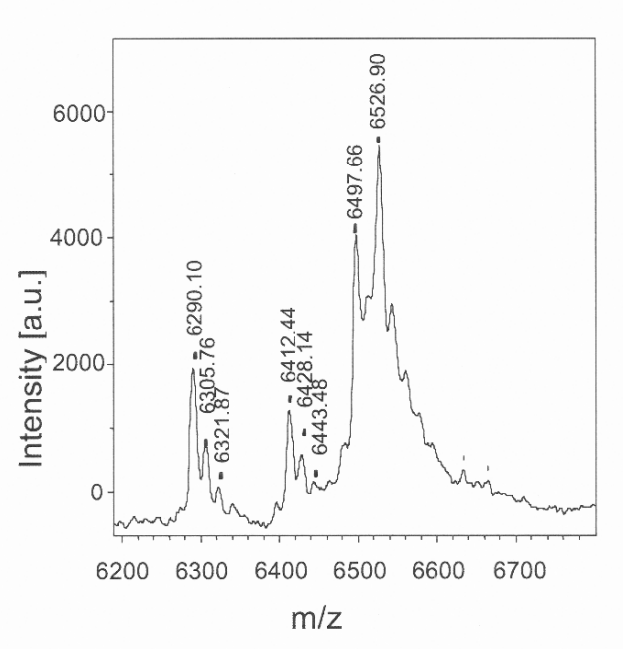

Figure 3. Mass spectrum of the LysC-fragments of β-tubulin from log phase BY2 cells. Acidic proteins were separated by one-dimensional SDS-PAGE, transferred to PVDF membrane and stained with ServaBlue G. β-tubulin band was excised and digested with LysC and analyzed by MALDI-TOF MS with linear negative mode.

The spectrum corresponding to the expected sizes of NTB1-5 C-terminal LysC-fragments (6,200–6,800 Da) is shown in Figure 3. The peak with the mass of 6,290.10 corresponds to NTB4 (calculated mass = 6,290.40 ($[M-H]^-$)). The successive two smaller peaks were spaced at approximately 16 Da increments, and they are probably the products of the oxidation of methionine residues. The B fragment in the AspN-digested

Cva has two methionine residues (Figure 1) and its spectrum was accompanied with two successive peaks separated by 16 Da (Figure 2). Similarly, the peak at 6,412.44 and the successive two peaks correspond to NTB2 (6,412.47 ($[M-H]^-$)) and their oxidized products. There was a less-well resolved large group of peaks between 6,490–6,600. The 6,497.66 peak corresponded to NTB1 (6,497.58 ($[M-H]^-$)), but the highest peak, 6,526.90, did not coincide with the mass of any known isotype. On the other hand, the peaks corresponding to NTB3 (6,521.56 ($[M-H]^-$)) and NTB5 (6,506.59 ($[M-H]^-$)) did not resolve.

The acidic proteins from the BY2 cell lysate were separated by 2-D PAGE. The separated proteins were transferred to a PVDF membrane and the five resolved spots were excised from the β-tubulin region (1–5 in Figure 4). The membrane was incubated with LysC and the digested products were analyzed by MALDI-TOF MS with linear negative mode (Figure 5).

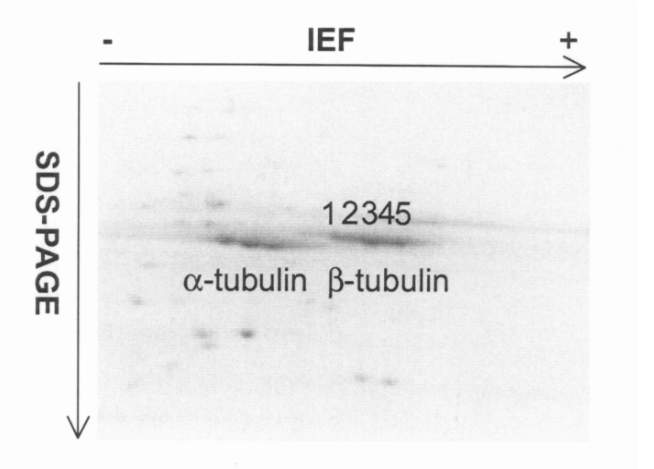

Figure 4. 2-D PVDF-blot of acidic proteins from log phase BY2 cells. Acidic proteins (600 μg) from log phase BY2 cells were separated by 2-D electrophoresis, transferred to PVDF membrane and stained with ServaBlue G. Spots 1–5 in β-tubulin region were excised and analyzed by MALDI-TOF MS after LysC or AspN digestion (Figures 5, 6).

Major peaks (#1-3) in the spectrum of spot 1 (top panel, Figure 5) corresponded to NTB4 and its oxidized products. The spectrum of spot 2 (2nd panel from the top) also contained NTB4 (peaks 1–3), but the major components were NTB3 (peak 5) and its oxidized products. In the spectrum of spot 3, NTB1 and its oxidized products (peak 1–3, 5) constituted the major peaks. The spectrum of spot 4 contained two groups of complex peaks. The group with smaller masses (peaks 1–3) contained NTB2 and its oxidized products and the group with larger masses (peaks 4–9) contained

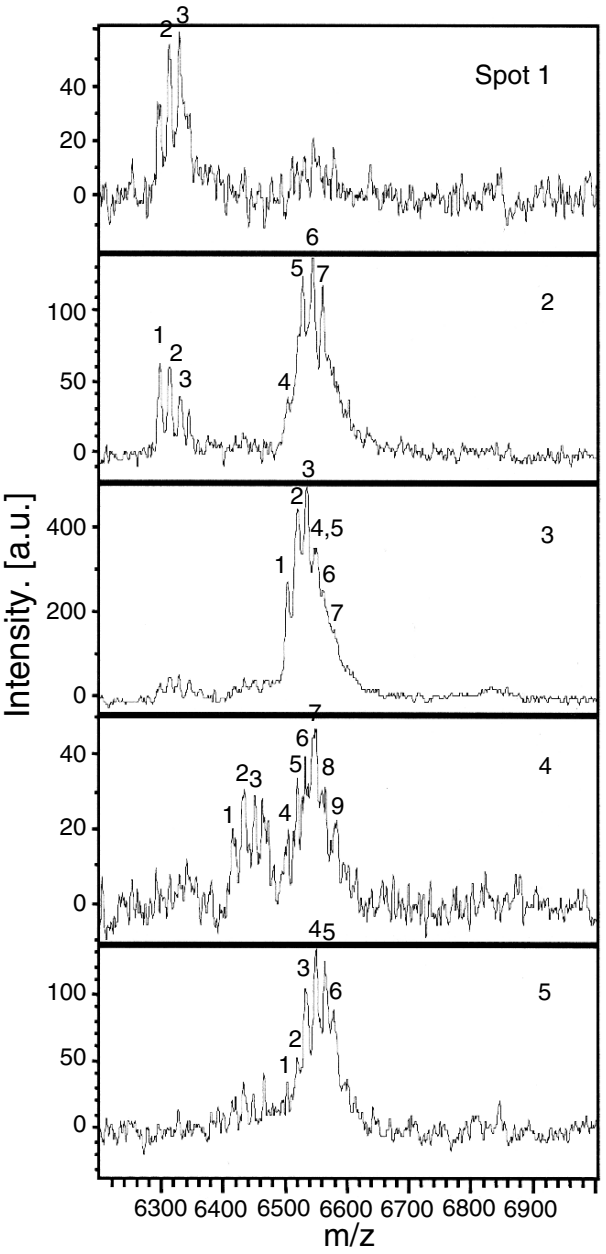

Figure 5. Mass spectra of the LysC-fragments of β-tubulin from 2-D spots in Figure 4. The five β-tubulin isoform spots were excised, digested with LysC and analyzed by MALDI-TOF MS with linear negative mode.

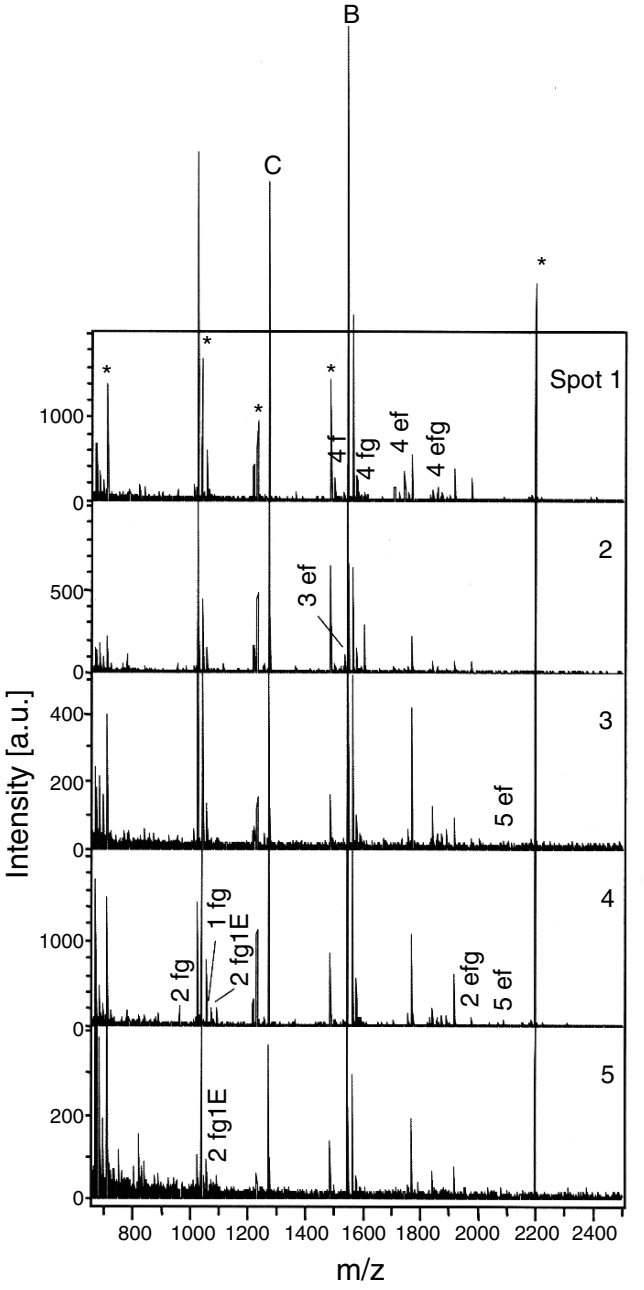

Figure 6. Mass spectra of the AspN-fragments of β-tubulin from 2-D spots in Figure 4. β-tubulin isoform spots in Figure 4 were excised and digested with AspN and analyzed by MALDI-TOF MS with reflector negative mode. Peaks marked with asterisks indicate the fragments from the N-terminal portion of β-tubulin.

unknown fragments except for peaks 4 and 5 that corresponded to NTB1 and its oxidized product. The mass of the highest peak (#7) coincided with NTB2 containing one additional glutamic acid. The peaks in the spectrum of the spot 5 were also complex. The mass of the major peak (peak 4) coincided with the oxidized product of NTB2 with the addition of two glycine residues. The peaks corresponding to its unoxidized form of this fragment were seen in Figure 3 (6,526.90), and peak 6 in spot 4 and peak 3 in spot 5 in Figure 5. It is also possible that these peaks were from unidentified isotype products or modified peptides with similar mass.

Figure 6 shows the spectra of the AspN-digestion products of the same sample as in Figure 5 within the mass range from 650 to 2,500 Da. The expected peaks from the common AspN fragments B and C and several peptides from the N-terminal portions of the ß-tubulins were evident. The peaks corresponding to the isotype-specific fragments could be found of lower intensities. The expected main fragments of NTB4 (f, fg, ef, and efg fragments in Figure 1) were found in spot 1. The peak derived from the "ef" fragment of NTB3 was found in spot 2 as a major peak among spot-specific ones. Similarly, the "fg" fragment of NTB2 was the spot-specific major fragment of spot 4 as well as the "efg" fragment. In the spectrum of spot 5, the major spot-specific peak coincided with the mass of the "fg" fragment of NTB2 with an additional glutamic acid residue, not NTB2 with two glycines as expected from the results of the LysC digestion.

2.3. QUANTITATION OF ß-TUBULIN ISOTYPES BY THE USE OF A STABLE ISOTOPE OF NITROGEN

Although mass spectrometry is very useful for qualitative analysis, it lacks reliability for quantitative analysis. However, there are several ways of quantitation using mass spectrometry based on the use of stable isotope labeling of the control.[30,31] Using heavy Nagata's medium (HNM), in which ^{15}N-NH_4NO_3 and ^{15}N-KNO_3 are substituted for ^{14}N-NH_4NO_3 and ^{14}N-KNO_3 in Nagata's medium (NM),[18] about 99% of the nitrogen in every component in BY2 cells can be substituted with ^{15}N following a week preculture of a 1/500 dilution of cells in NM into HNM. This ^{15}N-substitution method is easy to perform and is superior to other labeling methods in its efficiency.

Control culture was made in HNM and experimental culture was made in NM. In this experiment, log phase cells grown in HNM and metaphase cells in NM were mixed in a mortar filled with liquid N_2 and prepared for MS analysis. The tubulin fragments from each culture were detected as paired peaks corresponding to the difference in their nitrogen atom content. Because all of the conditions for the preparation and detection are common,

the ratio of the amount of components should accurately reflect the ratio of the intensities of the corresponding peaks.

Figure 7 shows a spectrum of LysC-fragments from whole β-tubulin separated by SDS-PAGE as a preliminary experiment. Several paired peaks appeared as expected. The mass difference between the pairs of peaks 2, 3, and 4 were 62 Da, 63 Da, and 64 Da, respectively, which coincided with the number of nitrogen atoms in the presumed fragments of NTB2, 1 and 2 with two glycines. The difference between the paired peaks 1 was 62. This value is 1 Da less than what was calculated from the number of nitrogen in the NTB4 fragment. Intensity ratios of the peaks of ^{14}N- and ^{15}N-fragments (front and back peaks, respectively, of each pair) should reflect the relative amount in metaphase and log phase cells. Therefore, the relative amount of NTB4-Cva was supposed to be high, and others low, in metaphase cells. The same sample was digested with AspN and the paired peaks were identified and the ratios of the intensities were calculated (Table 1). NTB4 ("ef") fragment (peak(8)) and NTB 5(C') (peak(9)) were high, whereas NTB3 ("ef") (peak(6)), 5 ("ef") (peak(10)) and 2 ("fg") (peak(3)) fragments were low in metaphase.

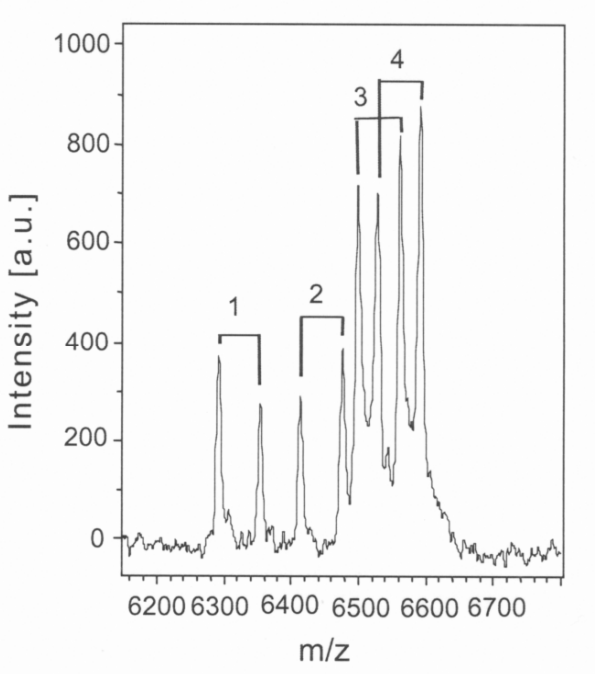

Figure 7. Mass spectrum of the LysC-fragments of ß-tubulin from ^{14}N-M phase and ^{15}N-log phase BY2 cells. Synchronized M phase cells grown in NM and log phase cells grown in HNM were mixed together and processed to isolate the acidic proteins. The ß-tubulin band on a PVDF membrane was excised and digested with LysC and analyzed by MALDI-TOF MS with linear negative mode.

3. Discussion

We demonstrated a method for the comprehensive analysis of β-tubulin Cva from tobacco BY2 cells and described some of the results of our analysis. Combining the results of LysC- and AspN-digestions, predicted C-terminal peptides corresponding to isotypes NTB 1, 2, 3, 4 are detected in spot 3, 4, 2, 1, respectively, as the main or sub-main components. Spot 5 may contain an

TABLE 1. Calculation of the ratio between the amount of the same fragments from ^{14}N-M phase and ^{15}N-log phase BY2 cells from the paired peaks in the spectrum of AspN fragments.

Peak	^{14}N M phase			^{15}N Log phase			^{14}N/^{15}N	Candidate		
	m/z	Intens.	R.I.	m/z	Intens.	R.I.		m/z	Sequence	N
N-terminal side										
1	722.246	5,739.5	0.275	729.226	5,041.5	0.241	1.14	722.300	[203-208]	7
2	760.283	3,928.6	0.188	767.262	3,384.2	0.162	1.16	760.319	[37-73]	7
3	1,039.391[a]	5,418.7	0.259	1,050.376	4,335.9	0.208	1.25	1,041.390	[295-303] -CM1Ox	11
4	1,231.588	6,915.8	0.331	1,246.557	5,321.9	0.255	1.30	1,231.480	[31-41]-1P	15
5	1,483.650	9,172.9	0.439	1,496.615	8,614.9	0.412	1.06	1,483.590	[197-208] -CM1Ox	13
6	1,590.850	510.1	0.024	1,611.793	497.8	0.024	1.02	1,590.803	[75-88]	21
7	1,767.987	2,140.6	0.102	1,786.931	1,694.4	0.081	1.26	1,767.910	[210-224]-CM	19
8	1,839.960	474.7	0.023	1,859.892	579.2	0.028	0.84	1,839.912	[161-176]	21
9	2,197.175	6,926.4	0.332	2,222.086	5,103.4	0.244	1.36	2,197.080	[177-196]	25
C-terminal common										
B	1,544.617	20,046.6	0.960	1,559.591	16,997.4	0.814	1.18	1,544.552	B	15
C	1,270.579	20,885.6	1.000	1,283.553	17,497.1	0.838	1.19	1,270.559	C	13
Isotype-specific										
(1)	1,045.416	513.4	0.025	1,056.385	419.0	0.020	1.23	1,046.39	NTB1gh-2G	11
(2)	1,089.378	454.2	0.022	1,097.365	434.3	0.021	1.05	1,089.353	NTB1fg	8
(3)	962.315	836.9	0.040	971.302	1,020.6	0.049	0.82	962.323	NTB2fg	9
(4)	1,031.367	963.6	0.046	*1,039.391	5,418.7	0.259	0.18	1,031.348	NTB2e	8
(5)	1,091.370	1,933.6	0.093	1,101.355	1,790.4	0.086	1.08	1,091.366	NTB2fg-1E	10
(6)	1,533.572	649.0	0.031	1,558.618	1,446.4	0.069	0.45	1,533.503	NTB3ef	12
(7)	1,706.845	157.9	0.008	1,723.794	160.1	0.008	0.99	1,706.539	NTB4Cd'-1P	17
(8)	1,741.675	424.2	0.020	1,757.641	222.4	0.011	1.91	1,741.599	NTB4ef	16
(9)	1,254.574	2,538.8	0.122	1,267.510	1,411.7	0.068	1.80	1,254.564	NTB5C'	13
(10)	2,086.807	249.2	0.012	2,107.019	412.4	0.020	0.60	2,086.716	NTB5ef	19

R.I.: Relative intensity, Ox: oxidized, E: glutamylated, G: glucylated, P: phosphorylated, CM: carboxymethylated

[a]Overlapped peaks

unknown isotype product or a modified known product such as diglycylated NTB2. Further analysis should clarify the identity including any post-translational modifications (PTM), of many of the other reproducible peaks in the spectra.

When a β-tubulin spot consisted of more than several percent of the whole β-tubulin, its digested peptides could be detected and the structure of the candidate molecule could be predicted. Changes in the abundance of the Cva could be grouped into two classes. There are two possible ways of contributing to the change in the PTM of microtubules for the participation in hormonal control of cellular activity.

One process is related to the process of the dynamic rearrangement of microtubule arrays as for example during mitosis, cytokinesis and cortical microtubule bundling. Tubulin phosphorylation at S-172 by Cdk1 in HeLa cells,[32] and PTM of cortical microtubules in *Tetrahymena* and its role as a recognition marker for katanin,[33] would be the case. Because severing of selective microtubules was shown to contribute to the proper alignment of the cortical arrays,[34] it is conceivable that PTM of microtubules also participates in controlling the plant cortical microtubule arrays. Through immunological studies, it was shown that tobacco α- and β-tubulins are extensively post-translationally modified.[35] The other forms of PTM of tubulin may participate in signal transduction. The Gli signal molecule is transferred from cytoplasm to nucleus after activation via microtubule traffic.[8] Recently, it was shown that Jak2 phosphorylated tubulin and associated with it. The phosphorylated tubulin interacted with STAT1 and contributed to its translocation.[36] Signal molecules related to plant hormones also require the translocation from cytoplasm to nucleus.[7] Interestingly, the Wnt signal pathway was shown to have dual effects on microtubules – controlling MT dynamics as well as the canonical pathway that requires translocation of β-catenin into the nucleus.[37]

It is possible that the "tubulin code" made by specific PTM of Cva[20] is related to the selective association of microtubules with specific cellular protein counterparts. In that case, the proportion of modified tubulin would be rather small. In order to detect such class of PTM, it is necessary to fractionate and concentrate Cva-containing fragments. The sensitivity of mass spectrometry is high enough to detect peptides at fmol concentrations. That means even a small change in several molecules of PTM in a cell would be detected if appropriate selective fractionation is applied on an experimental scale to cultured cells with sufficient recovery. However, if such PTM occurs only in a short period, it is hard to detect unless a very high level of synchrony of the event in the population is achieved.

4. Protocols

4.1. CELLS AND ^{15}N-LABELING

Tobacco BY2 cells (*Nicotiana tabacum* L. cv. Bright yellow 2) were maintained in Nagata's medium (NM).[18] Cells were regularly transferred (200 µl of 7 day-culture) to 100 ml of fresh NM in a 300 ml Erlenmeyer flask. The suspension was agitated on a rotary shaker at 150 rpm at 27°C. When the ^{15}N-labled cells were prepared, 200 µl of 7 day-culture in NM was transferred to 100 ml of fresh heavy Nagata's medium (HNM) in which 99% ^{15}N-NH_4NO_3 and 99% ^{15}N-KNO_3 (Nippon Sanso, Kawasaki, Japan) were substituted for the ^{14}N reagents (NH_4NO_3 and KNO_3), and pre-cultured for 7 days. For the preparation of ^{15}N-log phase cells, 3.6 ml of the preculture was transferred to 100 ml of fresh HNM and grown for 3 days. The cells were vacuum harvested, weighed, frozen in liquid N_2 and stored at -80°C.

4.2. PREPARATION OF M PHASE CELLS

For the preparation of M phase cells, double treatment with aphidicolin and propyzamide[38] was performed in NM. Ten milliliter of 7 day-old cells were transferred to 90 ml of fresh NM and cultured for 24 h with 5 mg/l aphidicolin. The cells were washed with 10 volumes of fresh NM and resuspended in fresh NM. At 6 h after the release from aphidicolin treatment, propyzamide was added to the culture for a final concentration of 3 µM. The cells were harvested at 4 h after the propyzamide treatment, washed with fresh medium, weighed, frozen in liquid N_2 and stored at -80°C.

4.3. PREPARATION OF TUBULIN-RICH EXTRACT (DEAE-TUBULIN)

A mortar was filled with liquid N_2 and polyvinylpolypyrrolidone (300 mg) was placed in it. Four milliliters of extraction buffer [STPE (1M sucrose, 0.2M Na-tartrate, 1 mM EDTA, 10 mM K-phosphate buffer, pH 6.9) supplemented with 0.015 volume of protease inhibitors (Sigma P9599), 30 µg/ml each of DNase I and RNase A and 1.5 mM dithiothreitol (DTT)] were added dropwise to freeze and then, frozen cells (2 g) were added and ground to fine, dry powder with a pestle. The powdered cells were transferred into centrifugation tubes with a cold spatula and immediately after the cells were thawed by hand warming, they were centrifuged at 20,000 x *g* for 10 min at 4°C. Then, the volume of the supernatant was checked and one third volume of DEAE-Sephacel suspension pre-equilibrated

with STPE was added and mixed. Proteins were adsorbed for 5 min at 4°C and centrifuged at 2,800 x *g* for 5 min at 4°C. The adsorption was repeated once more and the precipitates were combined. The precipitate was washed twice with 3 ml of 0.2M ammonium sulfate in PE (10 mM K-phosphate, 1 mM EDTA, pH 6.9) and once with 3 ml of 0.3M ammonium sulfate in PE. The acidic protein fraction was eluted with 0.4 ml of 0.8M ammonium sulfate in PE three times.

Aliquots were taken in 1.5 ml Eppendorf tubes and 250 mg of fine powdered ammonium sulfate per 1 ml of eluate was dissolved by mixing gently. The DEAE-tubulin samples were stored at -30°C.

4.4. ISOELECTRIC FOCUSING AND SDS-PAGE

The DEAE-tubulin was centrifuged at 15,000 x *g* for 20 min. The precipitate was washed carefully three times, with 0.5 ml of 70% ethanol that had been chilled at -20°C. The precipitate was dried in a desiccator for 20 min and suspended in the immobilized pH gradient (IPG) buffer [8 M urea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), 0.5% IPG buffer (GE Healthcare Bio-Sciences, Uppsala, Sweden), 18 mM DTT, 0.001% bromophenol blue] containing 1/100 volume of the protease inhibitors (Sigma P9599).

The suspension was placed in the IPG gel holder and rehydrated the IPG gel [18 cm gel (pH4-7), GE Healthcare Bio-Sciences, Uppsala, Sweden] for more than 12 h at 20°C. Then, isoelectric focusing was performed on IPGphor using the following schedule: 500 V 1 h, 1,500 V 1 h, 4,000 V 1 h, and 8,000 V 3 h. Then a section of the gel 2-9 cm from the cathode (+) end was cut out, equilibrated with sample buffer [25% glycerine, 10 mM DTT, 1.25% SDS (Nacalai Tesque Inc., Kyoto, Japan), 375 mM TrisHCl, 0.001% bromophenol blue, pH 6.8] at 37°C for 8 min twice, and applied on the SDS-10% PAGE gel (85 x 85 mm). Electrophoresis was performed at 150 mA for 2 h in the electrode buffer [25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3] .

4.5. DIGESTION OF ISOFORM SPOTS

Resolved isoform spots were transferred to PVDF membrane, stained with ServaBlue G, destained with 50% methanol, 10% acetic acid in milliQ water and washed with milliQ water three times and dried with dry air. The β-tubulin spots were excised, wet with ethanol, and denaturation and digestion of tubulin were performed on the membrane essentially following the method of Iwamatsu.[39] The membrane was equilibrated with 200 µl of reducing buffer [8 M Guanidine HCl, 0.5 M Tris, 0.3% Na_2EDTA, 5%

acetonitrile, pH 8.5] twice and 50 µl of reduction buffer containing 10 mg of DTT was added, and the reaction mixture was incubated at 27°C for 1 h. Then, 10 µl of 3 mg iodoacetamide in 10 µl NaOH were added and incubated at 27°C for 15 min in the dark. The membrane was washed in 100 ml of milliQ water, and then with 100 ml of 2% acetonitrile at room temperature for 5 min each. It was equilibrated with 200 µl of LysC buffer [20 mM TrisHCl (pH 8.0), 70% acetonitrile] three times and suspended in 25 µl of LysC buffer containing 1.5 ml of 0.1 µg/µl LysC (Mass spectrometry grade, Wako Pure Chem. Ind., Osaka, Japan) and incubated at 37°C overnight.

4.6. MASS SPECTROMETRY

One microliter of reaction mixture (Section 4.5) was mixed with 1 µl of THAP/DAC (0.3% trihydroxyacetophenone: 20 mM diammonium citrate = 1:1) matrix and their masses were analyzed in a MALDI-TOF mass spectrometer (AutoFLEX, Bruker Daltonics GmbH, Bremen, Germany).

For the digestion with AspN, the membrane was equilibrated with 200 µl of AspN buffer [20 mM Tris HCl (pH 7.4), 70% acetonitrile] by changing the buffer three times, and suspended in the AspN buffer containing 1 µl of 0.04 µg/µl AspN (Sequencing grade, Wako Pure Chem. Ind., Osaka, Japan). The mixture was incubated at 40°C for 1 h. One microliter of the reaction mixture was mixed with 1 µl of THAP/DAC and analyzed in a MALDI-TOF MS with negative reflector mode.

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PART 6

STRUCTURAL BIOLOGY AND BIOINFORMATICS FOR CYTOSKELETON RESEARCH

TUBULIN ISOTYPES AND THEIR ROLE IN MICROTUBULE DYNAMIC INSTABILITY, IMPLICATIONS FOR MODELING AND RATIONAL DESIGN OF INHIBITORS

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Abstract. The structural protein, β-tubulin is the target for a number of anti-mitotic compounds that bind to and inhibit microtubule dynamics, leading to apoptosis in all dividing cells. The existence of several isotypes of β-tubulin, coupled with their varied distribution throughout different organisms provides a platform upon which to construct novel agents, which are able to differentiate between cell types. Several examples of compounds that perturb microtubule dynamics, such as paclitaxel, are currently some of the most effective drugs used in cancer chemotherapy. Additionally, MT disrupting agents such as the dinitroanilines, disrupt plant but not animal microtubules and are therefore useful herbicides. However, even with their wide use and important function, the method of action of MT disrupting compounds is unknown. We have performed homology modeling on approximately 500 α- and β-tubulin sequences and identified an expected global, structural similarity of tubulin monomers. We have been able to calculate discernable differences in several properties, including their net electric charge, volume, surface area, electric dipole moment and dipole vector orientation. These are properties that may influence the functional characteristics of individual tubulin monomers, thereby resulting in a global effect on microtubule stability and assembly kinetics.

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Keywords: Tubulin, microtubule, isotype, ligand binding, colchicine, paclitaxel

1. Introduction

A key attribute that links the structure of tubulin to the function and stability of a microtubule (MT) is the existence and distribution of several isotypes of both α and β tubulin. At the molecular level, the role of tubulin is extremely complex and seems to be related to structural variations observed between α and β isotypes. In all eukaryotic organisms, several α and β tubulin isotypes have now been identified and characterized.[1] In addition to α and β tubulin, three other tubulin homologues are required for efficient MT assembly *in vivo*. The first, γ tubulin, is not essential for assembly, yet is found at MT-organizing centers and is thought to aid in initial MT nucleation.[2] Two other homologues, δ and ε tubulin, have uncertain roles in MT assembly, although models have been proposed that describe their possible involvement in MT self-assembly.[3] At the molecular level, the roles and interactions of tubulin within MTs are complex and differ between isotypes. For instance, MT dynamics appear to change with β tubulin isotype composition.[4] This implies that if MT assembly/ disassembly equilibria are disrupted, cells might respond by producing an appropriate isotype mix to restore normal balance. The functional heterogeneity of tubulin isotypes is also regulated by the expression of spatio-temporally distinct gene products, many of which are also subject to post-translational modification.[1,5] However, while the presence of numerous tubulin isotypes suggests that they perform a key role in MT function, there are still no precise models to describe their mechanisms of action.

1.1. TUBULIN ISOTYPES: THEIR STRUCTURE, FUNCTION, AND RELATIONSHIPS

An apparent conservation of the three-dimensional pattern of conserved amino acids within tubulin points to the existence of a conserved folding nucleus.[6] However, mapping evolutionary rates onto the sequence of the α/β tubulin dimer has demonstrated the opposite pattern.[7] The relative rates of evolution at sites within the core of the tubulin proteins seem to occur more rapidly than the rates for sites at the surface. This is most likely due to the large number of critical intermolecular interactions between tubulin molecules within the MT, leading to the conservation of binding sites on the protein surface. It is clear that much of tubulin's surface is indeed invariant, however those substitutions that do occur are clustered at positions that comprise the longitudinal interface between protofilaments (see Figure 1).

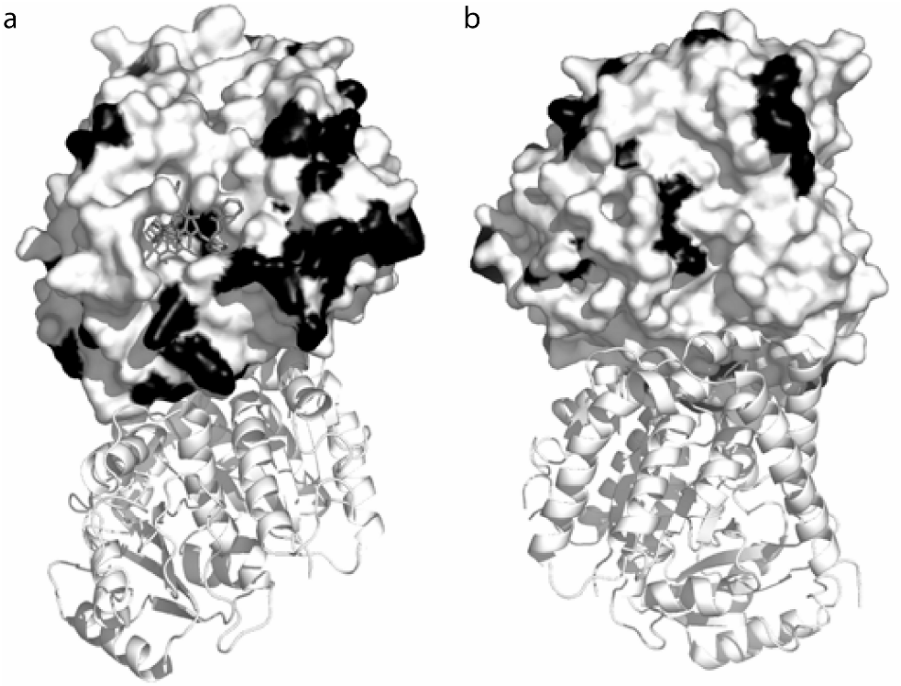

Figure 1. Surface Map of non-conserved Residues in all Human β-Tubulin Isotypes. A solvent accessible surface was drawn onto the β-tubulin monomer within the α/β-tubulin heterodimer as obtained by Nogales et al. 2001 (PDB identifier 1JFF). The α-tubulin monomer is depicted as a cartoon, while the β-tubulin surface colored white with non-conserved positions colored black. Panel a. Illustrates the MT from the luminal surface and shows the binding site for paclitaxel (grey). Panel b. Illustrates a 180° rotation about the y-axis to show the surface of the microtubule.

This observation implies that there must be a significant interaction at the inter-dimer interface, between the individual protofilaments, that is essential to understand the properties that each isotype contributes to overall MT stability.

1.2. ISOTYPES AND MICTOTUBULE DYNAMICS

Tubulin isotype composition has previously been demonstrated to have a measurable effect on MT assembly kinetics.[4,8] Small differences in the binding energies and chemical affinities of different tubulin isotypes may translate into significant deviations in the growth rates and catastrophe frequencies. Differences could also be due to changes in the electrostatic potential of each tubulin isotype, in particular, its net charge. This property although significantly screened by counter-ions does affect MT assembly by influencing dimer-dimer interactions over relatively short distances (approximately 5 nm). Short-range interactions, such as these, have been

studied by calculating the energy of protofilament-protofilament interactions demonstrating that two types of MT lattices (A and B lattices differ in the relative positions of the protofilaments relative to each other) corresponded to local energy minima in the context of an electrostatic potential.[9] As interest in the research of tubulin isotype expression grows, it is becoming evident that cells are capable of altering the expression of each tubulin isotype in response to external conditions that affect MT stability. There are several examples of this response, most recent of which is the overexpression of tubulin isotype βIII following exposure to MT stabilizing agents such as paclitaxel.[10–13] It has also been shown that the *Vinca* alkaloids bind best to βII,[14] providing an explanation of their particular efficacy in leukemia and Hodgkin lymphoma cases, since these types of cancer cells express βII while normal lymphocytes do not. The design of novel drugs based on isotype specific differences would therefore provide therapies targeted only to specific cells.[15]

1.3. PLANT ISOTYPES

Tubulin isotype expression has previously been documented in many plant species.[16–18] Phylogenetic analysis of these genes has revealed at least four distinct classes of plant β tubulin isotypes. Class I comprises the largest cluster, while class II consists primarily of β tubulins found mostly in dicots. Class III and class IV have a much narrower distribution and seem to be preferentially expressed in pollen.[19,20] Several examples of tubulin isotype expression have been documented and the overall family sizes vary in higher plants, with six α and nine β tubulin genes in Arabidopsis.[18] Arabidopsis βI and βV are preferentially expressed in roots and leaves, while βVIIII accumulates specifically in pollen.[18,19] Rice also contains eight β tubulin isotypes that show variable expression during plant development.[20] Other examples of spatio-temoral gene expression are also seen in cotton, where all of its five α tubulin genes are highly expressed in elongating root fibers.[21]

2. Structural modeling of tubulin isotypes

A major advance for our understanding of MT structure and function was the solution of tubulin's 3D structure.[22] Unfortunately, no data on the structural differences between tubulin isotypes exists, with the exception that protofilaments constructed of a mixture of tubulin isotypes showed no difference in electron density from isotypically purified βI,[22] suggesting that there is no major conformational change involved in differences to dynamic instability. Additionally, there are no structures of plant tubulin, which show the greatest differences from animal sources, available. However,

using homology modeling, we have previously been able to construct likely candidate structures for all tubulin sequences that are currently known. This has allowed us to produce coarse models of tubulin's bulk structure, as well as providing insight into possible differences between isotypes.

2.1. COMPARATIVE MODELING

Comparative, or homology modeling uses known protein structures, referred to as templates, to produce 3D models of a target protein with a related amino acid sequence. By assuming that parts of the target and templates with similar sequences have similar 3D structures, portions of the template structures may be copied and amino acids substituted, inserted, or deleted to produce target models. If more than one template structure is used, they must be fit together into a unified model. Furthermore, any part of the target not obtained from the templates might be constructed via *de novo* methods. Finally, the result must be optimized to a protein-like geometry. This technique has been verified for a small number of homologous protein families and can yield accurate results for template/target pairs with strong sequence identity.[23]

2.1.1. *Tubulin sequences*

The availability of numerous tubulin isotype sequences provides us with the opportunity to create a library of tubulin models, from which calculated physical characteristics can be correlated with biological roles for MTs. To produce our models, the Uniprot Knowledgebase, consisting of Swiss-Prot release 42.1 and TrEMBL release 25.12 (March, 15, 2004) was downloaded and filtered for amino acid sequences annotated with the term "tubulin", thereby producing an initial set of 2,294 entries.[24] This set was subsequently filtered to remove entries labeled as fragmentary, producing an intermediate subset of 652 sequences. Using default parameters within Clustal W (version 1.82) these sequences were aligned and using cluster analysis assembled into a guide tree, or dendrogram.[25] This step identified sequences that were not tubulin, but were actually unrelated proteins with tubulin in their name (e.g. tubulin tyrosine ligase or tubulin-folding cofactor D). Since these non-tubulin sequences differ significantly from the tubulins, they clustered separately in the dendrogram, providing a clear delineation between 557 "tubulin-like" and 95 non-tubulin sequences. A second dendrogram guide tree was then produced using only the "tubulin-like" sequences. We identified three major branches on this second tree, the first containing 190 α tubulin sequences and the second containing 285 β tubulin sequences which were used as our final dataset for comparative modeling.

2.1.2. *Tubulin structural templates*

A total of five unique structures, upon which we can construct homology models of β tubulin, are available in the Brookhaven Protein Databank. The first, and most obvious choice was the initial Nogales et al.[22] structure of the α/β tubulin heterodimer (PDB identifier 1TUB). However, this structure was produced at a comparatively low resolution and contains numerous omissions and misalignments due to difficulties in density fitting. A refinement of the 1TUB structure (PDB identifier 1JFF) was subsequently deposited with a slightly better resolution at 3.5 Å.[26] Along with increased resolution, the 1JFF structure also addressed a number of misalignment errors within the 1TUB structure and therefore makes a substantially better choice for a homology modeling template. Two additional structures of the tubulin heterodimer that are complexed with the stathmin-like domain, RB3-SLD, in the presence of colchicine (PDB identifier 1SA0) and vinblastine (PDB identifier 1Z20) are also available.[27,28] The 1SA0 structure was solved at 3.5 Å, while the 1Z20 structure was refined to only 4.1 Å. While the resolution of 1SA0 is slightly better than that in 1JFF, this structure contains a nine-residue gap in the β tubulin, ranging from Ser277 to Leu286. Finally, a template structure of the bacterial protein FtsZ, a prokaryotic tubulin homologue, was also available for use as model template.[29] When initially generating models of the β tubulin monomers, only the 1FSZ structure was used and we determined that the presence of several extended loops within models was a direct result of alignment anomalies between the structure and our β tubulin alignments. It was therefore decided to eliminate the FtSZ structure from our templates and because of the high sequence homology and the greatest structural resolution, we chose to only use 1JFF and 1SA0 as our templates for modeling all of the β tubulin isotypes.

Because the sequences within β tubulin family are more similar to each other than to those sequences belonging to the other tubulin isotypes, it is reasonable to believe that any given sequence should produce a structure very similar to another member of a given family. Further support for this comes from the published structures of Nogales et al.[22] and Lowe et al.[26] which are of a porcine sequence that were fit to structural data from an inhomogeneous bovine sample. Accordingly, by substituting appropriate amino acid side chains and properly adjusting other residues to accommodate insertions and deletions in the sequence, these crystallographic structures can be used as a framework to produce model structures with different sequences to a high degree of confidence.

2.1.3. *Model generation*

The tubulin templates used for comparative modeling are incomplete; each has missing and weakly defined residues. Modeller was used to create models of these missing regions.[30] For instance, one α tubulin loop is incomplete with residues Q35–L60 missing from the A chain in 1JFF and residues P37–D46 and S38–D46 missing from the A and C chains of 1SA0, respectively. Both of the 1SA0 β monomers, B and D chains lack residues R278–A285 and several side chains within these structures are also truncated prematurely. Further, all template structures lack a 20–30 residue region, commonly referred to as the C-terminal tail (from approximately residue 440 to the C-terminus); this region is believed to be highly flexible and was therefore omitted from our models.

3. Models and results

Having obtained models of the entire known collection of tubulin isotypes, we were able to study their physical properties and infer how these may affect MT structure and function. Not surprisingly, comparison of all the tubulin models shows that regions that are found within the templates have defined structures, and those without templates are more disordered (Figure 2).

The region missing from the 1JFF α tubulin template (Q35–L60) produces a large unstructured loop away from the core of the monomer in many models. The large variability of this region within the human repeats led us to reject the 1JFF α tubulin derived models from further consideration. In the 1SA0-based models, due to the resolved structure of the template, this region has a more compact structure, although it is more variable than most other regions of the models. In all the models, loops corresponding to residues 360–371 and 275–286 are also of high variability, owing to unresolved structure in the templates. Subtle variations within the structure of tubulin could have an effect on dimer interactions, which may translate into significant changes in assembly/disassembly rates for MTs. We believe that this library of α and β tubulin models is large enough to sample the diversity of tubulin variation. Indeed, our results suggest that deviation from the model template is the dominant source of variation that we see. For each of the resulting tubulin models, we have estimated net charges, surface areas and dipole moments and discuss their implications for MT assembly below.

Figure 2. Superposition of human β tubulin homology models. Several of the tubulin homology models were superimposed over one another to illustrate regions within the models that are more flexible. This generally includes solvent exposed surface loops which were either missing or have greater variability within the template structures.

3.1. TUBULIN ELECTROSTATICS

One contribution to the interactions between tubulin dimers in the protofilament is due to the electrostatics of each tubulin monomer, in particular, their net charges and dipole moments. It was previously demonstrated that tubulin electrostatics contribute to the energy of relative longitudinal offsets between two protofilaments, producing energy minima relating to the two MT lattices.[9] The net electrostatic charge is perhaps the simplest approximation to a complicated electrostatic field produced by tubulin. Slightly more complex is the electrostatic dipole moment, which is due to opposing positive and negative charges along a central axis, and which is radially symmetric about this axis. Like the monopole, the dipole is an average over a real system and is generally inadequate for representing

the complex electrostatic field near a protein surface. It is screened in a solvent by water and counter-ions, but having both a magnitude and an orientation, a dipole may give rise to more complex interactions than a monopole alone. With specific charges assigned to points in space, we can calculate a dipole moment centered about a chosen co-ordinate origin. If a system has zero net charge, this dipole moment is independent of the choice of origin, but is otherwise dependent upon the origin. The tubulin sequences generally contain more acidic than basic residues and accordingly have a negative overall electrostatic charge. In an overly simplistic analysis we could expect that these negative charges would repel, thereby preventing MT formation. Reality is more complex, as systems with ionic solutions are electrically neutral, and at long ranges counter-ions in the solvent balance the net solute charge. At short ranges charge is partially screened and other solvent-related screening and dielectric effects may be relevant. For short-range interactions between dimers, some regions will be non-trivially closer than others, and close charge pairs may dominate the electrostatic field over more distant charge pairs. The extreme of this occurs between monomers, where contacting regions have interactions strong enough to form a protein-protein bond.

3.2. DIPOLE MOMENT ESTIMATES

Having built our tubulin models, their total charge, and dipole moments were estimated using analysis tools in Gromacs v. 3.3.[31] Models were converted using pdb2gmx with the GROMOS96 43a1 force field, providing atomic charges, sizes, and some hydrogen atom locations.[32] As a side-effect, the net charge of the sequence is reported, assuming charges of +1 e for lysine and arginine, –1 e for aspartic and glutamic acids, and 0 e for all other residues. Connolly solvent accessible surfaces (SAS) were then calculated using the Gromacs g_sas program.[33a] The Gromacs program, editconf was used to shift the co-ordinate origin to the model's geometric center, which is expected to be a good approximation of the monomer center-of-mass. Dipole moments were then calculated using g_dipoles. To convert dipole components from g_dipole into a cylindrical coordinate system used in subsequent calculations, each 1SA0 monomer was α-carbon RMSD fit onto the corresponding 1JFF monomer to yield components in a 1JFF coordinate system; 1JFF coordinates were rotated by 63° about the protofilament axis. The dipole moment of each model was transformed identically to that of the template.

The α tubulins are estimated to have dipole magnitudes about their centers in the range 1,000–2,000 D, while β tubulin magnitudes are generally higher, in the range 2,000–3,000 D (Figure 3).

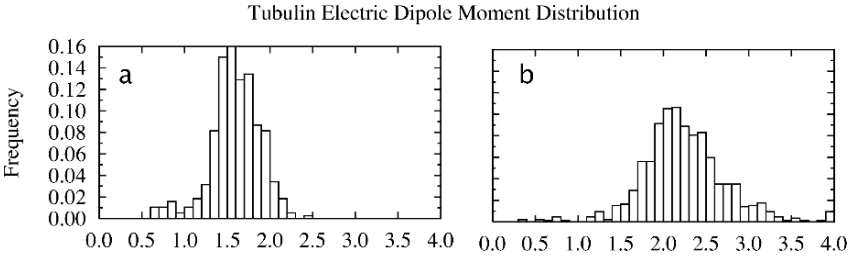

Figure 3. Distribution of the estimated dipole moment magnitudes for α tubulin (Panel a) and β tubulin (Panel b).

The β tubulins are, on average, more highly charged with an approximate mean of –24 e, compared with –22 e for the α tubulins. The β tubulins also have a slightly broader net charge distribution than α tubulins (standard deviation of 3.0 e versus 2.6 e). There appears to be little if any correlation, in the scatter plots, between the dipole moment and the net charge as might be expected since polynomial expansions coefficients such as the electrostatic potential generally have no dependence on one another (Figure 4).

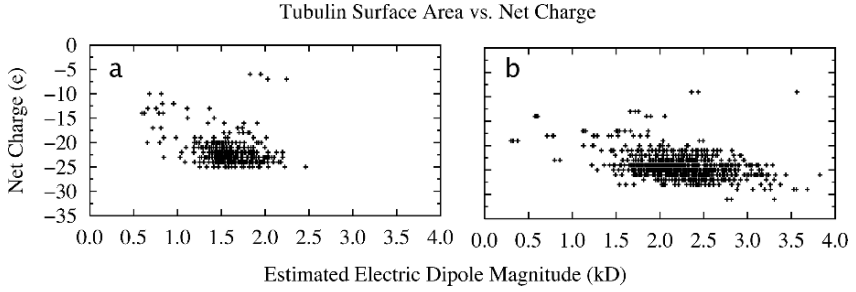

Figure 4. Comparison between the dipole moments and net charges for α tubulin (Panel a) and β tubulin (Panel b).

Sharply contrasting to these numbers are experimentally observed unscreened charges of approximately –0.2 e per tubulin dimer (with pI ~ 5) in 1–5V/cm electric fields applied externally at pH6.8.[33b] Despite the large difference between the charges reported here and the measured numbers, these values are still appropriate for comparisons since in solution, electrostatic fields are screened by counter-ions and affected by hydration shells of water molecules. Furthermore, the complicated surface electrostatic fields control polymerization of tubulin and protein-ligand binding.[34] It is important to note that while no single, absolute value can be stated for the charge of an atom, as it depends on surrounding charges, many similar models of atomic charge have been developed for molecular simulations.

Since the dipole-dipole energies of our calculations depend on a uniform dielectric constant for both protein and medium, which depends on solution conditions (pH, ionic concentrations, etc.), it is impossible to give precise and meaningful numbers, but relative comparisons may make sense. Schoutens estimated dipole-dipole interactions in a MT and concluded that they have a destabilizing effect amounting to as much as 50–60% of the elastic free energy of the lattice.[35]

The dipole moments of tubulin dimers may have a direct role in MT assembly. For example, the dipole could be instrumental in the docking of ligands to tubulin and in creating the proper steric configuration of a tubulin dimer as it approaches an MT for binding. In an MT, individual dimers have near parallel dipole moments relative to their nearest neighbors. The greater the individual dipole of each dimer, the less stable the MT will generally be since these interactions will be repulsive, pushing apart the MT. Note the strength of these interactions is proportional to the square of the dipole moments. Hence MTs made up of tubulin with larger dipole moments should be more prone to disassembly than those with lower dipole moments. For cells expressing more than one isotype, one can conceive MT dynamics regulated by altering relative amounts of different isotypes. However, these effects are moderated by local interactions between two dimers, which produce more complicated electrostatic field than an assembly of dipoles suggests; for details see.[36]

3.3. SOLVENT ACCESSIBLE SURFACE AREA

The solvent accessible surface areas are largely the same for α and β tubulins with mean areas of approximately 220 nm², and standard deviations of 10 and 30 nm², respectively. This difference in variation is visible in Figure 5 where surface areas for α and β tubulin are compared with the

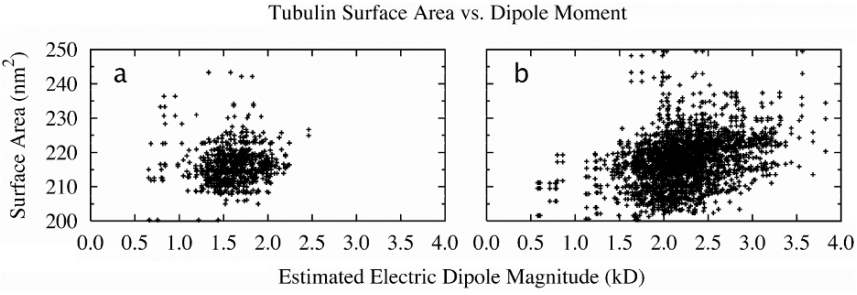

Figure 5. Comparison between tubulin isotype models solvent accessible surface areas and estimated dipole moment magnitudes. Panel a illustrates correlation within α tubulin models. Panel b illustrates correlation within β tubulin models.

estimated dipole moments. Little correlation between these two properties is evident.

4. Tubulin binding drugs and their role in MT dynamics

Due to the quantity of data that would need to be analyzed when examining discrete differences between all of the isotypes, we have chosen to focus only on the human β tubulin isotypes for simplicity. We feel that this is an appropriate approximation, as all characterized MT disrupting drugs bind to β tubulin and most of the available data deals with the human isotypes. When displayed on the surface of the 3D tubulin structure, it becomes apparent that most of the differences found between the various isotypes are restricted to the lateral and longitudinal surfaces that are involved in protein-protein interactions with the neighboring tubulin dimers within the MT (Figure 1). Differences at these locations may provide the cell with the ability to alter expression patterns for tubulin isotypes and in this way affect the overall kinetics of MT assembly and disassembly. This could be in response to drug exposure. Interestingly, several differences can be observed on the exterior face of the β tubulin when it is assembled into the MT, while there are significantly fewer observed differences on the inner surface. This may be a result of selective differences between isotypes that are meant to accommodate Microtubule Associated Protein (MAP) binding, thereby leading to altered MT kinetics.[37] However, the underlying molecular mechanism of tubulin isotype expression, in particular βIII over-expression, in paclitaxel resistance is not well understood. More likely, it is a critical change within surfaces that interact during MT assembly. Therefore, the expression of tubulin isotypes would result in the direct modification of MT stability and a requirement for less stable MTs would result in the over-expression of isotypes such as βIII, which is more dynamic than others and which seems to have several differences at the longitudinal and lateral interaction surfaces.

The dynamic properties of MTs are especially critical during mitosis, when a delicate equilibrium of distances and forces is necessary for proper chromosome alignment prior to segregation.[38–43] MTs comprise much of the mitotic spindle apparatus and its proper assembly is essential, as it likely provides the substantial mechanical force for mitotic chromosome segregation.[44] Assembly failure of the mitotic spindle generally results in mitotic arrest, apoptosis, and eventually cell death. As such, MTs have become the target for a large number of anti-mitotic agents including antitumor drugs such as the taxanes, epothilones, colchicine and *Vinca* alkaloids.[45] The method of action of these drugs is to either promote or inhibit MT polymerization by binding at specific sites on the surface of α/β-tubulin

heterodimers (Figure 1). Paclitaxel, which continues to be one of the most successful cancer therapeutic agents, binds to and results in the stabilization of MTs within all cells.[46,47] Derivatives of paclitaxel, such as docetaxel, have been synthesized to address the limited solubility of paclitaxel and show increased binding to β-tubulin.[48] Two additional families of similar tubulin-binding drugs are the epothilones and dolastatins. Interest in the epothilones over existing MT stabilizing agents such as paclitaxel is primarily due to their ability to retain cytotoxicity in multidrug resistant (MDR) cells.[49] Compounds, such as discodermolide and sarcodictyin have been shown to inhibit the proliferation of human cells through a mechanism similar do that of the taxanes. These compounds seem to selectively stabilize MTs during cell division, and out-compete paclitaxel for tubulin binding. In the absence of paclitaxel, it has been predicted that the energy stored in longitudinal contacts is greater than that stored in lateral contacts.[50] Recent results using hydrogen/deuterium exchange coupled with mass liquid chromatography have demonstrated that increased rigidity in paclitaxel stabilized MTs was distinct from stabilization as a result of GTP-induced polymerization.[51] The authors suggest that paclitaxel increases the overall energy associated with polymerization while maintaining the same differential between lateral and longitudinal contacts in the MT lattice.

In plants, the dinitroaniline herbicides (such as trifluralin, ethafluralin and oryzalin) have been developed for the selective control of weeds. However, prolonged use of these chemicals has resulted in the selection of resistant plants. The dinitroanilines method of action is identical to that for the MT binding compounds previously discussed. They disrupt the MTs of plants, ranging from single-celled alga to higher plants.[52–54] Dinitroanilines also disrupt the MTs of protozoa, including both free-living species such as Tetrahymena and protozoan parasites such as Trypanosoma.[55–57] Remarkably, the activity of dinitroanilines is restricted to only plants and protozoa; these compounds are ineffective against vertebrate or fungal MTs.[58,59] Given that dinitroanilines do not disrupt vertebrate MTs, its activity is of particular interest for the development of anti-parasitic drugs.

4.1. PACLITAXEL AND EPOTHILONE BINDING SITE

While arguably one of the most important MT disrupting agents, the binding of paclitaxel or epothilone within β tubulin is unable to fully explain the effect of these drugs on MT stabilization. This is because the dynamics of an individual tubulin heterodimer, within the greater context of the MT is not fully understood. This conclusion directly impacts our ability to create a precise model of the paclitaxel or epothilone stabilizing effect. Lowe et al.[26] have previously suggested that the stabilization of the M-loop,

upon paclitaxel binding, plays a role in the underlying dynamics of a MT. Based on this hypothesis, it is reasonable to assume that differences between the βIII and βVI isotypes (Ser275, Arg276) may prove to be essential when addressing paclitaxel or epothilone binding on MT stabilization. Several residues have been identified that are involved in its interaction with β tubulin. First, Val23 was shown to make hydrophobic contacts with both the N' and 3' phenyl rings. Asp26 was shown to be within hydrogen bonding distance to the nitrogen group in paclitaxel's C13 side chain. Additionally, leucines 215, 217, 228 and His227 make hydrophobic contacts with the 2-phenyl ring, while Ala231, Ser234 contact the 3' phenyl group. While Phe270 does not directly contact paclitaxel, it was identified as a key residue within the hydrophobic binding pocket. Several residues within the M-loop were also identified as essential for the taxanes interaction with tubulin. These residues include Pro272, Leu273, Thr274 (which contacts the essential oxetane ring) as well as Ser275 and Arg276. Additionally, Pro358, Arg359, Gly360 and Leu361 were also shown to not interact directly with paclitaxel, but form the binding pocket itself. Through our analysis of the human β tubulin isotypes, we have identified six positions

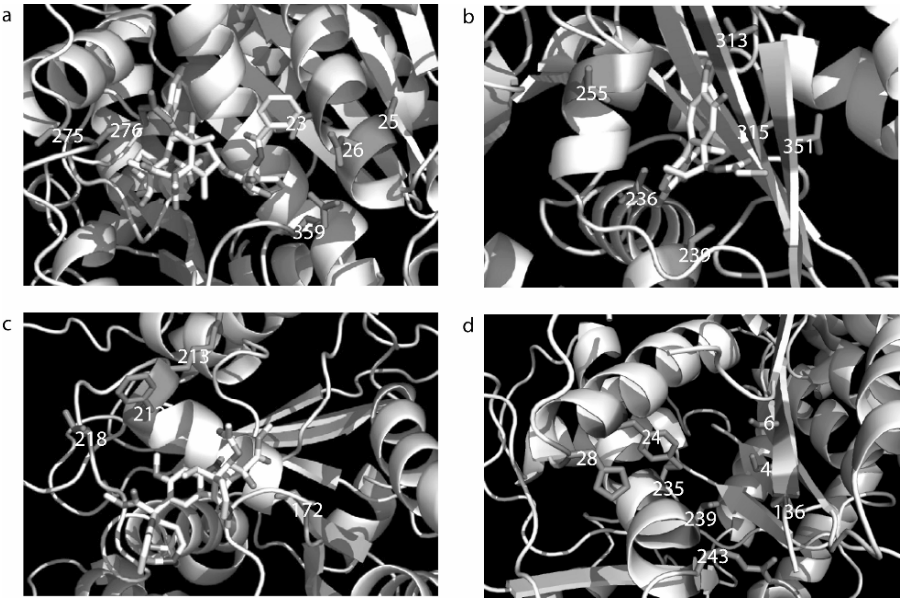

Figure 6. Schematic diagram of isotype differences in the paclitaxel, colchicine and vinblastine and the putative dinitroaniline binding sites. Residues containing any atom within 6°A of the bound drug molecule were selected and the amino acid side chain differences between each of the isotypes, within this cut-off, are shown as grey sticks over white cartoon representations of the protein backbone. (a) The paclitaxel binding site from 1JFF. (b) The colchicine binding site from 1SA0. (c) The vinblastine binding site from 1Z2B. (d) Mutated residues found within the putative dinitroaniline binding site.[69]

show any difference between each of the β tubulin isotypes, all of which occur within the βIII, βVI and βVII sequences (Figure 6a). Unfortunately, most of these substitutions are conservative, with the exception of βVI position Ser25-Gly and βVII position Arg359-Trp.

4.2. COLCHICINE

Unlike paclitaxel, the binding of colchicine does not result in MT stabilization, but results in their destabilization. While the binding site for colchicine was shown to be at the interface between α and β tubulin, the majority of the interaction is restricted to the β tubulin monomer. Ravelli et al. (2004) identified several principal interactions between the bound colchicine and β tubulin.[60] First were interactions with strands S8 and S9, helix H7 and H8 and loop T7 (see Nogales et al.[22]). These interactions occur as a result of the colchicine binding site being buried within the intermediate domain of β tubulin. The authors also suggest that interactions between colchicine and α tubulin's T5 loop could result in the observed stabilization and bending of the α/β heterodimer.[61] They also point out that upon binding colchicine, the displacement of residues Asp249 and Lys349 are thought to interfere with filament formation. Additional biochemical data has also suggested that Val316 and Cys239 are also involved in colchicine binding.[62] Observations have also been made that suggest the chemical sensitivity of Cys239 and 354, within tubulin, are altered upon binding of colchicine.[63] We identified seven positions that contained differences between the β tubulin isotypes. All of the observed substitutions occur within the H7 and H8 helix, as well as the S8 and S9 sheets. These positions were initially identified by Ravelli et al.[60] as those that are displaced upon colchicine binding to the β tubulin. In contrast to the paclitaxel binding site, differences here occur over a wider range of β isotypes, encompassing βIII, βV, βVI, βVII and βVIII (Figure 6b). Many of these substitutions are conservative, with the exception of βVII position Val255-Met and βIII, βVII and βVII position Ala316-Thr/Cys.

4.3. THE VINCA ALKALOIDS

The *Vinca* alkaloids include vincristine vinblastine and vinorelbine and are used most commonly in combination chemotherapy regimes. Vinblastine was shown to bind at the inter-tubulin dimer interface, ultimately resulting in the net reduction of polymerized tubulin concentration.[28] At high concentrations the *Vinca* alkaloid, vinblastine, binds to MTs and results in their depolymerization. However, at low concentrations, vinblastine is thought to bind to MT tips and suppress their dynamic instability.[64] This

may be a result of vinblastine's ability to maintain the tubulin dimer at the end of the growing MT in a slightly bent conformation.[65] If the tip of a protofilament was maintained with a slightly bent conformation, due to the positioning of vinblastine between the dimers, then at low enough concentrations, a small amount of drug bound to the end of the MT would mimic the presence of a GTP cap, thereby resulting in MT stabilization. At high concentrations vinblastine binds to MTs and results in their depolymerization.

The only available tubulin structure containing vinblastine identified its binding site at the interface between the α/β heterodimers within the protofilament (PDB identifier 1Z2B). Gigant et al. (2005)[28] identified several important interactions between β tubulin and vinblastine. First, several contacts with residues Val175, Asp177, Tyr208 and Phe212 were shown to be in agreement with previous photoaffinity labeling experiments that identified residues 173–211 to be involved in vinblastine binding.[66] Of these four residues, Asp177 was shown to become displaced from a position, close to the bound GTP molecule, to a position that is near vinblastine. Residue Tyr222 was also identified as becoming captured between the base of the bound GDP and the D' ring within vinblastine. We have identified only four positions show any variation within each of the β tubulin isotypes, encompassing βIII, βVII and βVIII (Figure 6c). Interestingly, unlike the binding sites for paclitaxel and colchicine, substitutions within the vinblastine-binding site are somewhat less conserved.

4.4. DINITROANILINES

While the precise binding sites for the dinitroanilines has not been determined, molecular modeling has demonstrated that these compounds most likely associate with α tubulin.[67] Subsequently, through analysis of mutations that confer dinitroaniline resistance, it was proposed that these compounds act through a novel mechanism, by disrupting M-N loop contacts within protozoan MTs.[68] Mutation of specific residues has been shown to lead to dinitroaniline-resistance.[69] Most of these residues are found near the N-loop at the interdimer interface, however several are also located within the M-loop at the intradimer interface. Those residues identified in the N-loop include His28, Phe49 and Phe52, which are identical to residues found in vertebrate α tubulin. However, within the 1JFF structure of bovine tubulin, most of the residues found within the N-loop are missing due to poor resolution and presumably greater flexibility in this region of the protein. Within the proposed dinitroaniline binding site, several mutations have also been identified (Figure 6d). The first of these mutations that show differences in vertebrate α tubulin is Val4, which is Ser. Next, Phe24 is a Tyrosine and Leu136 is a Serine in vertebrate α tubulin. Interestingly, all of these

residues form a rather tight ring at the surface of the binding pocket and differences may lead to altered drug entry into the binding site. Several other residues including Ser6, His28, Ile235, Thr239 and Arg243 are identical to their counterparts in vertebrate α tubulin.

5. Conclusions

Even with the vast quantity of data available to us, it is still not entirely clear what effect the expression of a particular β-tubulin isotype, or acquisition of point mutations has on the stability of cellular MTs and their relationship to drug resistance. Two possible models may explain the role that these two processes play in drug resistance. First, it may be possible that certain isotypes or mutations exhibit decreased drug binding to β-tubulin. However, our previous observations suggest that while this may contribute to drug resistance, it most likely plays a minimal role due to the lack of observable differences within the drug binding sites.[70] A second mechanism could produce global conformational differences between β-isotypes or mutants, thereby attenuating MT dynamics.

A number of studies suggest that altered paclitaxel binding is not responsible for differences in MT stability. For example, β-tubulin is reported to contain the taxane binding site, yet mutations conferring paclitaxel resistance occur with equal frequency in both α and β-tubulin and both groups of mutants exhibit similar properties.[12] Many paclitaxel-resistant cells require the presence of the drug for cell division and, therefore, must retain the ability to bind the drug. Also, paclitaxel-resistant cells frequently exhibit increased sensitivity to drugs such as colchicine and vinblastine that bind to distinctly different sites. Instead of altered drug binding, we favor a mechanism in which binding of compounds such as paclitaxel alter the conformation of both the M-loop and the short loop connecting H6 and H7 of β-tubulin in such a way as to facilitate and stabilize protein-protein interactions important in the formation of MTs. Recent fitting of the crystal structure of tubulin to a lower resolution map of the MT is consistent with the participation of the H6-H7 loop in both longitudinal and lateral contacts.[71] This hypothesis is also supported by the recent observations that suggest paclitaxel-binding may act to increase the energy associated with lateral and longitudinal interactions between tubulin dimers in the MT.[51] Therefore changes within these surfaces would mitigate the action energetic contributions of paclitaxel binding. In general we can consider that cells resistant to MT stabilizing drugs, such as the taxanes or epothilones, will produce less stable MTs in response. This is demonstrated by decreased drug induced MT stability and often a requirement on the drug for growth. Conversely, cells that become resistant to MT destabilizing drugs such as

colchicine or vinblastine will produce MTs with increased stability. We must, however, be cautious when examining the data available in the literature, as much of the experimental results have been obtained through *in vivo* screening and do not give us specific information about paclitaxel binding to β tubulin. We believe that the key to developing new drugs that target tubulin and disrupt MT dynamics is through a better understanding of MT dynamics at the molecular level and the role mutations and isotype differences have on the dynamic properties of tubulin and the MT. With this idea in mind, we feel that the colchicine-tubulin interaction may have the greatest pharmacological importance, yet this interaction is poorly understood in terms of its biological significance. Colchicines apparent discrimination between isotypes of β tubulin, coupled with its increased affinity for animal tubulin over plant tubulin makes it an ideal candidate for future drug development.

Acknowledgements

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DINITROANILINE INTERACTIONS WITH TUBULIN: GENETIC AND COMPUTATIONAL APPROACHES TO DEFINE THE MECHANISMS OF ACTION AND RESISTANCE

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Abstract. The dinitroanilines are small molecules that selectively bind to plant and protozoan tubulin dimers and disrupt microtubules in these organisms. Despite the high degree of sequence conservation among all tubulins, these compounds do not bind to tubulins from fungi or vertebrates, nor are microtubules in these organisms disrupted by dinitroaniline treatment. Studies on tubulin affinity for dinitroanilines, dinitroaniline effects on microtubule dynamics and the genetics of dinitroaniline resistance in the unicellular alga *Chlamydomonas reinhardtii* and in the higher plants *Eleusine indica* and *Setaria viridis* are summarized here. We also describe our ongoing research which exploits computational methods and the genetics of resistance in the protozoan parasite *Toxoplasma gondii* to describe the action of the dinitroanilines on tubulin. We conclude that the dinitroanilines bind beneath the H1-S2 (N) loop of α-tubulin and cause this loop to be drawn inwards, disrupting protofilament interactions within the microtubule. *Toxoplasma* mutations that confer resistance to these compounds appear to act by increasing microtubule stability or by decreasing affinity for dinitroanilines. Moreover, resistance mutations impose a fitness cost on *Toxoplasma*, and growth of resistant parasites in the absence of dinitroanilines leads to the spontaneous appearance of suppressor mutations. Many of these secondary mutations are point mutations located in the α- or β-tubulin genes. The

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suppressor mutations act to improve the growth of *Toxoplasma* and to decrease resistance to dinitroanilines, indicating that there is a trade-off between dinitroaniline resistance and tubulin function.

Keywords: Docking, fitness, herbicide, microtubule, molecular dynamics, mutant, parasite, protozoa, resistance, *Toxoplasma*

1. Discovery of the "yellow" herbicides

The action of dinitroanilines, informally called the "yellow" herbicides, was first reported in 1960 by scientists at Eli Lilly who observed that trifluralin inhibited plant root growth.[1] Further studies established that growth inhibition by these compounds (Figure 1) was associated with inhibited cell division reflected by abnormal mitotic figures observed in dinitroaniline-treated root tips of various plants. By drawing parallels with contemporary

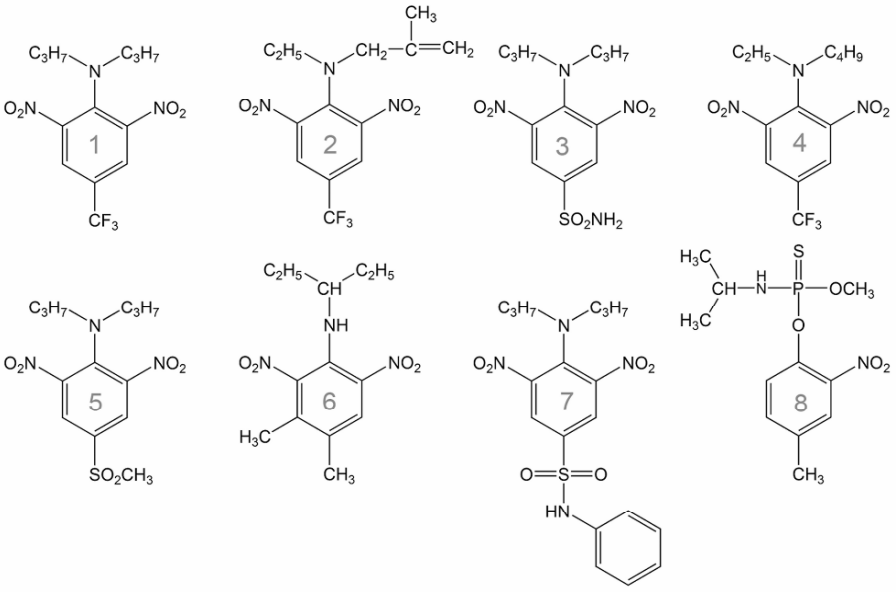

Figure 1. Dinitroaniline compounds include (1) trifluralin, (2) ethalfluralin, (3) oryzalin, (4) benfluralin, (5) nitralin, (6) pendamethalin, and (7) GB-II-5, an oryzalin derivative with increased activity against tubulin from kinetoplastid parasites. Amiprophosmethyl (APM, 8) is categorized as a phosphorothioamidate herbicide rather than a dinitroaniline but is likely to interact with tubulin in a similar fashion. It competes with dinitroanilines for binding to plant tubulins and dinitroaniline-resistant organisms are cross-resistant to APM.

studies of the action of colchicine on cell division, researchers correlated inhibition of mitosis with a reduction or total disruption of spindle microtubules in dividing plant cells treated with dinitroanilines.[2–4] Studies of the effect of dinitroanilines on land plants established that root and shoot elongation are inhibited and that cells in meristematic regions are arrested during mitosis with an enlarged and multinucleate phenotype correlated with dinitroaniline-induced microtubule disassembly.[4–9]

The earliest studies to investigate the interaction of dinitroanilines with tubulin used pig tubulin and these experiments indicated that trifluralin did not bind to or inhibit polymerization of tubulin.[10] In hindsight, it is obvious that these experiments failed to demonstrate binding because dinitroanilines selectively bind to plant and protozoan tubulin but not to vertebrate tubulin.[11–14] In 1977, Hess and Bayer established that dinitroanilines interacted with tubulin from the unicellular alga *Chlamydomonas eugametos*.[15] When combined with other cell biological studies, it was established that dinitroaniline compounds disrupt microtubules in diverse plants, ranging from the single-celled alga *Chlamydomonas* to higher plants such as the monocot *Eleusine indica*.[2,4,15–18] Dinitroanilines were also shown to disrupt microtubules in protozoa, including free-living species such as *Tetrahymena* and protozoan parasites *Trypanosoma* spp., *Leishmania* spp., *Entamoeba* spp., *Plasmodium falciparum*, *Cryptosporidium parvum* and *Toxoplasma gondii*.[11,19–35] However, these compounds were found to be inactive in fungi and in metazoans.[11,16]

2. Tubulin, microtubules and interacting drugs

Tubulins are an extensively studied group of highly conserved and essential eukaryotic proteins which function to build microtubules and regulate their stability and organization.[36] Microtubules are built by the polymerization of α-β-tubulin heterodimers and typically contain 13 protofilaments, a substructure formed by the longitudinal head-to-tail association of the heterodimers.[37–40] The lateral association of protofilaments forms a microtubule, which consists of a 24 nm wide cylinder with a hollow lumen (Figure 2). Since α- and β-tubulins are ancestrally related proteins, they contain many domains with similar structure and function, such as the H1-S2 (N) and M loops which coordinate contacts between adjacent protofilaments. However, α- and β-tubulins also have important differences. For example, α-tubulin contains an eight amino acid insert missing from the analogous region of β-tubulin. This region stabilizes the α-tubulin M loop to promote lateral association between protofilaments.[41,42] Moreover, although both α- and β-tubulins bind to GTP, only β-tubulin GTP can be exchanged or hydrolyzed.[43–45] β-tubulin subunits are stimulated to hydrolyze GTP in a

polymerization-dependent fashion by the association of a GTPase activating domain in the α-tubulin subunit of the adjacent dimer in the microtubule protofilament. Structural evidence indicates that GTP-bound tubulin dimers have a conformation that facilitates M loop to H1-S2 (N) loop contacts in both α- and β-subunits within the microtubule lattice. GDP-tubulin dimers have a kinked conformation that is believed to weaken lateral contacts to promote protofilament fraying and microtubule disassembly.[46–48]

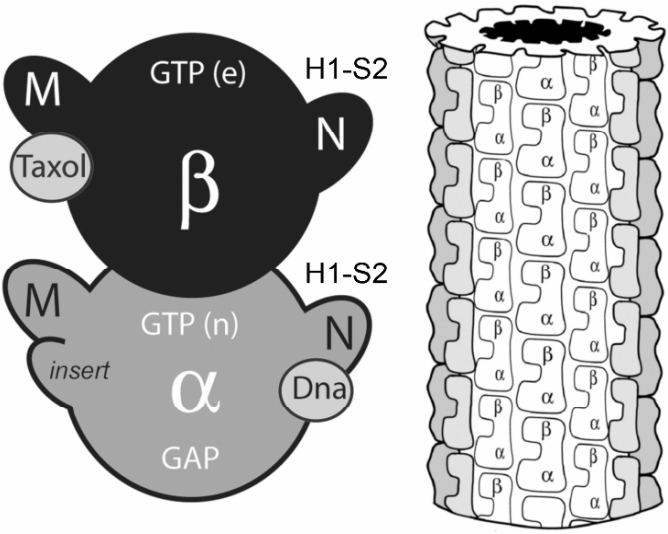

Figure 2. (Left) The tubulin dimer is composed of α- and β-tubulin subunits which are ancestrally related proteins that share many structural features. Both α- and β-tubulin bind to GTP and both have M and N (H1-S2) loops which coordinate lateral interactions. The β-bound GTP is exchangeable and hydrolysable (e) whereas the α-bound GTP is non-exchangeable (n) and non-hydrolysable. The α-tubulin subunit has an eight amino acid insert missing in β-tubulin which stabilizes the α-tubulin M loop. Taxol stabilizes microtubules by binding to the corresponding region of β-tubulin. The α-tubulin subunit also has a GTPase activating domain (GAP) that activates β-tubulin GTP hydrolysis in a polymerization dependent fashion. Dinitroanilines (Dna) bind under the α-tubulin N loop. (Right) Microtubules are built by the head to tail assembly of dimers into a longitudinal protofilament and the lateral association of these protofilaments. Most microtubules are built of thirteen protofilaments.

Pharmacological agents affect microtubule function by shifting the normal equilibrium between tubulin dimers and polymers to destabilize (e.g. colchicines) or stabilize (e.g. taxol) microtubules.[49] Until recently, all known compounds that influence the tubulin equilibrium bound to β-tubulin. The β-tubulin subunit has at least three distinct drug binding sites: the taxol, colchicine and vinca sites. A variety of structurally diverse compounds bind to each of these sites. For example, taxol (isolated from

yew trees) and epothilones (isolated from myxobacterium) bind to the same region of β-tubulin.[42,50] Both compounds stabilize microtubules by binding to β-tubulin and acting in part to mimic the α-tubulin insert that stabilizes α-tubulin lateral contacts. By compensating for these missing residues in β-tubulin, these drugs stabilize the weaker β-tubulin M loop protofilament interaction. The dinitroanilines bind to the α-tubulin subunit, as does peloruside A, a recently characterized sponge metabolite that stabilizes microtubules.[51–54] Since these compounds are the first drugs to affect α-tubulin function they are likely to provide important insights into the role of the α-tubulin subunit in the tubulin dimer.

3. Characterization of dinitroaniline binding and microtubule dynamics

The earliest characterization of the interaction of dinitroanilines with purified tubulins was carried out using tubulin isolated from pig brain and *Chlamydomonas eugametos*.[10,15] In 1990 Chan and Fong showed specific binding of trifluralin to *Leishmania* (protozoan) tubulin but not to mammalian tubulin.[11] These studies defined the specificity of dinitroanilines; they bind to plant and protozoan tubulins but not to vertebrate tubulins. Later studies quantified binding to purified plant tubulin. Oryzalin binds to purified *Zea mays* tubulin in a rapid and pH dependent fashion with a K_D of 95 Nm.[12–14] Oryzalin binds to both dimers and microtubules, and slows microtubule assembly by a substoichiometric poisoning mechanism. In parallel experiments, oryzalin did not bind to purified vertebrate tubulin, indicating that the observed inactivity of dinitroanilines on vertebrate microtubules can be attributed to an absence of an oryzalin-binding site on mammalian tubulin. *Arabidopsis* epidermal cells expressing GFP-tagged tubulin have been exploited to follow the effect of oryzalin on microtubules *in vivo*.[55] Microtubules in 50–100 nM oryzalin exhibited little net change in length; that is, they spent more time in a paused state. Oryzalin slowed microtubule growth and shortening velocities, increased catastrophe, and caused an overall decrease in microtubule dynamic turnover.

4. The genetics of dinitroaniline resistance

Distinct biotypes of dinitroaniline-resistant *Eleusine indica* (goosegrass) are characterized by high resistance (R) or intermediate (I) resistance.[43,56–59] Reciprocal crosses between biotypes associated with sensitivity, intermediate resistance and high resistance indicate that dinitroaniline resistance is inherited as a single gene with three alleles (S, I and R). Susceptibility is dominant to intermediate resistance, and intermediate resistance is dominant

to high resistance. Resistant plants have a Thr239Ile or a Met268Thr point mutation to the TUA1 α-tubulin gene.[56–59] Both mutations confer dinitroaniline resistance when transformed into maize or tobacco and the level of resistance exhibited by the transformed plants is comparable to that in the original R or I biotype seedlings.[59–62] Maize calli expressing a Thr239Ile/Met268Thr double mutation show additive resistance. More recently, resistance in *Setaria viridis* (green foxtail) was associated with Leu136Phe and Thr239Ile point mutations to α-tubulin.[63]

Dinitroaniline resistance in the single cell alga *Chlamydomonas reinhardtii* is rarely due to mutations to tubulin.[64–69] The dearth of tubulin mutations may be explained by the recessive or semi-recessive nature of tubulin mutations. Although *Chlamydomonas* is haploid, it has two copies of both α- and β-tubulin genes.[70] Presumably, tubulin-based resistance does not arise in *Chlamydomonas* because these mutations are masked by expression of the other wild type tubulin gene. The most common mechanism of dinitroaniline-resistance in *Chlamydomonas* involves regulation of tubulin polymerization, rather than direct mutations to tubulin.[65,68] Although the specific genes associated with these resistance loci remain unidentified, they share the properties of being unlinked to tubulin genes and independent of each other. They cause Taxol hypersensitivity and show synthetic lethality, suggesting that the gene products function in the same process. Two distinct genetic loci (*apm1* and *apm2*) are resistant to both amiprophosmethyl and oryzalin and are synthetically lethal in combination.[64,68] Some alleles of *apm-1* are also synthetically lethal with temperature-sensitive alleles of *FLA10*, a kinesin required for flagellar assembly.[68] The oryzalin-resistant allele *ory1* displays a lethal genetic interaction with *cor1*, a colchicine resistance mutation in *Chlamydomonas*.[65] In a few cases, dinitroaniline resistance can be conferred by multi-drug resistance mechanisms via non-specific efflux pumps. Two such independent drug-resistance loci (*pdr1* and *pdr2*) were identified in an oryzalin resistance screen.[66] These resistance mutations confer cross-resistance to unrelated inhibitors. Tubulin mutations that confer dinitroaniline resistance in *Chlamydomonas* include the α-tubulin mutation Tyr24His, which was identified as a "step-up" mutation in a strain with low levels of dinitroaniline resistance.[67] This semi-dominant mutation doubled resistance to oryzalin and increased taxol hypersensitivity, suggesting that the mutation enhanced microtubule stability. Two β-tubulin mutations Lys350Met and Lys350Glu confer hypersensitivity to Taxol and resistance to dinitroanilines and other microtubule-disrupting drugs such as colchicine and amiprophosmethyl.[69] These mutations hyperstabilize microtubules and are therefore not dinitroaniline-specific. The Lys350Met mutation also confers dinitroaniline resistance in the ciliate *Tetrahymena thermophila*.[34]

TABLE 1. Alpha-tubulin mutations associated with dinitroaniline resistance.

Substitution	Organism	Location	Reference
Val4Leu	Toxoplasma gondii	Binding site	54, 71
Ser6Ile	Toxoplasma gondii	Binding site	54, 71
His8Tyr	Toxoplasma gondii	β-sheet 1	54, 71
Tyr24His	C. reinhardtii	Binding site	67
Phe24His	Toxoplasma gondii	Binding site	75
His28Gln	Toxoplasma gondii	H1-S2 loop/binding site	54, 71
Phe49Cys	Toxoplasma gondii	H1-S2 loop	54, 71
Phe52Ile	Toxoplasma gondii	H1-S2 loop	54, 71
Phe52Leu	Toxoplasma gondii	H1-S2 loop	54, 71
Phe52Tyr	Toxoplasma gondii	H1-S2 loop	54, 71
Leu136Phe	Toxoplasma gondii	Binding site (β-sheet 4)	54, 71
Leu136Phe	Setaria viridis	Binding site (β-sheet 4)	63
Asn139Lys	Toxoplasma gondii	β-sheet 4	54
Gly142Ser	Toxoplasma gondii	GTP binding	83
Leu154Arg	Toxoplasma gondii	α-helix 4	54, 71
Ser165Ala	Toxoplasma gondii	β-sheet 5	54
Ser165Pro	Toxoplasma gondii	β-sheet 5	54
Ser165Thr	Toxoplasma gondii	β-sheet 5	54
Gln176Glu	Toxoplasma gondii	β-sheet 5 to α-helix 5	54, 71
Ser178Thr	Toxoplasma gondii	β-sheet 5 to α-helix 5 loop	54, 71
Ile231Thr	Toxoplasma gondii	α-helix 7	54
Ile235Thr	Toxoplasma gondii	Binding site (α-helix 7)	75
Ile235Leu	Toxoplasma gondii	Binding site (α-helix 7)	75
Ile235Val	Toxoplasma gondii	Binding site (α-helix 7)	54, 71
Leu238Val	Toxoplasma gondii	α-helix 7	54
Thr239Ile	Eleusine indica	Binding site (α-helix 7)	56, 59
Thr239Ile	Toxoplasma gondii	Binding site (α-helix 7)	54, 71
Thr239Ile	Setaria viridis	Binding site (α-helix 7)	63
Arg243Cys	Toxoplasma gondii	Binding site (after α-helix 7)	54, 71
Arg243Ser	Toxoplasma gondii	Binding site (after α-helix 7)	54, 71
Val252Leu	Toxoplasma gondii	α-helix 8 (GAP domain)	54, 71
Met268Thr	Eleusine indica	near M loop	56, 59
Met268Thr	Toxoplasma gondii	near M loop	74
Ile275Thr	Toxoplasma gondii	M loop	54, 71
Ala295Val	Toxoplasma gondii	α-helix 9	54
Met301Thr	Toxoplasma gondii	after α-helix 9	54
Ile378Met	Toxoplasma gondii	β-sheet 10	54, 71
Met391Leu	Toxoplasma gondii	α-helix 11	Unpublished Observation
Met391Ile	Toxoplasma gondii	α-helix 11	54, 71

Identical mutations found in different dinitroaniline resistant species.

Same residue mutated to different amino acids in Toxoplasma gondii.

A diverse set of point mutations to α-tubulin are associated with dinitroaniline resistance in the protozoan *Toxoplasma gondii*.[54,71] *Toxoplasma* is an obligate intracellular parasite that exists as a haploid organism during most of its life cycle. The *Toxoplasma* genome contains three α-tubulin genes.[72] The α2-tubulin gene is most likely a gamete-specific gene and does not appear to be expressed during the proliferative stages. The α3-tubulin gene has a divergent and extended carboxy terminal region (40 residues) and may be used to build the conoid, an invasive structure constructed of flattened tubulin sheets.[73] The α1-tubulin gene is dominant during growth of the asexually replicating form of the parasite which causes acute disease and is likely to represent the primary source of α-tubulin in this developmental stage. The simplicity of *Toxoplasma* tubulin genetics may explain why such a large set of novel α1-tubulin mutations can function as resistance alleles (Table 1). At present, no mutations in the three β-tubulin genes or in the α2- or α3-tubulin genes have been associated with resistance.[71] Rather, each resistant line harbors a single mutation to the α1-tubulin gene. Several amino acids within α1-tubulin are "hot spots" and are mutated in several independently derived lines. These include Ser165 which can be mutated to threonine, proline or alanine, Ile235 which can be mutated to leucine, threonine or valine, Phe52 which can be mutated to isoleucine, leucine or tyrosine and Arg243 which can be mutated to cysteine or serine.[54,71] Since novel α1-tubulin mutations that confer dinitroaniline resistance continue to be identified in *Toxoplasma*, the screen is not saturated at this time.

5. Dinitroaniline resistance in *Toxoplasma*

Diverse protozoan parasites are sensitive to dinitroanilines, which disrupt microtubules and consequently inhibit replication. Since the dinitroanilines do not disrupt vertebrate tubulins, this specificity is of particular interest for the development of new anti-parasitic drugs. Our studies have focused on using the genetics of dinitroaniline resistance to better understand the action of the dinitroanilines on the protozoan parasite *Toxoplasma gondii*. *Toxoplasma* is a member of the Apicomplexa, a phylum of obligate intracellular parasites.[74,75] These protozoan pathogens have a profound impact on human welfare. Infection by *Toxoplasma gondii* can cause life-threatening illness in immunocompromised individuals and birth defects or miscarriage during fetal infection. Other apicomplexans include *Plasmodium* (agent of malaria) and *Cryptosporidium* (an opportunistic, pathogen) which are parasites of considerable medical importance and *Theileria* and *Eimeria*, animal pathogens with extensive impact on food production. Apicomplexans are named for their polarized apex which contains a number of unique

organelles that coordinate invasion of host cells. The proliferative stages of apicomplexan parasites have two microtubule populations: subpellicular microtubules and spindle microtubules.[19,74] Subpellicular microtubules are

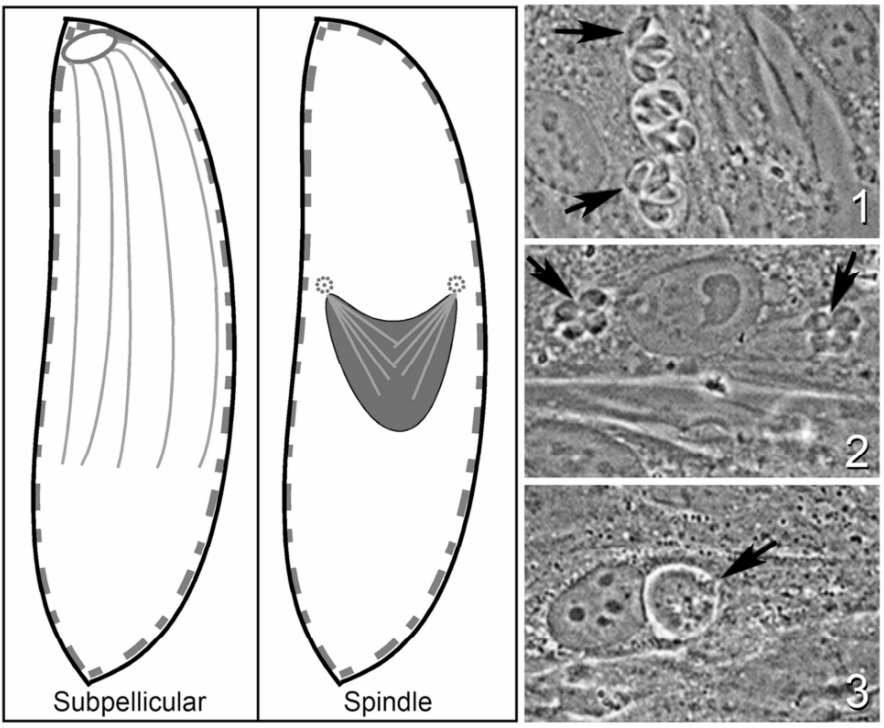

Figure 3. (Left) *Toxoplasma* parasites have two discrete microtubule populations. Subpellicular microtubules are 5 μ long and impose an elongated shape and apical polarity on *Toxoplasma*. Spindle microtubules are 1 μ long and are used to segregate chromosomes during nuclear division. (Right) The effect of oryzalin treatment on intracellular *Toxoplasma* (arrows) imaged with phase-contrast microscopy. (1) Without oryzalin, both subpellicular and spindle microtubules are intact. Arrows indicate two vacuoles within a host cell, each containing four crescent shaped *Toxoplasma*. (2) In 0.5 μM oryzalin, *Toxoplasma* have intact spindle microtubules but subpellicular microtubules are disrupted, so parasites are round but replicate (arrows). (3) In 2.5 μM oryzalin, both spindle and subpellicular microtubules are disrupted. The arrow indicates a single growing but non-replicating parasite.

non-dynamic; they maintain both apical polarity and the characteristic crescent shape of the parasite by interacting with the pellicle, a composite structure assembled from the plasma membrane and associated inner membrane complex. Spindle microtubules form an intra-nuclear spindle to coordinate chromosome segregation (Figure 3). Both populations are critically important to parasite survival and replication.

Since dinitroanilines do not disrupt vertebrate microtubules, these compounds have been useful to study tubulin in *Toxoplasma*, which only has dynamic (disruptable) microtubules when it is replicating inside vertebrate cells (Figure 3). Wild type *Toxoplasma* parasites are resistant to <0.2 μM oryzalin, but increased concentrations first shorten (0.5 μM), then eliminate (2.5 μM) parasite microtubules, while leaving host cell microtubules intact.[19,20] Resistant *Toxoplasma* parasites display distinct behavior with respect to specific dinitroaniline ligands in *Toxoplasma*. For example, a trifluralin resistant mutant is completely cross-resistant to oryzalin but only partially resistant to ethafluralin.[20] Several years ago, we carried out a genetic screen to identify oryzalin resistant *Toxoplasma* parasites after chemical mutagenesis. We selected for resistance to either 0.5 or 2.5 μM oryzalin, since both concentrations induce parasite phenotypes that inhibit reinvasion of host cells and are therefore lethal. We chose to select at these two different concentrations with the expectation that we might obtain different types of resistance mutations in conditions that shorten microtubules in contrast to treatment that eliminates microtubules. To our great surprise, all of the lines that we have characterized to date have mutations to α-tubulin, making the results of this screen quite different from the basis of resistance in the unicellular alga *Chlamydomonas*. The resistant lines have diverse mutations to the α1-tubulin gene and exhibit a range of resistance levels, ranging from 0.5 to >50 μM oryzalin. We developed genetic strategies to engineer homologous replacement of wild type α1-tubulin allele with transgenes that bear specific point mutations to α1-tubulin and have demonstrated that the point mutations are sufficient to confer resistance when reintroduced into wild type (sensitive) parasites.[54,71] We were also able to use this strategy to determine that resistance mutations identified in other species confer resistance when introduced into the α1-tubulin gene in *Toxoplasma*. For example, the Met268Thr mutation observed in *Eleusine* confers dinitroaniline resistance in *Toxoplasma* as does a Phe24His substitution which is analogous to the Tyr24His mutation in *Chlamydomonas*.[71]

The α1-tubulin point mutations associated with oryzalin resistance are distributed throughout the linear sequence of α-tubulin. However, when mapped onto a model of *Toxoplasma* α-tubulin based on the structure of the vertebrate tubulin dimer, many of the mutations cluster in specific domains of the protein (Figure 4). Specifically, many mutations are located in or near domains that are associated with microtubule stability or in the core of α-tubulin, adjacent to or within a dinitroaniline binding site identified by computational methods (see below). The dinitroaniline binding site is located beneath the H1-S2 (N) loop and is formed by residues of strand S1,

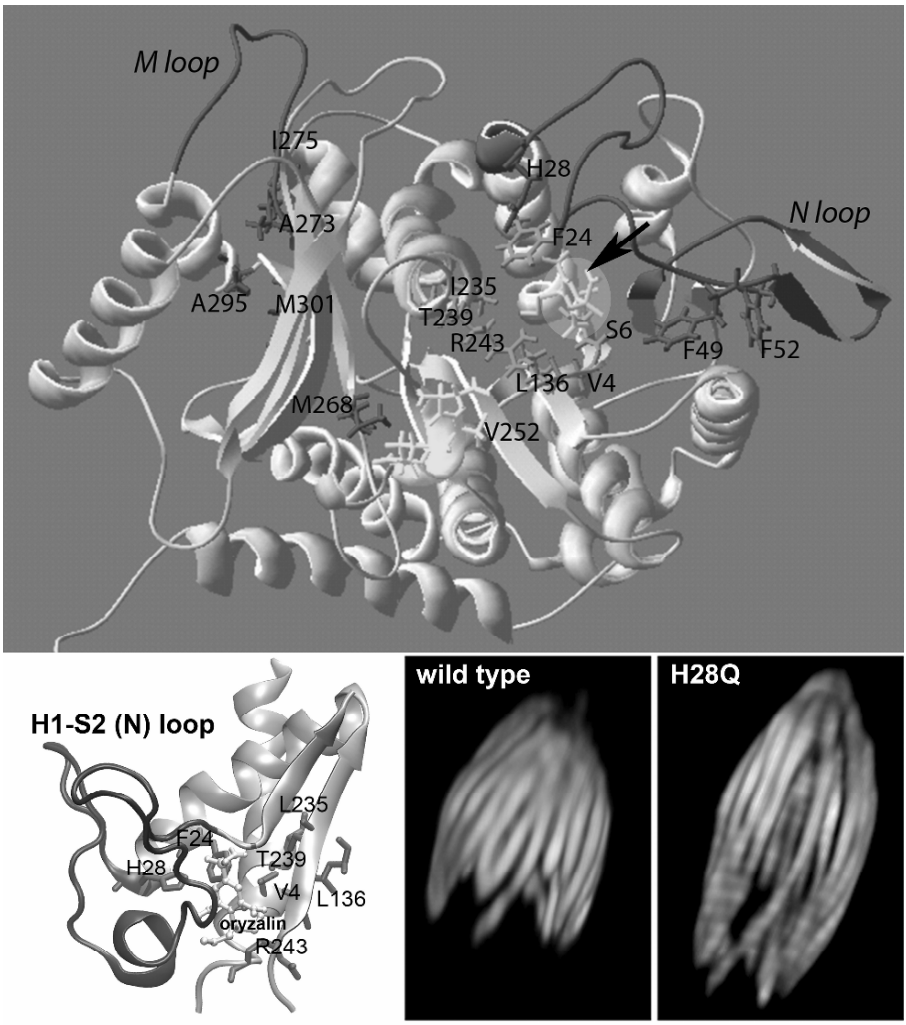

Figure 4. (Top) Dinitroaniline resistant point mutations mapped on a ribbon model of *Toxoplasma* α-tubulin show clustering of the mutations in the loops and in a core domain. Bound dinitroaniline is indicated with an arrow. (Bottom left) A number of mutations localize in or near the dinitroaniline binding site. (Bottom right) Relative to wild type parasites, *Toxoplasma* expressing the His28Gln α1-tubulin mutation have elongated microtubules.

helix H1, the H1-S2 (N) loop, strand S2, strand S4, helix H7 and the T7 loop. Eight of the binding site residues (Val4, Ser6, Phe24, His28, Leu136, Ile235, Thr239 and Arg243) have been identified as point mutations (Figure 4) in the oryzalin resistance screen.[54,71] The Thr239Ile mutation has also been identified in resistant *Eleusine indica* (goosegrass) and *Setaria viridis* (foxtail) and the Leu136Phe was also identified in foxtail.[56,59,63]

Many previous structural and genetic studies have established the critically important role of the H1-S2 (N) and M loops in microtubule assembly. Studies docking the tubulin dimer structure into high-resolution images of microtubules have established that M loops interact with H1-S2 (N) loops of laterally adjacent subunits.[76] Alanine scanning studies in budding yeast have identified M and H1-S2 (N) loop mutations that are benomyl resistant, presumably due to increased microtubule stability.[77,78] Moreover, the α-tubulin H1-S2 (N) loop mutation Glu55Lys and the M loop mutation His283Tyr confer colcimid and vinblastine resistance in human cells.[79] Nine *Toxoplasma* resistance mutations map to the H1-S2 (N) and M loops, which coordinate protofilament interactions in the microtubule lattice (Table 1). These α-tubulin loop mutations may compensate for the destabilizing effects of the dinitroanilines by increasing the intrinsic stability of *Toxoplasma* microtubules. In the absence of oryzalin, some of these tubulin mutations result in *Toxoplasma* that are thinner and have an elongated shape relative to the dinitroaniline-sensitive parental strain. This is most obvious in *Toxoplasma* bearing the His28Gln mutation.[71] Three-dimensional reconstructions of subpellicular microtubules using deconvolution microscopy suggests that microtubule length is increased in parasites expressing α-tubulin with the His28Gln point mutation (Figure 4). This increased length is likely to result from a shift in the equilibrium between tubulin dimers and microtubule polymers. We have also identified a mutation (Val252Leu) that maps to the GTPase activating domain of α-tubulin which serves to destabilize the microtubule lattice by stimulating the polymerization dependent hydrolysis of β-tubulin bound GTP. In this case, we postulate that a substitution that attenuates the ability of the GTPase activating domain to initiate hydrolysis would increase microtubule stability. This hypothesis awaits further experimental evidence.

We have generated allelic replacements for 20 of the point mutations that localize to domains that are predicted to act by altering the dinitroaniline binding site or by increasing microtubule stability. Our observations (1) that M and H1-S2 (N) loop mutations have relatively low resistance, a high incidence of replication defects and some have elongated microtubules, and (2) that a subset of binding site mutations have extremely high resistance, are consistent with a model that invokes two major mechanisms of dinitroaniline resistance. In the case of loop mutations, protofilament affinity is increased to non-specifically provide resistance. In the case of binding site mutations, substitutions to this location influence the ability of the dinitroanilines to bind to tubulin. Increased microtubule stability has been shown to be associated with chromosome segregation defects and other aberrant replication events.[80,81] It was therefore not surprising that resistance mutations that are predicted to increase microtubule stability are

associated with increased incidence of replication defects. However, we discovered that expression of dinitroaniline binding site mutations also causes parasites to have a greater frequency of aberrant replication.[71]

Toxoplasma replicates by endodyogeny, a process of internal budding which only occurs in host cells. This process requires the coordinated action of the spindle microtubules to drive nuclear division and the subpellicular microtubules to drive cytokinesis (Figure 5). After growing *Toxoplasma* lines without oryzalin, we quantified extracellular (lysed out) parasites for overt replication defects. Approximately 4% of wild type parasites are abnormal and we observed an increased proportion of malformed *Toxoplasma* "monsters" in the oryzalin resistant lines (Figure 5). In many cases, these monsters can be attributed to a failure of mutant parasites to synchronize nuclear division (driven by spindle microtubules) and the formation of daughter buds (driven by subpellicular microtubules) during endodyogeny. Parasites that lack a rigid elongated crescent shape are incapable of invading host cells; therefore, abnormal forms that fail to complete scission cannot invade new host cells and are non-viable organisms. Other parasites that appear normal at the level of the light microscope may have lost single chromosomes. Although we attempted to evaluate the frequency of chromosome mis-segregation using flow cytometry, we were not able to resolve any reproducible differences in DNA content given the variation in this assay and the resolution required for this measurement. Enhanced replication defect rates are particularly obvious in H1-S2 (N) loop mutants, which have 10–13% of extracellular parasites that are grossly altered and incapable of continued cycles of invasion and replication. However, some binding site mutations, such as Arg243Ser also have high rates of replication defects. Ultimately, we determined that all of the point mutations are associated with some degree of increased rates of replication defects that negatively influence parasite fitness. Substitutions predicted to hyperstabilize microtubules are generally associated with the highest rates of replication defects, consistent with previous studies that have associated decreased microtubule dynamics (the α-tubulin mutation Val260Ile or loss of EB-1) with chromosome loss.[80,81] Since we do not believe that the putative binding site mutations influence microtubule stability, we hypothesize that they alter some other aspect of tubulin function such as tubulin folding or subunit flexibility in *Toxoplasma*. Ultimately, the significant issue is whether resistant parasites could be sufficiently fit to be spread throughout natural populations in competition with wild type parasites in the absence of drug selection. Studies that have exploited growth competition assays to assess the fitness of *Toxoplasma* strains bearing wild type or pyrimethamine resistant dihydrofolate reductase genes have concluded that even strains that behave

identically *in vitro* can display growth defects *in vivo*.[82] Our dinitroaniline-resistant strains have overt replication defects *in vitro*, suggesting that they have reduced fitness and would fare poorly in competition with wild type *Toxoplasma*.

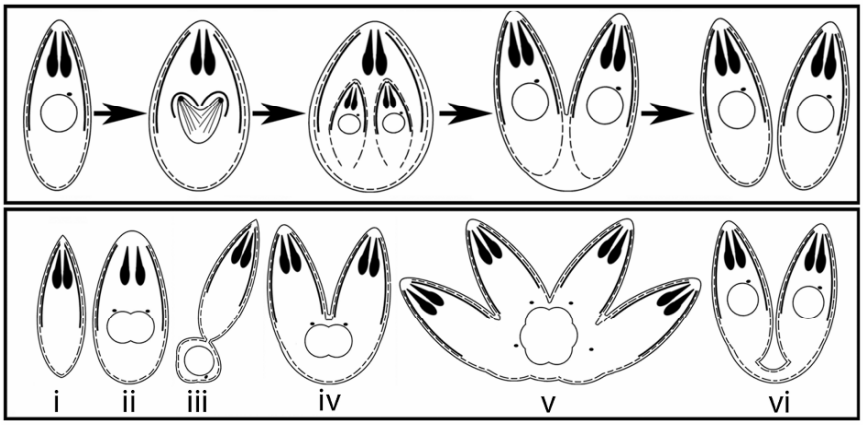

Figure 5. (Top) *Toxoplasma* parasites replicate by endodyogeny, a process of internal budding that creates two daughter parasites within a mother parasite. This process permits an apically polarized "mother" parasite to undergo mitosis while building two polarized daughter buds, each consisting of inner membrane complex, subpellicular microtubules and apical organelles around the daughter nuclei. When the daughter parasites are mature, they emerge from the mother, adopting her plasma membrane. (Bottom) Illustration of the aberrant forms of extracellular parasites: (i) anucleate and (ii) diploid parasites result from completion of cytokinesis in the absence of nuclear division; (iii) parasites that complete cytokinesis in the absence of nuclear division can exclude nuclear material; two-headed (iv) or multiple headed (v) parasites result from arrest during cytokinesis in the absence of nuclear division; (vi) and parasites with late completion of nuclear division can fail to complete septation (cytokinesis).

Our observation that spontaneous suppressor mutations arise in the absence of oryzalin selection suggests that the oryzalin resistant lines have significantly reduced fitness. When parasites bearing the H1-S2 (N) loop mutation Phe52Tyr were grown without oryzalin, they acquired second site mutations at either Ala273Val or Asp367Val in the α1-tubulin gene.[71] Our evidence indicates that the secondary mutations increase *Toxoplasma* fitness by decreasing microtubule stability. Parasites with the double mutations have decreased both dinitroaniline resistance and replication defects (Figure 6). We suspect that the Phe52Tyr strain has a high rate of chromosome segregation defects that provides a strong selective pressure for second site mutations to correct for tubulin function in the absence of dinitroaniline selection. The location of the second site mutations is consistent with a role in decreased protofilament interactions. The Ala273Val mutation is in the M loop and the Asp367Val mutation is in an

insert that stabilizes the α-tubulin M loop. The substitution of Val for Asp at this location disrupts a salt bridge between Asp367 and Arg229, presumably destabilizing the insert, which in turn destabilizes the M loop protofilament contact.

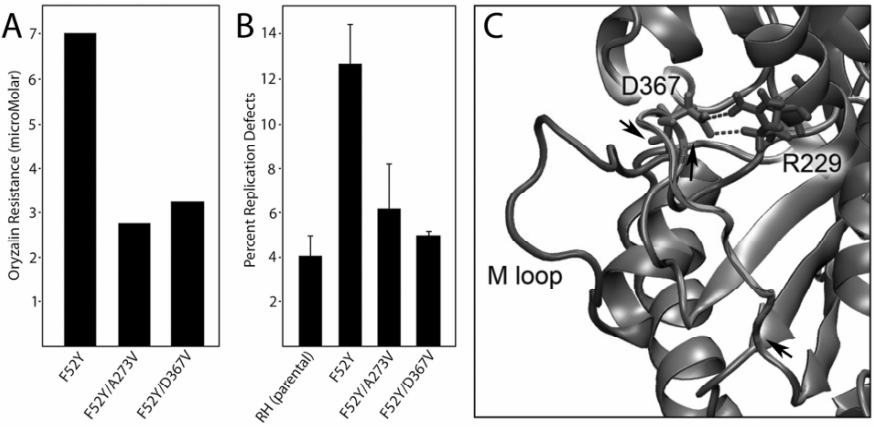

Figure 6. Dinitroaniline resistance is sufficiently deleterious that in the absence of oryzalin selection, *Toxoplasma* parasites harboring the Phe52Tyr mutation acquire compensatory suppressor mutations at either Ala273Val (the M loop) or Asp367Val (the α-tubulin insert). (A) Oryzalin resistance of the single mutant Phe52Tyr is greater than that of the double mutants. The single substitutions Ala273Val or Asp367Val do not confer any oryzalin resistance. (B) Replication defects associated with the single mutant Phe52Tyr are greater than those in the double mutants. (C) Substitution of Val for Asp at residue 367 disrupts a salt bridge that links Arg229 to Asp367 within the α-tubulin-specific insert (arrows). The eight amino acids of the insert stabilize the M loop of α-tubulin.

These observations have inspired our current studies which explore the balance between fitness (tubulin function) and resistance in parasites containing either the G142S or the F52Y point mutation in the α1-tubulin gene.[83] Both mutations cause *Toxoplasma* to be resistant to dinitroanilines but also cause them to have profound replication defects. *Toxoplasma* parasites bearing the F52Y mutation are resistant to 7.0 μM oryzalin and have a demonstrated capacity to spontaneously acquire mutations that decrease both resistance levels and replication defects. Parasites bearing the G142S mutation are resistant to 1.0 μM oryzalin and are largely dependent on the presence of oryzalin for viable growth in culture. By growing these *Toxoplasma* strains in the absence of dinitroaniline selection, we were able to rapidly isolate 54 suppressor lines that have robust growth in the absence of dinitroanilines. Diverse mutations that suppressed the growth defects include 29 intragenic mutations (pseudorevertants) in α1-tubulin, 16 extragenic mutations that introduce a point mutation into β1-tubulin, and 9 other unidentified extragenic mutations. Preliminary growth

competition assays indicate that the suppressor lines are better able to compete with wild type parasites than the original mutant lines; however, dinitroaniline resistance is apparently reduced or eliminated in these parasites, indicating that there is a trade-off between replication fitness and drug resistance.

6. Models of the dinitroaniline binding site and action

We have used computational methods to build models of protozoan (sensitive) and vertebrate (insensitive) tubulins and have employed these structures to generate multiple tubulin conformations from a molecular dynamics analysis.[53,54] To prepare α-tubulin for the docking studies, the missing residues (Gln35 to Lys60) of bovine α-tubulin (1JFFA) were modeled based on the analogous bovine β-tubulin N loop structure (1JFFB). This homology modeling was followed by energy minimization and the *Toxoplasma* α-tubulin structure was created by mutating the completed bovine structure to the *Toxoplasma* α-tubulin sequence. The resulting complexes (*Toxoplasma* α-tubulin with GTP and bovine α-tubulin with GTP) were minimized, heated and equilibrated prior to molecular dynamics simulations. Representative structures were taken every 500 ps from the 2.5-ns trajectory for use in docking simulations. Flexible docking of oryzalin to each snapshot taken from the MD trajectory was performed using AutoDock3.0. Thirty trial runs were carried out for each α-tubulin conformation followed by cluster analysis of the results. These studies predict that oryzalin binds with an average affinity of 23 nM to a site located beneath the H1-S2 (N) loop of *Toxoplasma* α-tubulin (Figure 7A).[54] Parallel analysis of *Bos taurus* (vertebrate) α-tubulin with oryzalin docking to structures obtained from the molecular dynamics trajectories indicated that oryzalin has non-specific, low affinity interactions in single tubulin snapshots, significantly decreased overall affinity and no true binding site, consistent with *in vivo* and *in vitro* observations that indicate the dinitroanilines do not bind to vertebrate tubulin or disrupt vertebrate microtubules.

We later extended this analysis to models for apicomplexan (*Toxoplasma gondii* and *Plasmodium falciparum*) and kinetoplastid (*Leishmania major*) α-tubulin interacting with the dinitroanilines oryzalin, trifluralin and GB-II-5.[54] All compounds dock to an equivalent binding site in the *Toxoplasma*, *Plasmodium* and *Leishmania* models of α-tubulin. These refined studies indicate that the *Leishmania* dinitroaniline binding pocket is defined by Arg2, Glu3, Ala4, Ile5, Cys6 (β- strand 1), Cys20, Trp21, Phe24 (helix H1), His28, Met36, Asp39, Lys40, Cys41, Ile42, Asp47, Pro63, Arg64 (H1-S2 (N) loop), Cys65 (β-strand 2), Met136 (β-strand S4), Val235, Ser236,

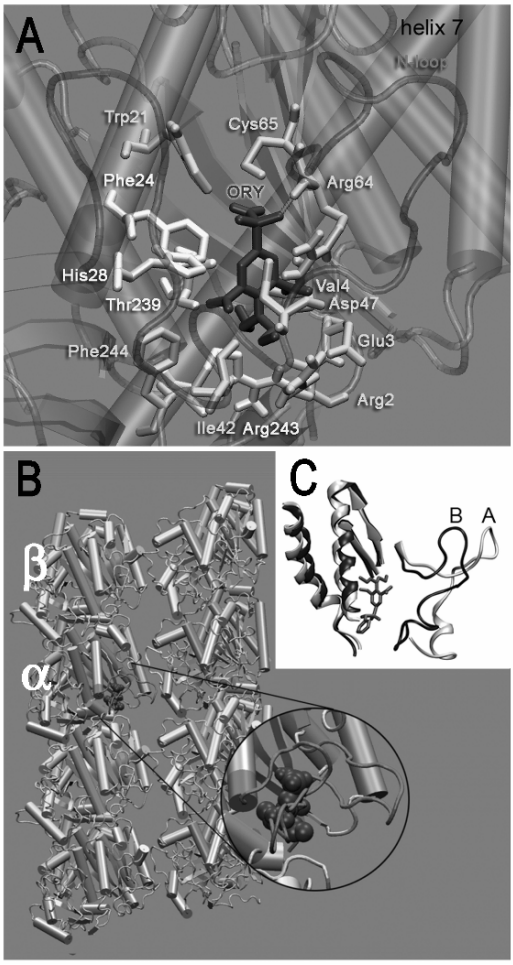

Figure 7. (A) A representative structure of oryzalin bound to α-tubulin. The binding site lies beneath the H1-S2 (N) loop. (B) Two protofilaments, each consisting of two α-β heterodimers, with bound oryzalin. Oryzalin binds to α-tubulin beneath the H1-S2 (N) loop; this is predicted to interfere with lateral interactions between the N loop and the M loop of the adjacent protofilament. The inset shows oryzalin beneath the N loop. (C) Binding of the dinitroaniline GB-II-5 to α-tubulin causes the N loop to be pulled inwards (right) relative to the N loop of α-tubulin in the absence of bound dinitroaniline. B: dinitroaniline bound form; A: apo form.

Thr239, Ala240, Ser 241 (helix H7) and Arg243, Phe244, Asp245 (the T7 loop). When molecular dynamics simulations were used to compare the mobility of *Leishmania* α-tubulin with and without the bound dinitroaniline GB-II-5, compound binding beneath the α-tubulin H1-S2 (N) loop profoundly limits the flexibility of this loop, which is drawn inwards

towards the protein core (Figure 7). These studies also indicate that long range allosteric effects enhance M loop flexibility in the dinitroaniline-bound tubulin complex, further disrupting protofilament contacts. When the α-tubulin subunit is docked into two adjoining protofilaments of a 13-protofilament microtubule, residues Phe52-Val62 of the H1-S2 (N) loop of *Leishmania* α-tubulin directly interact with amino acids Lys280-Ser287 of the M loop of the neighboring α-monomer. Collectively, these results indicate that dinitroanilines inhibit tubulin polymerization by disrupting protofilament contacts mediated by H1-S2 (N) and M loops of laterally adjacent α-tubulin subunits in the microtubule lattice.

7. Summary

Dinitroanilines have many functions in addition to their commercial use as pre-emergence herbicides. They are invaluable tools for the study of microtubule function in plants and protozoa. Moreover, the discovery that these compounds are selectively active against protozoan parasites but not vertebrate microtubules suggests that by understanding their selective action, we can develop new anti-parasitic therapeutics. Lastly, the recent appreciation that dinitroanilines act on α-tubulin rather than β-tubulin function suggests that these compounds will help elucidate the specific role that α-tubulin plays in the tubulin heterodimer and in regulation of microtubule polymerization.

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DOMAIN COMPLEXITY OF PLANT KINESINS

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Abstract. The *Arabidopsis* genome contains 61 kinesins, the largest kinesin family of any fully sequenced and annotated genome. Characterizing each member of a large protein family is a difficult and laborious task, not only due to the size, but also the inherent diversification and/or redundancy associated with gene duplication. An alternative approach to better understand the *Arabidopsis* kinesin family is to study the domain composition of these proteins. Domains are defined as the structural and functional units of proteins. Understanding the domains and domain structure of an unknown protein often allows for a reliable prediction of function when experimental evidence is unavailable. Kinesins share a highly conserved motor domain with low sequence similarity outside of this domain, and many members contain additional protein domains in the nonconserved regions. New arrangements of domains, by means of genetic duplication, replication, or loss, result in novel proteins with unique functions. Using both the *Arabidopsis* and other sequenced genomes, it was found that several kinesins have protein domain arrangements specific to different lineages, providing compelling evidence for the evolution of kinesins with novel functions. A phylogenic analysis of plant-specific kinesin domains, as well as a comparative review of the molecular/ biochemical functions of these domains, is presented in this chapter. Combined, they provide an alternative means to understanding and developing more direct and relevant hypotheses concerning novel kinesins.

Keywords: Kinesins, plant genomes, protein domains

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1. Genomes and kinesins

Sequencing projects have expanded to include many branches of the taxonomic tree. Sequenced genomes afford the ability to identify proteins and protein families in many organisms, based on sequence homology, and this method has been used to identify kinesins in organisms from all kingdoms of the Eukarya domain. The kinesin superfamily, consisting of over 600 proteins, can be organized into 14 families.[1,2] Angiosperm genomes contain kinesins from at least ten of these families. Currently no clear representative plant sequences from Kinesin-2, Kinesin-3, Kinesin-9 and Kinesin-11 families have been identified.[3]

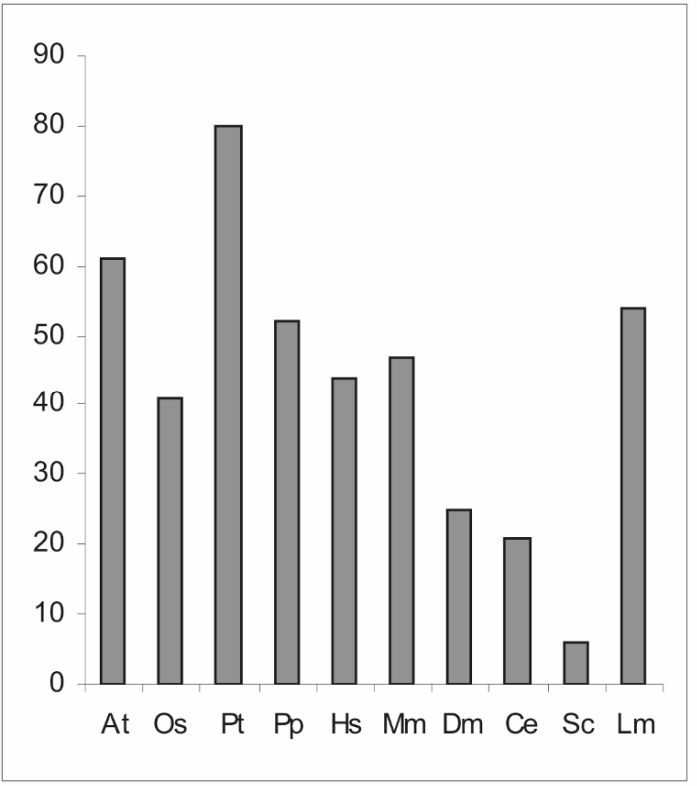

Figure 1. Number of kinesins in sequenced genomes. Abbreviations are as followed: At – *Arabidopsis thaliana*; Os – *Oryza sativa* ssp. *Japonica*; Pt – *Populus trichopcarpa*; Pp – *Physcomitrella patens*; Hs – *Homo sapiens*; Mm – *Mus musculus*; Dm – *Drosophila melanogaster*; Ce – *Caenorhabditis elegans*; Sc – *Saccharomyces cerevisiae*; Lm – *Leishmania major*.

Notably, angiosperms contain some of the largest kinesin families. *Arabidopsis* has the largest family of annotated kinesins, with 61 members (Figure 1). The unannotated *Populus trichocarpa* genome contains a predicted 80 kinesins. It is unclear why flowering plants have larger kinesin families than compared to protists or mammals. One hypothesis is that these organisms require a larger repertoire of kinesins to organize plant-specific microtubule arrays (i.e., the preprophase band, phragmoplast, and cortical array). An alternative hypothesis is that the lack of dyneins, minus-end-directed motor proteins found in other organisms, is compensated by an increase in minus-end-directed kinesins.[4] The Kinesin-14 family consists of minus-end-directed kinesins and is expanded in plant genomes.[3] In either scenario, characterization of kinesins in flowering plants, including *Arabidopsis*, is a first step to understanding these families.

Only a fraction of the members of the *Arabidopsis* kinesin family have been functionally characterized by traditional means (Table 1). The characterization of these few proteins and/or their genes has provided valuable insight into kinesin functions in plants, and reveals the importance of kinesins to normal plant functions; furthermore, it also reflects the difficulties associated with working with large protein families. Several of the mutant phenotypes require double knockouts, which presumably reflects the redundancy of large protein families due to paralogous duplication events. Protein domain identification and phylogenic analysis are two bioinformatic tools that can utilize the large amount of sequential information to develop more direct and relevant hypotheses concerning protein function.

TABLE 1. *Arabidopsis* kinesins functionally characterized by means of a mutant phenotype.

Name	Locus	Family	Function	Reference
KRP125C	At2g28620	5	Spindle formation	5
HINKEL	At1g18370	7	Gametogenic cytokinesis	6
TETRASPORE	At3g43210	7	Gametogenic cytokinesis	6
POK1[a]	At3g17360	12	Spatial control of cytokinesis	7
POK2[a]	At3g19050	12	Spatial control of cytokinesis	7
PAKRP1[a]	At4g14150	12	Phragmoplast formation	8
PAKRP2[a]	At4g14330	12	Phragmoplast formation	8
ATK1	At4g21270	14	Meiotic spindle formation	9
ATK5	At4g05190	14	Mitotic spindle formation	10
KCBP	At5g65930	14	Trichome morphogenesis	11
MRH2	At3g54870	UG	Root hair morphogenesis	12

[a]Mutant phenotypes are observed only in double mutants of POK1/POK2 and PAKRP1/PAKRP2.

2. The process of molecular tinkering

Proteins consist of one or more distinct structural units, referred to as domains.[13,14] Domains are continuous stretches of amino acids that can fold and function independent of the complete protein sequence.[13,15] It is from individual domains that proteins derive the majority of their molecular functions.[15] Therefore, understanding the domain architecture of a protein is a valuable tool in predicting its overall function. Essentially, domains are the structural and functional building blocks of proteins, and they play a role in protein evolution.

The evolution of new proteins has been described as a "tinkering process".[16] In this scenario, protein evolution is driven by the rearrangement of preexisting genetic material, rather than *de novo* synthesis.[17] Duplication and recombination are two of the main mechanisms of genetic rearrangement. Both mechanisms can result in a phenomenon called gene fusion, an event where a gene or part of a gene is inserted into an open reading frame of another gene, resulting in a larger and more complex protein.[18] Gene fusion is often mediated by transposons or retrotransposons, or unequal recombination (recombination at different loci during meiosis). Rearrangement of genetic material throughout the genome can be extended to the concept of protein domains. As the building blocks of proteins, the rearrangement of domains in a genome is a method by which proteins with novel functions may evolve.

The tinkering of preexisting genetic material has played, and continues to play, an important role in protein evolution.[16,18,19] In animal proteomes, only 7% of known protein domains are estimated to be mammalian-specific. On the other hand, twice as many unique domain arrangements are observed in mammals when compared to *Drosophila melanogaster* or *Caenorbabditis elegans*.[19] This observation emphasizes the importance of molecular tinkering in protein evolution over *de novo* synthesis. A similar study found that 30% of domain arrangements in the *Arabidopsis thaliana* genome are not observed in *Homo sapiens*, *Drosophila melanogaster*, or *Caenorbabditis elegans*, providing evidence of molecular tinkering in plant genomes.[20] The kinesin protein family is unified by the presence of a highly conserved motor domain, but areas outside of this domain are less conserved and exhibit characteristics of molecular tinkering (as determined by domain analysis).

3. Kinesins and domains

Kinesins are force-generating proteins that typically utilize the energy released from ATP hydrolysis for unidirectional movement and transport of

cargo on microtubules. Cargo can include chromosomes, organelles, vesicles, and even other microtubules.[21] A typical kinesin consists of a motor domain, an α-helical coiled-coil domain referred to as the stalk, and a globular tail domain that associates with cargo. Many kinesins function as homodimers or heterodimers, but some also function as monomers or tetramers.[22]

Motor domains can be divided into two functional regions: the catalytic core responsible for ATP- and microtubule-binding, and a short neck region that confers directionality.[23,24] The majority of motor domains are located at the N- or C-terminus, but a few motor domains are internal. The position of the motor domain, in conjunction with the neck region, dictates kinesin function. Kinesins with motor domains in the N-terminus typically move toward microtubule plus ends, whereas kinesins with C-terminal motor domains typically move toward microtubule minus ends; internally positioned motor domains do not exhibit motility, but affect microtubule dynamics.

TABLE 2. Classification of organisms analyzed and the number of kinesin sequences used in the analysis. See notes for descriptions of identifying kinesin sequences.

Organism	Kingdom	Kinesins
Giardia lamblia	Diplomonadida	24
Trichomonas vaginalis	Parabasala	21
Leishmania major	Euglenzoa	54
Plasmodium falciparum	Alveolata	9
Thalassiosira pseudonana	Stramenopila	22
Phytophthora sojae	Stramenopila	47
Cyanidioschyzon merolae	Rhodophyta	5
Chlamydomonas reinhardtii	Chlorophyta	23
Volvox cartri	Chlorophyta	18
Physcomitrella patens	Plantae	52
Arabidopsis thaliana	Plantae	61
Oryza sativa	Plantae	41
Populus trichocarpa	Plantae	80
Sorghum bicolor	Plantae	41
Dictyostelium discoideum	Mycetozoa	13
Saccharomyces cervisae	Fungi	6
Schizosaccharomyces pombe	Fungi	9
Phanerochaete chryosporium	Fungi	7
Caenorhabditis elegans	Animalia	21
Drosophila melanogaster	Animalia	25
Homo sapiens	Animalia	44

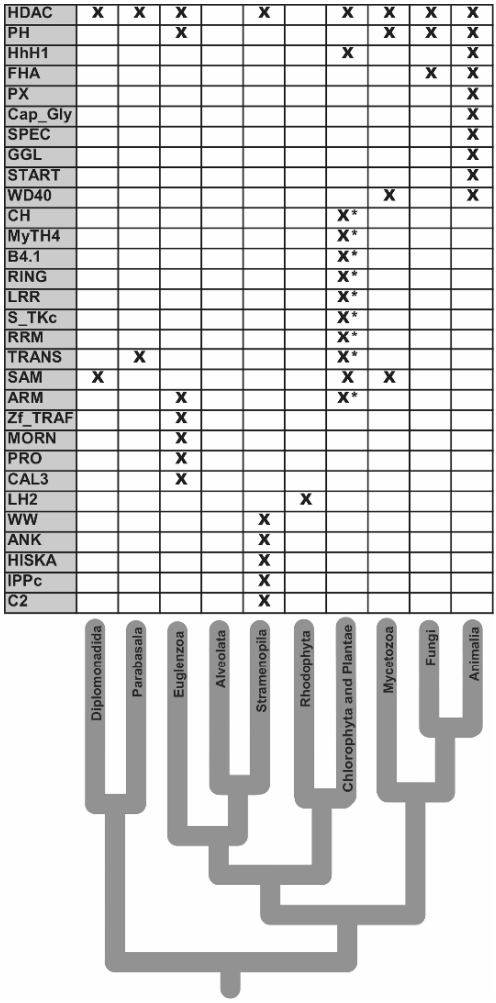

Figure 2. Distribution of domains associated with kinesins across the tree of life. SMART database was used to identify domains in kinesin sequences.[27] Domains with an asterisk are found in land plants. Domain abbreviations are as followed: HDAC – HDAC-interacting; PH – pleckstrin homology; HhH1 – helix-hairpin-helix DNA-binding; FHA – forkhead associated; PX – PhoX homologous; Cap_Gly – glycine-rich domain found in cytoskeletal-associated proteins; SPEC – spectrin repeats; GGL – G-protein-like gamma; START – in StaR and phosphatidylcholine transfer protein; WD40 – WD40 repeat; CH – calponin homology; MyTH4 – myosin and kinesin tails; B4.1 – band 4 homologues, RING – RING zinc-finger; LRR – leucine-rich repeats; S_TKc – serine/threonine protein kinase; RRM – RNA recognition; TRANS – transmembrane; SAM – sterile alpha motif; ARM – armadillo repeat; Zf_TRAF – TRAF-type zinc finger; MORN – membrane occupational and recognition motif; PRO – protamine P1; CAL3 – calpain subunit 3; LH2 – lipooxygenase; WW – two conserved Trp residues; ANK – ankyrin repeat; HISKA – HIS kinase A; IPPc – inositol polyphosphate phosphatase; C2 – protein kinase-C conserved.

The catalytic portion of the motor domain represents the ancestral core of all kinesins and confers ATP- and microtubule-binding.[25] Predictably, these sites are conserved throughout the family. However, nucleotide- and microtubule-binding account for only part of kinesin function. Complete kinesin functionality includes the associations with various cargo in different cellular locations. Variations of protein structure outside of the motor domain, like the presence of additional protein domains, help explain the diverse roles that kinesins play in cells.

A kinesin superfamily, extracted from 21 diverse organisms, was analyzed in this work (Table 2). A total of 30 different kinesin-associated domains were identified and the distribution of domains varies from ubiquitous to lineage specific (Figure 2). The HDAC_Interact domain is found in eight of the ten lineages sampled, and it represents the most ubiquitous of kinesin-associated domains. This domain interacts with histone acetylases to assist with transcription regulation, however, its function in kinesins is undetermined.[26] Several domains are found in multiple lineages, either in closely related organisms (e.g., the FHA domain in Fungi and Animalia) or in more distantly related organisms (e.g. the SAM domain in Diplomonadida, Plantae, and Mycetozoa). It is not clear if domains common to disparate phylogenic lineages are examples of convergent protein evolution, or rather, the existence of ancestral kinesins that have been lost in some lineages and maintained in others. The majority of domains are found in only one of the ten lineages. Representative species from these lineages contain kinesins with novel domain arrangements, which may convey a clade-specific function.

There are 12 kinesin-associated domains in the Chlorophyta/Plantae branch, 7 of which are unique to plants. One *Arabidopsis* kinesin, KCBP, contains MyTH4 and B4.1 domains; it has been thoroughly characterized and will not be further addressed in this chapter (see [28] for a review). The characterization of kinesins with armadillo (ARM) repeat domains has been initiated,[12,29,30] and will be discussed in more detail here. Additionally, kinesins with calponin homology domains and RING zinc-finger domains will also be addressed.

4. Kinesins with Armadillo repeats

The *Arabidopsis* genome contains three, N-terminal kinesins that have an ARM repeat domain in the C-terminal region. An ARM repeat consists of about 42 amino acids, repeated up to 12 times to make a complete structural domain.[31] In *Arabidopsis*, the ARM domain consists of either three or four repeats (Figure 3). This domain was originally identified in the *Drosophila melanogaster* polarity gene ARMADILLO, which influences developmental patterning.[32] The ARM repeat domain is widely distributed and mediates

protein-protein interactions in a variety of cellular processes in both animals[33] and plants.[34]

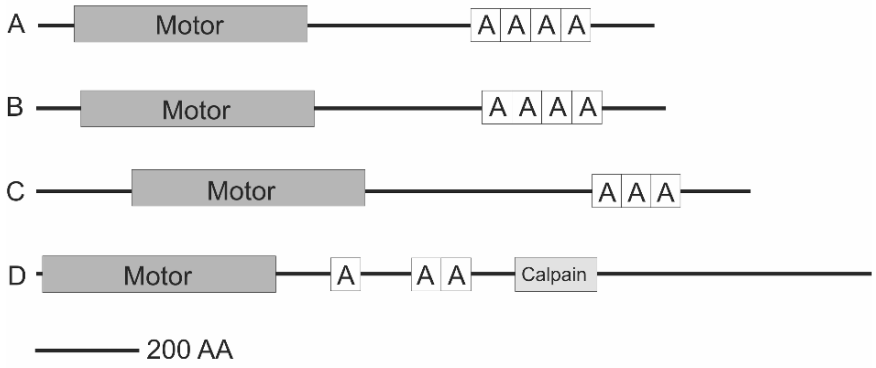

Figure 3. The domain architecture of ARM kinesins. A – At1g01950; B – At1g12430; C – At3g54870; D – LmjF17.1110. White boxes with "A" indicate a single armadillo repeat.

In earlier phylogenic analyses, ARM kinesins were grouped with Kinesin-1, Kinesin-2, and Kinesin-9, but now they are recognized as an ungrouped, plant-specific clade.[2,3,35] To better understand the evolutionary history of ARM kinesins, a phylogenic tree was created using full-length sequences (Figure 4).

Within the plant-specific ARM kinesin family, there are two different subtrees: one consists of kinesins from all plant species, and the other is more deeply rooted with kinesins from tracheophytes only. In the first subtree, there have been several duplication events: first, between kinesins of *Physcomitrell*a; second, after the split of dicots from monocots; and third, between *Arabidopsis* and poplar. In the second subtree, a duplication event is again seen between monocots and dicots, but more interesting is the lack of a bryophyte kinesin. Because bryophytes represent an earlier stage of plant evolution, it is likely that the ancestral ARM kinesin was lost from the bryophyte lineage, while it persisted and diverged under different selection pressures in some tracheophytes.

The *Arabidopsis* kinesin in the second subtree influences root hair morphology through potential interactions with Rho-related GTPases or "never in mitiosis" (NIMA)-related kinases.[29,30] The other two *Arabidopsis* ARM kinesins do not appear to play a role in root hair morphology, but may be important for other epidermal cell functions.

Armadillo domains are observed in a variety of other *Arabidopsis* proteins. The ARABIDILLO-1 (At3g44900) and ARABIDILLO-2 (At3g60350) proteins contain ARM repeat domains most similar to those observed in ARM kinesins. The presence of similar ARM domains in the ARABIDILLO proteins and ARM kinesins likely represents an instance of

molecular tinkering in the *Arabidopsis* genome. ARABIDILLO-1 and ARABIDILLO-2 double mutants have reduced lateral root branching.[37] Overexpression of truncates has a dominant negative effect, providing evidence that ARABIDILLO-1 and ARABIDILLO-2 function through protein interaction. These proteins were originally identified as the closest plant homolog to the mammalian ARMADILLO protein, β-catenin. β-catenin influences gene transcription, microtubule organization, and cell polarity through interactions with Wnt signaling proteins and Kinesin-2 members.[38,39] Based on the function of ARM repeat domains in plants and animals, it is exciting to ponder that a unique kinesin with an ARM domain already incorporated into its structure may form protein complexes that regulate the cytoskeleton, and ultimately plant morphology.

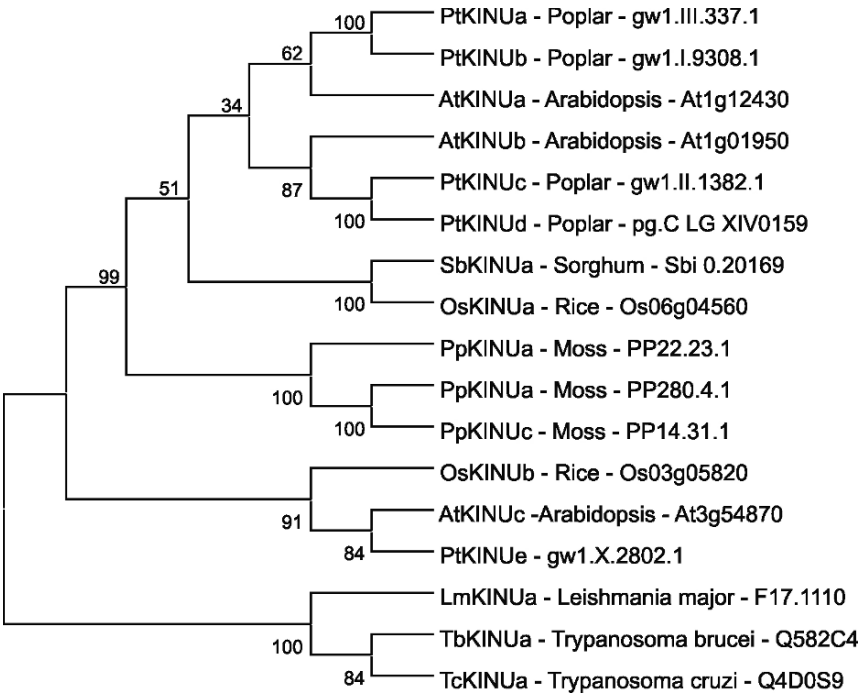

Figure 4. Phylogenic tree of ARM kinesins created by the neighbor-joining method. All sequence alignments and trees were created using MEGA 4.0 software.[36] Bootstrap values represent the resampling of 1,000 replications.

4.1. PROTIST ARM KINESINS – CONVERGENT EVOLUTION?

Kinesins that contain ARM repeat domains are also found in *Leishmania major* and other kinetoplastids in the Euglenozoa lineage. The euglenzoan ARM kinesins have N-terminal motor domains, but the ARM domains are

more internally located with fewer clustered repeats, compared to the plant ARM kinesins (Figure 3). Additionally, the euglenzoan ARM kinesins contain a calpain III domain adjacent to the ARM domain, which is not observed in the plant proteins. Calpain III domains are part of multi-domain calpain proteases. These domains may function in binding phospholipids, although it is typically seen with a calpain-like protease domain and an EF-hand domain.[40]

The eulgenozoan ARM kinesins are either ancestral proteins, examples of horizontal gene transfer, or examples of convergent evolution. Like plant ARM kinesins, the euglenozoan ARM kinesins are ungrouped in the kinesin superfamily. Despite the similar domain architecture and ungrouped categorization, the two sets of sequences do not form a clade.[3] The euglenozoan ARM kinesins are more similar to each other than to any of the plant ARM kinesins and form an outgroup of the ARM kinesin tree (Figure 4). This phylogenic analysis is consistent with other kinesin trees made with motor domains only.[2,3] Additionally, kinetoplastids are animal parasites that do not infect plant organisms, decreasing the possibility of horizontal gene transfer. Because of the lack of a phylogenic relationship and the absence of ARM kinesins in all other taxonomic lineages, it is more likely that these two classes of proteins are a result of convergent evolution.

Kinetoplastids are parasitic protists that cause a variety of diseases in humans, including Chagas disease, sleeping sickness, and leishmansiasis. Their invasion into host cells is dependent on the complex reorganization of their microtubule cytoskeleton, allowing rapid transport of lysosomes to the site of host/parasite contact.[41] This mechanism may rely on a diverse repertoire of kinesins (e.g., ARM kinesins). Plants also rely on complex microtubule arrays not seen in other organisms. The presence of ARM kinesins may increase the success of these lineages due to the selection pressures of complex cytoskeletal organization. Additionally, the presence of calpain III domains in kinetoplastid ARM kinesins may confer a benefit not needed in plant species. Therefore, independent events of domain shuffling and further evolution of ARM kinesins would be beneficial in these two divergent lineages.

5. Kinesins with calponin homology domains

The largest kinesin family in the *Arabidopsis* genome, consisting of 21 members, is the Kinesin-14 family. Kinesin-14 members typically have C-terminal motor domains, which have a higher sequence similarity to each other than to N-terminal or internal motor domains.[4] Plants have a unique Kinesin-14 family because many members have C-terminal type motor

domains that are shuffled internally or to the N-terminus of the protein (Figure 5). Because these motor domains share sequence homology, it is predicted they exhibit a similar motility to the other Kinesin-14 members; however, the characteristics of shuffled C-terminal motor domains have not been experimentally addressed.

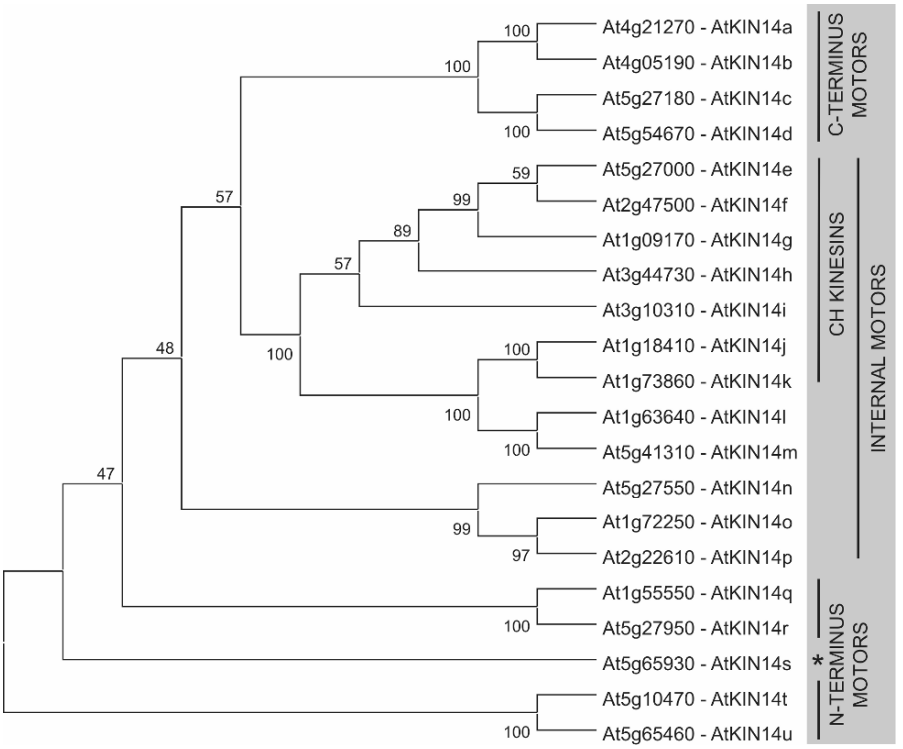

Figure 5. Phylogenic tree of the *Arabidopsis* Kinesin-14 family, created by the neighbor-joining method. Bootstrap values represent the resampling of 1,000 replicates. The location of the motor domain within the protein is indicated in the shaded box. Asterisk indicates KCBP, which has a C-terminus motor. KIN nomenclature is as suggested by Bannigan et al.[5]

Seven *Arabidopsis* Kinesin-14 family members have an internal motor domain and a C-terminal calponin homology (CH) domain. ATK4 was one of the first kinesins isolated from *Arabidopsis* and it is a representative member of this group.[42] Other plant species have CH kinesins in the following distributions: *Physcomitrella patens* (3), *Oryza sativa* (6), and *Populus trichocarpa* (13). Due to the large number of these kinesins, it is likely that they have undergone several duplication events. Cholorphytes do not have any representative CH kinesins, therefore, this domain architecture is unique to land plants.

The CH domain consists of approximately 100 amino acids. It is found in cytoskeleton-associated proteins, like actinin, spectrin, and utrophin, and signaling and regulatory proteins, like calponin, VAV3, and IQGAP1. A common link among this diverse collection of proteins is the ability to bind actin. The CH domain was originally isolated from calponin and is believed to function as an actin-binding domain due to sequence homology with α–actinin.[43] Further characterization of proteins containing a CH domain reveals that it has a far more complex function than simply binding to actin.

Calponin homology domains can be divided into three main categories, based on sequence similarity and function. Type 1 and type 2 CH domains (i.e., CH1 and CH2) make up the tandem N-terminal domains seen in most actin cross-linking proteins like actinin, spectrin, and utrophin.[44] Collectively, CH1 and CH2 are referred to as the actin-binding domain (ABD).[45] Although CH1 and CH2 work together to bind actin, CH1 alone can bind actin at a lower affinity than the complete ABD, whereas CH2 has no actin-binding activity.[44] This observation suggests that CH domains may have diverged from an ancestral domain to complete specific tasks. Fimbrin is a unique example of a tandem ABD, resulting in four CH domains. These observations suggest that CH domains might have diverged from an ancestral domain to complete specific tasks.

Type 3 CH domains are found in proteins associated with actin and cell signaling. The CH domain of CH kinesins falls into this group based on sequence comparison (Figure 6). There is some debate as to the exact role of the single CH domain in these proteins. The CH domain of calponin is not sufficient to mediate binding to actin and requires an additional site in the protein, similar to the tandem CH domains in actin-bundling proteins.[46] Alternatively, the CH domain in IQGAP, a protein that links signaling events to actin reorganization, does bind actin and at an affinity similar to actin-bundling proteins with tandem CH domains.[47] Type 3 CH domains may represent a more diverged state from CH1 and CH2 domains.

It is interesting that the Kinesin-14 family is expanded in plant species, and that the CH kinesins are a subgroup in this expansion. Because these kinesins have the potential of binding to microtubules and actin, the evolution and proliferation of CH kinesins in plants may represent lineage-specific selection pressures for complex co-regulation of the cytoskeleton. The only CH kinesin experimentally examined is the cotton homolog of ATK4, GhKCH1. This kinesin binds to actin *in vitro* and associates with actin microfilaments *in vivo*.[48] The large N-terminal piece of GhKCH1 used in this experimentation does not preclude the possibility of another actin-binding site that works cooperatively with the CH domain.

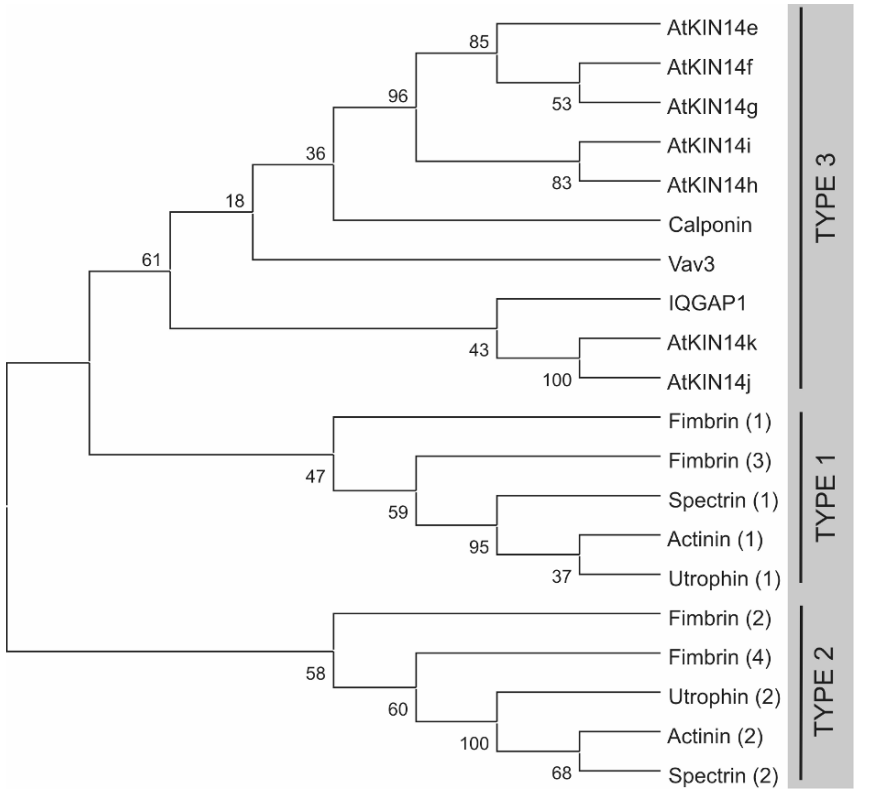

Figure 6. Phylogenic tree of CH domains created by the neighbor-joining method. Bootstrap values represent the resampling of 1000 replicates. The type of CH domain is indicated in the shaded box. Parenthetic numbers represent the order of CH domains in proteins with repeated copies. Accession numbers for the proteins are as follows: actinin (ABF50047), spectrin (NP_001020029), utrophin (EAW47848), calponin (NP_001290), VAV3 (AAD20349), IQGAP1 (AAI39732), fimbrin (NP_194400).

6. Kinesins with RING zinc-finger domains

Two members of the *Arabidopsis* Kinesin-7 family contain C-terminal "really interesting new gene" (RING) zinc-finger domains. This domain architecture appears to be a recent innovation to plant genomes, as it is only found in the angiosperms analyzed; *Physcomitrella patens* does not have any representative members. RING kinesins may be found to be a tracheophyte-specific domain arrangement, rather than just angiosperm-specific, depending on the eventual availability of gymnosperm sequential data.

The RING domain is a treble-cleft zinc finger that binds two zinc molecules in a cross-brace fashion; that is, the zinc molecules are bound in a single site rather than a tandem repeat. They range in size from from 30–100 amino acids and are characterized by a specific cysteine-rich region: C1-X2-C2-X(9-27)-C3-X(1-3)-H-X(2-3)-C4-X2-C5-X(4-48)-C6-X2-C7, where X represents any amino acid.[49] Cysteines 1, 2, 4, and 5 coordinate with one zinc molecule and cysteines 3, 6, 7 and histidine coordinate with another zinc molecule, forming the cross-braced tertiary structure.[50] Although the mechanism of zinc-binding is conserved in RING domains, the length of the linker regions is highly variable. It is hypothesized that RING domains mediate protein/protein interactions; however, they are found in a variety of proteins with different functions. Therefore, the specific function of this domain is limited.[49,50]

Plant kinesin RING domains are 35 amino acids in length and highly conserved among species (Figure 7). They follow the general RING pattern, and the linker lengths are conserved between monocots and dicots. A BLASTp search using the peptide sequence for the RING domain of At4g39050 reveals that the most closely related, functionally analyzed protein is the S-RNase-binding protein (SBP1) from *Petunia integrifolia*. The SBP1 associates with members of a novel E3 ligase complex that is responsible for ubiquitin-mediated degradation of the S-RNase proteins in self-incompatibility.[51]

The high sequence similarity between the RING kinesin domain and SBP1 RING domain provides evidence of molecular tinkering in these proteins. It is possible that the RING domain in SBP1 represents a conserved eukaryotic E3 ligase function, and its existence in a kinesin may link motor protein function with the ubiquination pathway in plants. Through this mechanism, kinesins may be able to sequester proteins destined for degradation to specific subcellular regions. Functional characterization will provide valuable insight into this hypothesis.

7. Kinesin domains beyond *Arabidopsis*

As more plant genomes are sequenced, there is the possibility of uncovering kinesins with domains that are not observed in *Arabidopsis*. In fact, there are several examples of unique domain arrangements in *Oryza sativa* and *Populus trichocarpa*. These kinesins may represent recent protein innovations in the kinesin superfamily.

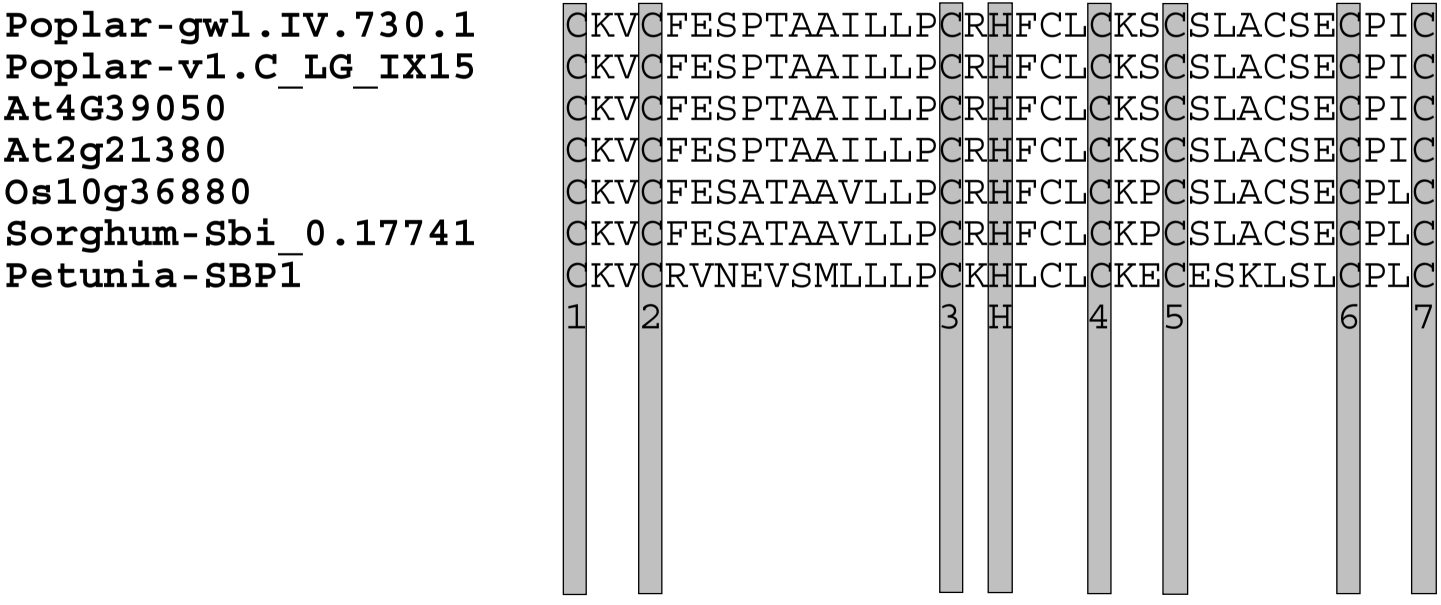

Figure 7. Alignment of RING domains in plant RING kinesins and related S-RNase binding protein 1 (SBP1) using CLUSTALW[52]. Cysteine residues are labeled (1–7), and the histidine residue is labeled (H).

7.1. RICE KINESIN DOMAINS

The rice genome contains one kinesin with an RNA recognition motif/domain (RRM). The RRM is a highly abundant, eukaryotic protein domain. It plays a role in many post-transcriptional events, including pre-mRNA processing, splicing, and mRNA stability.[53] In plants, proteins containing RRM domains function in mRNA processing in chloroplasts and mitochondria.[54,55] Additionally, localization of RNA is important for creating protein gradients that result in cell polarity.[56] A Kinesin-1 member in mice associates with large RNA complexes and functions in RNA movement, although an RRM domain is not responsible for the RNA binding.[57] A similar kinesin may assist in plant cell polarity in the monocot lineage.

7.2. POPLAR KINESIN DOMAINS

There are two kinesin-associated domains specific to the *Populus trichocarpa* genome: a leucine-rich repeat (LRR) domain and a serine/threonine protein kinase (S_TKc) domain. LRR domains are found in all forms of life, including bacteria and viruses. Despite the diversity of organisms in which LRR domains are found, they are all believed to function in protein/protein interactions. LRR domains consist of 20–30 amino acids, which are repeated up to 42 times.[58] The kinesin-associated LRR domain in *Populus trichocarpa* consists of a single repeat and may represent a novel LRR domain. In the case of a domain as ubiquitous as the LRR domain, it is difficult to ascertain a function based solely on its presence in a protein.

One kinesin in the poplar genome contains a serine/threonine protein kinase domain. This domain represents the catalytic core of kinases, which is responsible for binding ATP, binding the donor protein, and transferring a phosphate from the ATP to a serine or threonine of the protein.[59] It is interesting that two ancestrally related nucleotide-binding domains, a motor domain and a kinase domain, are found in the same protein. Possibly, a protein with both domains transports or sequesters phosphorylated proteins to specific subcellular locations to establish polarity. Another possibility is that the motor domain transports signaling molecules throughout the cell.

8. Conclusions

The kinesin families of the *Arabidopsis* and other sequenced genomes provide a unique opportunity to examine the role of molecular tinkering in protein evolution across diverse lineages. They also represent the challenges of working with large families that have undergone multiple duplication

events, preventing the benefit of single knockout phenotypes. Understanding the domain architecture and function of the domains is an approach to analyze these protein families. Using this information, it was found that many kinesin-associated domains are specific to different lineages and may represent the evolution of novel proteins. Plant genomes contain a variety of kinesins with unique domain compositions that have yet to be functionally characterized. Because protein domains dictate the function of a protein, using the role of these domains in other proteins can assist in the development of suitable hypotheses to functionally analyze unknown plant kinesins. Combining bioinformatic techniques with experimental methods will be the final step in obtaining a comprehensive understanding of the function of kinesins in plants.

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NOTES: IDENTIFICATION OF KINESINS

Kinesin sequences from unannotated organisms were extracted through BLASTp searches using motor domains of human KIFC (NP_004513), mouse KIF2A (BAA02165), and *Arabidopsis* ATK1 (AT4G21270). The following list details the individual databases used and the manual editing that was completed:

1. *Populus trichocarpa* – 88 sequences were extracted using Version 1.1 at the Joint Genome Institute (JGI) website (http://genome.jgi-psf.org). Two truncated sequences were removed. An additional six sequences were removed because they were shorter than 300 amino acids.
2. *Phanerochaete chryosporium* – 11 sequences were extracted using Version 2.0 at JGI. One had a motor domain below the threshold of SMART and was removed. An additional three sequences were removed because they were shorter than 300 amino acids.
3. *Caenorhabditis elegans* – 21 sequences were extracted using WWW.wormbase.org.
4. *Homo sapiens* – 45 sequences were extracted using the NCBI refseq database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). One was removed

because it was shorter than 300 amino acids. All others were hand annotated to ensure correct naming.

5. *Phytophthora sojae* – 56 sequences were extracted using Version 1.1 at JGI. five sequences were removed because they were shorter than 300 amino acids.
6. *Physcomitrella patens* – 70 sequences were extracted using Version 1.1 at JGI. One was removed because it was a duplication. An additional 17 sequences were removed because they were shorter than 300 amino acids.
7. *Dictyostelium discoideum* – 13 sequences were extracted using http://www.dictybase.org.
8. *Plasmodium falciparum* – Nine sequences were extracted using http://www.plasmodb.org, Release 5.2.
9. *Leishmania major* – 54 sequences were extracted using http://www.genedb.org.
10. *Cyanidioschyzon merolae* – Five sequences were extracted from http://merolae.biol.s.u-tokyo.ac.jp/.
11. *Thalassiosira pseudonana* – 23 sequences were extracted using Version 3.0 at JGI. One sequence was removed because it was shorter than 300 amino acids.
12. *Giardia lamblia* – 24 sequences were extracted using GiardiaDB (http://gmod.mbl.edu/perl/site/giardia14?page=intro).
13. *Chlamydomonsa reinhardtii* – 23 sequences were extracted using Version 3.0 at JGI.
14. *Oryza sativa ssp. japonic* – 51 sequences were extracted from TIGR Rice Genome Database (http://www.tigr.org/tdb/e2k1/osa1/). Eleven sequences were removed because they were variant splice products.
15. *Trichomonas vaginalis* – 21 sequences were extracted using TrichDB (http://trichdb.org/trichdb/).
16. *Volvox carterii* – 23 sequences were extracted from Version 1.0 at JGI. Five sequences were removed because they were less than 300 amino acids.
17. *Sorghum bicolor* – 45 sequences were extracted from Phytozome (www.phytozome.com). Four sequences were removed because they were shorter than 300 amino acids.

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BIOINFORMATIC SEARCH FOR PLANT HOMOLOGUES OF ANIMAL STRUCTURAL MAPs IN THE *ARABIDOPSIS* THALIANA GENOME

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Abstract. Although various plant microtubule-associated proteins (MAPs) have been described because of their role in modulating microtubule functions, the full complement of these important sequences has not yet been described. Taking into account the high level of homology between animal and plant tubulins, and the potential conservation of their MAP binding domains, we carried out a bioinformatic investigation of the MAP1, MAP2, tau protein, and MAP4 families from animals and humans. The gene-identification strategy, utilizing site-based and comparative methods, allowed us to identify amino acid motifs that are conserved within each MAP family. According to these amino acid motifs we designed corresponding nucleotide motifs in IUPAC code, accommodating potential codon polymorphism by applying the universal symbols representing all possible substitutions. Scanning the complete sequences of all five *Arabidopsis thaliana* chromosomes, we identified candidate coding sequences for plant homologues of animal structural MAPs. Furthermore, we identified 200 loci (i.e., 178 of MAP1 and 22 of MAP2 and tau) that are of interest as potential chromosomal regions for plant structural MAPs. Interestingly, consensus regions for MAP4 were not identified in *A. thaliana*. Chromosomal locations for three previously known plant homologues of MAP1 (i.e., AtEB1a, AtEB1b and AtEB1c) were also identified in the *A. thaliana* genome.

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1. Introduction

Structural microtubule-associated proteins (MAPs) are the fibrillar proteins, which play an important role in the regulation of microtubule functions. They increase the rate of polymerization and assembly, and stabilize microtubules.[1,2] MAPs, binding reversibly to microtubules, promote both their polymerization, organization into bundles, as well as their resistance to depolymerizing factors.[2–5]. Thus structural MAPs stabilize microtubules and support their interactions with other cell components.[6,7] Numerous specific families of plant MAPs, such as MAP18[2] in *A. thaliana*; MAP60 in *Daucus carota*[8]; MAP65 in *A. thaliana*,[9–12] *Nicotiana tabacum*[13] and *D. carota*[14]; MAP70 in *A. thaliana*[15]; MAP120 in *D. carota*[16]; MAP190 in *A. thaliana*[9] and *N. tabacum*,[17,18] etc. have been identified. Most of the plant MAPs discovered so far have homologs in eukaryotes, but not all animal or fungal MAPs are present in plants.[6,11,19]

The identified animal and human structural MAPs can be divided into two groups: Type I includes MAP1 proteins and Type II includes MAP2, tau and MAP4 proteins (Figure 1). MAP1a, MAP1b and MAP1c, which make up the MAP1 family, bind to microtubules differently than others MAPs, namely, by the force of charge interactions. Microtubule binding by MAP1 is closely associated with EB1 domain, which interacts directly with tubulin.[20] While the COOH-termini of these MAPs binds the microtubules, the NH_2-termini bind other components of the cytoskeleton or the plasma membrane to control the distribution of microtubules within the cell. As a rule animal analogs of the MAP1 are observed mainly in axons and dendrites of neurons.[21] It was shown previously, that the *A. thaliana* genome contained three EB1 genes designated AtEB1a, AtEB1b, and AtEB1c. Each of the predicted proteins contains conserved domains and shares significant sequence similarity with EB1 proteins from others eukaryotes. It was predicted that all three AtEB1 proteins would contain a conserved amino-terminal calponin homology domain required for MT binding in addition to a conserved coiled-coil (EB1) domain, which is thought to mediate protein-protein interactions.[20,22] So, among hypothetical plant homologues, they seem to be the most similar to animal structural MAPs.[23,24]

All Type II proteins have microtubule-binding repeats near their carboxyl ends (Figure 1),[25] each containing a conserved KXGS motif, which can be phosphorylated.[26,27] Reversible phosphorylation of the KXGS motif induces its association/dissociation with microtubules.[26,28,29] Also,

each protein of Type II family contains an amino-terminal NH-projection domain of varying size. Moreover, each protein has several isoforms, accounting for variations in the length of their projecting domain and the number of microtubule-binding repeats.[30] So, the main isoforms of MAP2 are MAP2c, which is relatively short, and MAP2a and MAP2b, which have longer projection domains.[30]

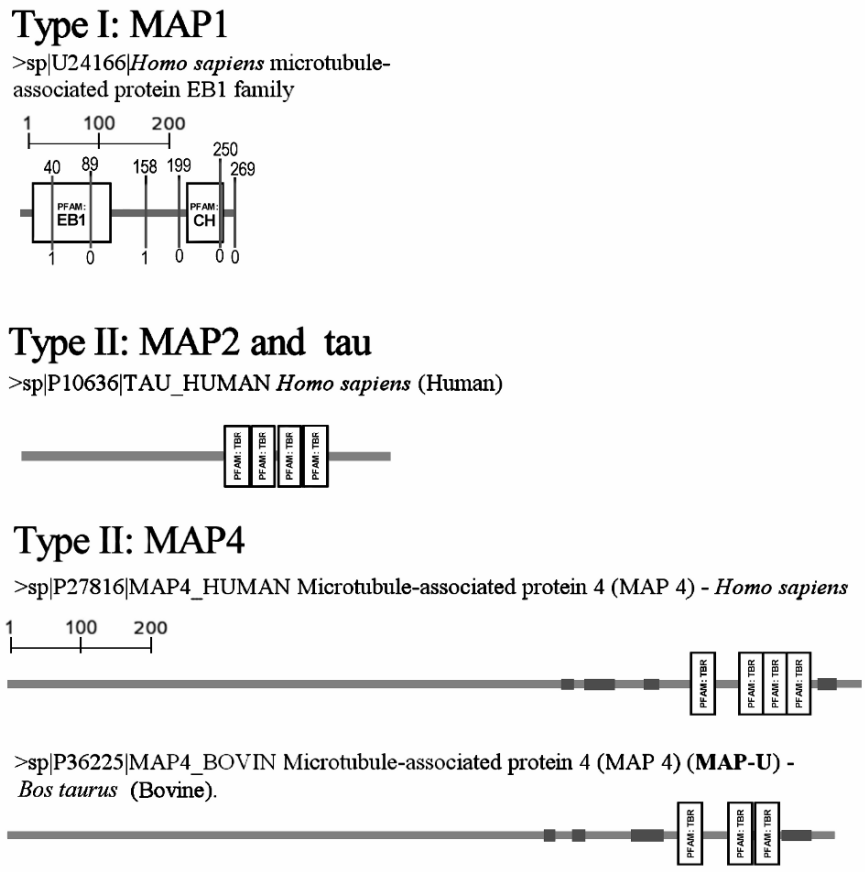


Figure 1. Examples of domain organization of typical structural MAPs representatives from Type I and Type II families. The illustration of Type I MAPs shows the conserved coiled-coil domain (EB1), and that of the Type II, shows the location of the Tubulin Binding Repeats (TBR) containing the conserved KXGS sequence.

The microtubule-binding domains of MAP2, tau and MAP4 proteins are structurally similar.[31] MAP2 and tau proteins are the most studied MAPs, and are found exclusively in neurons: MAP2 mainly in dendrites and tau protein in axons. They participate in determining the internal structure and

overall morphology of these neural cells. As mentioned previously, these proteins have a conserved COOH-terminal microtubule-binding domain and NH_2-terminal variable domains projecting outwards, potentially interacting with others proteins. MAP2 and tau stabilize microtubules, shift the reaction kinetics in favour of adding new tubulin heterodimer subunits, thus accelerating microtubule growth.[32] However, although MAP2, tau and MAP4 proteins share a high level of structural and sequence similarity, MAP4 proteins are present only in non-neuronal mammalian cells. These MAPs stabilize microtubules, promoting their frequent rescue. MAP4, binding to microtubules may be regulated by mapmodulin; a soluble protein that binds MAP4 and prevents binding of microtubules.[33] MAP4 has been proposed to play a role in regulating microtubular dynamics during metaphase.[30,34] It was shown that MAP65 protein, immunologically related to MAP4, is present in tobacco and thale cress.[35,36] Other evidence of the similarity between plant MAPs and MAP4 was provided by the cross-reactivity of a 90-kDa microtubule-binding protein from the freshwater alga *Dichotomosiphon tuberosus* with antibodies against vertebrate MAP4 – the widely occurring relative of brain MAP2 and tau proteins.[35] However, the obvious sequence and structural homology between candidate plant structural MAPs and animal MAPs containing 'PGGG' motifs (i.e., MAP2, tau and MAP4 proteins) was not detected.[35]

Traditionally, three major strategies have been employed to identify novel plant MAPs. These are: *immunological* – using antibodies raised against MAPs from other taxa; *biochemical* – isolating and identifying plant MAPs based upon their binding ability to tubulin or microtubules; and *genetic* – isolating mutants with defects in microtubule organization and identifying the affected proteins.[6] Now different bioinformatic methods are available for the discovery of MAPs and other cytoskeletal proteins.[6,10] Bioinformatic gene-identification strategies can be grouped into three major categories: *content-based methods* (based on the overall, bulk properties of a sequence), *site-based methods* (based on the availability of a specific sequence, pattern, or consensus) and *comparative methods* (which make determinations based on sequence homology).[37] Our present research, based on a combination of the site-based and comparative strategies, allowed us to identify the probable locations of plant homologues of animal structural MAPs. This was accomplished by scanning the complete sequences of the *A. thaliana* chromosomes, with the help of amino acid sequences of animal MAPs (TBLASTN) and nucleotide motifs (with substitution symbols).

2. Materials and methods

The amino acid sequences of known MAPs and their corresponding genes were investigated. The sequences were obtained from such databases as Swiss-Prot/TrEMBL (www.expasy.org),[38] GenBank (http://www.ncbi. nlm.nih.gov,[39] PDB (Protein Data Bank – http://www.rcsb. org/pdb/,[40] and DDBJ (DNA Data Bank of Japan – http://www.ddbj.nig.ac.jp/.[41] The sequences of the structural MAPs were sorted according to their types and families (basing on the data from www.cytochemistry.net/Cell-biology/microtubule_structure) and saved in FASTA format for subsequent analysis.[42] The information on domain architecture, presence of functional regions and their amino acid sequences was obtained from the Swiss-Prot data and was analysed with a help of web-tools SMART (Simple Modular Architecture Research Tool – http://smart.embl.de/)[43,44] and CDART (Conserved Domain Architecture Retrieval Tool – http://www.ncbi.hih.gow/ Structure/cdd/).[45] Alignment was performed by means of the local version of the multiple sequence alignment programme – Clustal X (1.8)[46–49] applying BLOSSUM matrix series (BLOSUM 80 (80–100%), BLOSUM 62 (60–80%), BLOSUM 45 (30–60%) and BLOSUM 30 (0–30%)).[50] The patterns of primary domains were created on the basis of alignment results according to the PROSITE recommendations (ScanProsite tool manual: http://cn.expasy.org /tools/ scanprosite /scanprosite-doc.html).

Motifs (not less than six amino acid residues) were considered conservative if an absolute (100%) identity was observed only with sequences of typical representatives of animal MAPs under scanning in the SIB BLAST (http://www.expasy.org/tools/blast/).[51–53] But motifs having 100% coincidence with other proteins were excluded from further analysis. Identification of structural MAP homologues in *A. thaliana* was also accomplished with the help of the TBLASTN tool using complete protein sequences, as well as sequences of domains or conserved TBRs (tubulin binding repeats). The sequence handling, assembly, translation and search of the *A. thaliana* genome were carried out with the help of different tools, integrated in program DNAMAN (V.5.2.9 Lynnon BioSoft). Analysis and visualization of localization sites of consensus regions, corresponding to the candidate genes of structural MAPs were carried out using the on-line tool NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview/) and data represented in TAIR (http://www.arabidopsis.org), TIGR (http://www.tigr.org/), KEGG (http://www.genome.jp/kegg/), ExpPASy (www.expasy.org) and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/ index.html).

3. Results and discussion

Sequence-homology search algorithms are important computational tools in molecular biology. There exist at least three general classes of techniques used to search for protein homologues, namely pair wise sequence comparisons such as basic local alignments search tool (BLAST), profile-based searchers such as HMM SEARCH, and motif- or pattern-based analyses such as pattern-hit-initiated BLAST (PHI-BLAST).[54]

We applied the different sequence comparison methods, based on TBLASTN algorithm in present investigation to search plant homologues of animal MAPs in *A. thaliana* genome. The search for putative genes and their products was performed according to a common scheme developed by us and which we followed during analyses.

First, we carried out a search of known sequences for the eukaryotic structural MAPs in the main bioinformatic databases and selected only animal MAPs of known amino acid sequences and sequences of the corresponding genes. As a result, we have selected 46 MAPs [(21 proteins of the Type I (MAP1) and 24 of Type II (11 proteins of MAP2 and tau and 13 proteins of MAP4)]:

MAP1: *Bos taurus* (Q3ZBD9_MARE1), *Brachydanio rerio* (Q6P020; Q6NUY9; Q803W2), *Coturnix coturnix* (Q66T82_MARE1; Q6V291_MARE1), *Gallus gallus* (Q5ZLC7_MARE), *Homo sapiens* (Q3KQS8; Q15691_MARE1; Q15555_MARE2; Q9UPY8_MARE3), *Ictalurus punctatus* (Q9W6M6), *Mus musculus* (Q61166_MARE1), *Pongo pygmaeus* (Q5R7Z5_MARE1), *Rattus norvegicus* (Q66HR2_MARE1), *Xenopus tropicalis* (Q6P848_MARE1; Q8UW77; Q8UW78) and *Arabidopsis thaliana* (Q7XJ60_AtEB1a; Q9FJJ5_AtEB1b; Q9FGQ6_AtEB1c).

MAP2 and tau: *Bos taurus* (P29172), *Capra hircus* (O02828), *Gorilla gorilla gorilla* (Q5YCW0), *Homo sapiens* (P10636), *Hylobates lar* (Q5YCV9), *Macaca mulatta* (P57786), *Mus musculus* (P10637), *Pan troglodytes* (Q5YCW1), *Pan pygmaeus* (Q5S6V2), *Papio hamadryas* (Q9MYX8), *Ratus norvegicus* (P19332).

MAP4: *Bos taurus* (Q8MJX3; Q867A8; Q8MJX2_MAPU; P36225_MAPU), *Homo sapiens* (P27816; Q86Y04; Q59FT2; Q68D74), *Mus musculus* (Q7TPC6; Q7TPD4; Q3UIS2; P27546), *Ratus norvegicus* (Q5M7W5_MAPU).

The analysis of the results of the multiple alignments revealed the absence of motifs common to all structural MAPs. At the same time, analysis in SMART, Pfam and PROSITE confirmed the different modular structure of different MAP families, and the high degree of similarity of domain composition, organization and sequences in the case of the definitive MAP family (MAP1, MAP2, tau and MAP4 proteins) (Figure 1).

Taking into account peculiarities of sequence analysis of proteins with different domain architecture and composition,[55,56] another search of conservative sites was carried out separately for MAP1, MAP2, tau and MAP4.

The comparison of amino acid sequences of known animal MAP1 from Swiss-Prot and putative EB1 proteins from TrEMBL (Q7XJ60_AtEB1a; Q9FJJ5_AtEB1b; Q9FGQ6_AtEB1c) showed that the human MARE2 possesses the highest resemblance to the plant putative homologues (UniProtKB/Swiss-Prot entry Q15555). These MAPs share 34% identity and 54% similarity with AtEB1c from *A. thaliana* (UniProtKB/Swiss-Prot entry Q9FGQ6) (Figure 2).

CLUSTAL X (1.8) sequence alignment: Q9FGQ6_ARATH / MARE2_HUMAN

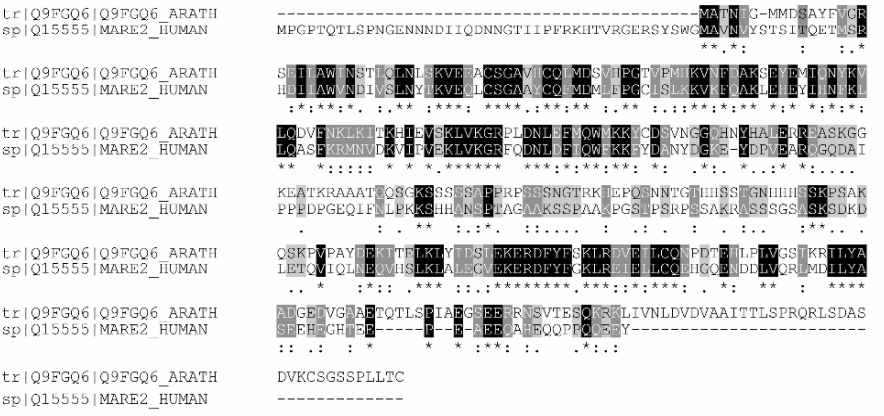


Figure 2. Sequence alignment Q9FGQ6_ARATH/MARE2_HUMAN. Identity = 34%; similarity = 54%.

This allows us to use the human MARE2 as the most suitable model sequence for homology searches using TBLASTN. The TBLASTN search was carried out against the *A. thaliana* whole genome nucleotide sequences database (referenced only) with default filter and Expect = 0.01. As a result, we identified nine Blast hits in the query sequences, which were distributed in chromosomes III (Score = 40.8; E-value = 2e-08) and V (Score = 60.1; E-value = 2e-08). Analysis of the identified consensus regions showed that they correspond to the coding regions (CDSs) of the known EB1 homologues of animal MAPs: AtEB1a (UniProtKB/TrEMBL: Q7XJ60 (Q9STU4))[23,24] (Table 1), AtEB1b (Q9FJJ5) (Table 2) and AtEB1c (Q9FGQ6)[23] (Table 3). Each of the identified genes corresponds to three discretely located coding DNA sequences with 42–77% similarity. The identity was at the level of 30–60% (Tables 1–3). The regions of similarity in all genes have similar topology, corresponding to the similar coding regions and localization in proteins. So, Region I was located in the center

of CH domain, Region II was located in the C-terminal region of the same domain and Region III was mainly located at the beginning of EB1 domain, extending partly into the interdomain region. Other consensus regions were not revealed in the *A. thaliana* genome. The check/control search, using the AtEB1c sequence, also identified only these three genes.

TABLE 1. *A. thaliana* chromosome 3, AtEB1a (TAIR: At3g47690; UniProtKB/TrEMBL: Q7XJ60).

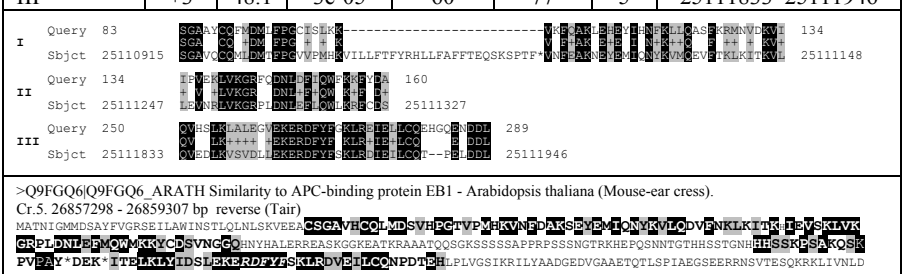
Consensus subsequence	Frame	Score (bits)	Expect	Identities (%)	Positives (%)	Gaps (%)	Consensus coordinates (bp)
I	+3	40.8	2e-08	32	42	36	17592825–17593064
II	+2	37.0	2e-08	51	77	0	17593166–17593246
III	+3	45.1	3e-04	55	75	5	17593737–17593850

>Q7XJ60|Q7XJ60_ARATH At3g47690 - *Arabidopsis thaliana* (Mouse-ear cress). Chr.3. 17592081 - 17594571 bp forward

MATNIGMMDSAYFVGRNEILTWINDRLHLNLSRVEEAASGAVQCQMLDMTFPGVVPMHKV*NFDAKNEYDMIQNYKVLQDVFNKLKITKPLEIN
RLVKGRPLDNLEFLQWLKRFCDSINGGIMNENYNPVERRSRNGKERSVKGSNKIPKSLQTNNNHPPPNSSSVGLSKASGPKSAKAAEVQALSKELVDLKK
ISTDLLEKERDFYFSKLRDVEILCQTPELDDLPIVVAVKKILYATDANESALEDAQEYLNQSLGVEDDEAEGNGEQLEEEKTQA

TABLE 2. *A. thaliana* chromosome 5, AtEB1b (TAIR: At5g62500; UniProtKB/TrEMBL: Q9FJJ5).

Consensus subsequence	Frame	Score (bits)	Expect	Identities (%)	Positives (%)	Gaps (%)	Consensus coordinates (bp)
I	+3	43.9	2e-09	30	46	33	25110915–25111148
II	+2	37.4	2e-09	55	77	0	25111247–25111327
III	+3	48.1	3e-05	60	77	5	25111833–25111946



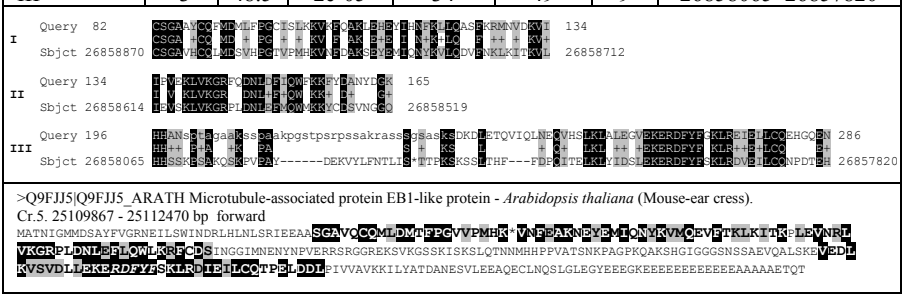
>Q9FGQ6|Q9FGQ6_ARATH Similarity to APC-binding protein EB1 - Arabidopsis thaliana (Mouse-ear cress).
Cr.5. 26857298 - 26859307 bp reverse (Tair)

MATNIGMMDSAYFVGRSEILAWINSTLQLNLSKVEEACSGAVHCQLMDSVHPGTVPYHKVNFDAKSEYEMIQNYKVLQDVFNKLKITKPLEVSKLVK
GRPLDNLEFMQWMKKYCDSVNGGQHNYHALERREASKGGKEATKRAAATQQSGKSSSSSAPPRPSSSNGTRKHEPQSNNTGTHHSSTGNHHESSKPSSAKQSK
PVPAY*DEK*ITELKLYIDSLEKERDFYFSKLRDVEILCQNPDTPHLPLVGSIKRILYAADGEDVGAAETQTLSPIAEGSEERRNSVTESQKRKLIVNLD
VDVAAITTLSPRQLSDASDVKCSGSSPLLTC

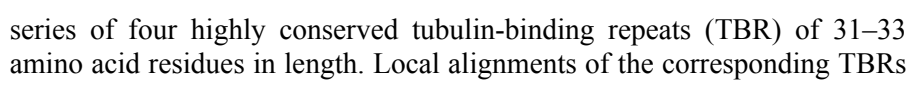
Because animal MAP2 and tau proteins demonstrate common domain organization, functional similarity and share a high level of sequence homology,[28,29] we analyzed them together (MAP2_tau). As mentioned earlier, all 11 MAP2_tau proteins (as the typical Type II MAPs) have at their COOH-end region a tubulin-binding domain, represented by a tandem

TABLE 3. *A. thaliana* chromosome 5, AtEB1c (TAIR: AT5G67270; UniProtKB/TrEMBL: Q9FGQ6).

Consensus subsequence	Frame	Score (bits)	Expect	Identities (%)	Positives (%)	Gaps (%)	Consensus coordinates (bp)
I	-2	60.1	1e-14	47	67	0	26858870–26858712
II	-3	39.3	1e-14	56	71	0	26858614–26858519
III	-3	48.5	2e-05	34	49	9	26858065–26857820



>Q9FJJ5|Q9FJJ5_ARATH Microtubule-associated protein EB1-like protein - *Arabidopsis thaliana* (Mouse-ear cress).
Cr.5. 25109867 - 25112470 bp forward



series of four highly conserved tubulin-binding repeats (TBR) of 31–33 amino acid residues in length. Local alignments of the corresponding TBRs showed that they share a very high level of similarity (identity = 97–100%). TBR2 was absolutely identical in all 11 MAP2_tau proteins: VQIINKKL DLSNVQSKCGSKDNIKHVPGGGS. Therefore, we used it for scanning the *A. thaliana* genome with the help of TBLASTN program. All parameters of the search were identical to those used for the MAP1 search. As a result, no hits or significant similarities were revealed. Scanning with a longer motif: APVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKL DLSN VQSKCGSKDNIKHVPGGGSV, including neighbouring conservative positions from TBR1 and TBR3 gave the same results. However, the control scanning of the human genome with the help of these motifs identified 20 hits, located in the chromosomes 2, 3 and 17, which correspond to genes of Type II MAPs: MAP2 (NM_001039538), tau (Gene: NM_016835) and MAP4 (Gene: NM_002375). The last case confirms ones more high level of similarity of the tubulin binding domain of MAP2_Tau and MAP4.

Like MAP2_Tau, MAP4 has a tubulin-binding domain represented by a tandem series of 4 (MAP4) or 3 (MAP-U) highly conserved tubulin-binding repeats. Analysis of alignments of the tubulin-binding domains and groups of the repeats showed that TBR3 and TBR4 have the highest degree of similarity of all animal MAP4s. Tubulin-binding repeat 3 was identical in all 13 MAP4 – VQIQNKKVDISKVSSKCGSKANIKHKPGGGD, and TBR4 varies only in two positions – VKIESQKLNFKEKAQAKVGSLDN VGH[LF]PAGG[AT]. Searching the *A. thaliana* genome, using the amino acid sequences of TBR3 and TBR4, resulted in the absence of any hits. A similar result was obtained when we used a longer motif: GN

VQIQNKKVDISKVSSKCGSKANIKHKPGGGDVKIESQKLNFKEKAQ AKVGSLDNVGH, including neighbouring conservative positions from TBR2, TBR3 and TBR4.

However, BLAST family programs, using comparative-search algorithms, may be ineffective in the case of weak similarities[6,35,57]. So, we applied another search method based on a screen of probable homologues with the help of the nucleotide motifs in which variable positions were compensated for by universal bases in compliance with IUPAC code.

Relying on the results of multiple alignments, we identified conserved motifs, which were 6–10 amino acid residues in length. SIB BLAST scanning of the available databases (www.expasy.org/tools/blast) has confirmed the absence of any proteins (with the exception of structural MAPs) with sequences corresponding to these conserved motifs. Thus, we have identified motifs specific to only the structural microtubule associated proteins (Table 4).

TABLE 4. Conserved motifs, their short variants and their correspondence to the coding regions of *A. thaliana* genome.

Family	Conserved peptide motif	Short variant of motif	% of correspondence to MAP[a]	Chromosome	Gene
MAP1	EKERDFYF	ERDFY	57.7	2	AT2G34410
		RDFYF	65.2	1, 4, 5	Not specified
MAP2_tau	HVPGGG	HVPGG	61.5	1	AT1G15750
				2	AT2G37650
				4	Not specified
				5	AT5G03480
	DHGAEI	HGAEI	88.2	1	AT1G34355
				2, 4	Not specified
		DHGAE	60.0	2	AT2G07190
	LSNVSSSGSI	LSNVSS	78.9	2	AT2G26920
				4	AT4G08108
		LSNVSSS	100	5	YLS8
	SPQLATLA	QLATLA	83.3	1	AT1G58766
		SPQLAT	75.0	2	AT2G29165
				5	AT2G12130
	LAKQGL	AKQGL	53.6	1	AT1G54780H
				3	Not specified
				5	EMB1006
MAP4	CPGPPAGEE	PPAGEE	75.0	4	ASY2
	QTLDSQIQET	QTLDSQ	75.0	5	AT5G10250
	PAGGAVKTEG	AVKTEG	60.0	5	AT5G26775

[a]According to data analysis using BLAST

The NCBI web-site (www.ncbi.nlm.nih.gov/mapview/map_search.cgi) affords access to complete sequence information concerning the *A. thaliana* genome. At the present time, for the five thale cress chromosomes 31,762 genes (31,897 including mitochondrion) have been identified: 8,057 in chromosome I; 5,216 in chromosome II; 6,382 in chromosome III; 4,887 in chromosome IV and 7,220 in chromosome V. For reverse translation and determination of potential sequence variants of the conserved amino acid motifs, we applied different search tools, integrated in the DNAMAN programme. We searched for these consensus coding DNA sequences in the databases of each of the five *A. thaliana* chromosomes. We identified 32 coding DNA sequences corresponding to the shortened motifs. Some of the detected coding DNA sequences were duplicated but had different coordinates, or were located on different chromosomes. After additional verification of the conserved motifs in BLAST, we chose only those that occurred in the corresponding MAPs (53.6–100%). As a result, we identified 13 highly conserved motifs (Table 4).

Analysis of the corresponding chromosomes' coding regions, by means of NCBI's MapViewer on-line tool (www.ncbi.nlm.nih.gov/mapview/), has resulted in identification of the sites within *A. thaliana* genome containing these conservative motives, but which were located outside the borders of the known genes.

By scanning the *A. thaliana* genome for the universal MAP1 motif (HGNGAYTTYTAYTTY), we identified 221 consensus regions: 55 in chromosome I, 38 each in chromosomes II, III and IV, and 52 in chromosome V. Analysis of the identified motifs using the MapViewer tool showed that 43 corresponded to genes encoding proteins containing the RDFYF motif. Among these consensus regions are those corresponding to the previously known homologues of EB1 MAPs: AT3G47690.1 (AtEB1a, Q7XJ60, chromosome III, 17592081–17594571 bp, forward)[23,24] AT5G62500.1 (AtEB1B, Q9FJJ5: chromosome V, 25109867–25112470 bp, forward)[23] and AT5G67270.1 (AtEB1c, Q9FGQ6: chromosome V, 26857298–26859307 bp, reverse)[23] (Table 5). The remaining 178 consensus regions correspond to the *Escherichia coli* BAC clones marked in the articles of TAIR and TIGR databases as: unknown products, unknown proteins, hypothetical proteins or pseudogenes (Table 6).

The significance of these findings is that the conserved RDFYF motif was identified as specific for the EB1 domain, which plays a very important role in MAP1-tubulin interactions in human and animal cells.[58,59] Taking into account the high degree of similarity between animal and plant tubulins,[60,61] and the fact that the majority of the consensus regions correspond to the insufficiently explored regions of the *A. thaliana* genome, makes it possible that these predicted sites are located in regions containing new still

TABLE 5. The known proteins of *A. thaliana*, corresponding to the location of identified consensus regions.

	Protein	TAIR	Location of consensus (bp)	+/-	Gene location (bp)
Chromosome I	GSK1 (GSK3/SHAGGY-LIKE PROTEIN KINASE 1)	AT1G06390.1	1947221-TGTGATTTTTACTTC-1947235	+	1946787–1950754 bp forward
	Myosin-related, F21J9.12	AT1G24460.1	8666163-TGAGATTTCTATTTC-8666177	+	8666060–8672346 bp forward
	RLM1 (Resistance to *Leptosphaeria maculans* 1)	AT1G64070.1	23785433-AGTGATTTTTATTTT-23785447	+	23783612–23787112 bp forward
	Hydrolase, hydrolyzing O-glycosyl compounds; F25C20.1	AT1G11820.1	3991121-AAAATAAAAATCACG-3991135	-	3991039–3993524 bp reverse
	Protein kinase, F5M15.3	AT1G20650.1	7160446-AAAATAAAAATCACG-7160460	-	7158234–7160516 bp reverse
	Ulp1 protease family protein, F14D7.7, F14D7_7	AT1G35770.1	13276676-AAAATAAAAATCCCA-13276690	-	13270778–13276890 bp reverse
	UBIQUITIN-SPECIFIC PROTEASE 6, UBP6, F19C24.8, F19C24_8	AT1G51710.1	19180768-GAAATAAAAGTCGCA-19180782	-	19179310–19183635 bp reverse
	Shaggy-related protein kinase kappa, putative / ASK-kappa, putative	AT1G57870.1	21439539-AAAGTAAAAATCACG-21439553	-	21434783–21439767 bp reverse
Chromosome II	ATCHX16 (CATION/H+ EXCHANGER 16); monovalent cation:proton	AT1G64170.1	23818990-GAAGTAGAAATCTCA-23819004	-	23818902–23821956 bp reverse
	Ulp1 protease family protein	AT2G06860.1	2766553-TGGGATTTTTATTTT-2766567	+	2766353–2772442 bp forward
	Pectinesterase family protein	AT2G19150.1	8313268-AGAGATTTTTATTTT-8313282	+	8312182–8314526 bp forward
	LCR60, Low-molecular-weight Cysteine-Rich	AT2G20208.1	8728377-TGTGATTTCTACTTT-8728391	+	608727871–8728504 bp forward
	33 kDa secretory protein-related	AT2G01660.1	291406-AAAATAAAAATCTCA-291420	-	290602–292589 bp reverse
	Mutator-like transposase family	AT2G06870.1	2778950-AAAGTAAAAATCACT-2778964	-	2775700–2779184 bp reverse
	Glycosyltransferase family protein	AT2G28080.1	11968912-AAAGTAAAAGTCCCT-11968926	-	11967648–11970370 bp reverse
	Peroxidase 21 (PER21)(P21)(PRXR5)	AT2G37130.1	15605132-AAAATAAAAATCCCA-15605146	-	15605000–15607156 bp reverse
Chromosome III	BTB/POZ domain-containing protein	AT2G40440.1	16897482-GAAGTAGAAATCGCA-16897496	-	16896290–16898370 bp reverse
	Armadillo/beta-catenin repeat family protein	AT3G09350.1	2872370-TGTGATTTCTATTTT-2872384	+	2871051–2873318 bp forward
	Glycerol-3-phosphate dehydrogenase, putative	AT3G10370.1	3216383-AGAGATTTCTATTTC-3216397	+	3216373–3219275 bp forward
	NRPD2b (nuclear RNA polymerase D 2b)	AT3G18090.1	6196034-TGGGATTTCTATTTT-6196048	+	6195003–6200243 bp forward
	Plastocyanin-like domain-containing protein	AT3G28958.1	10984327-AGTGATTTTTATTTC-10984341	+	10983943–10984769 bp forward
	Hydrolase, hydrolyzing O-glycosyl compounds	AT3G47010.1	17330239-TGTGATTTTTATTTT-17330253	+	17327638–17330516 bp reverse
	AtEB1a (Q7XJ60_ARATH)[a]	AT3G47690.1	17593782-AGAGACTTTTACTTC-17593796	+	17592081–17594571 bp forward
	DEL1 (DP-E2F-like 1)	AT3G48160.2	17794924-TGTGATTTTTATTTT-17794938	+	17794316–17797084 bp forward
	NHX2 (sodium proton exchanger 2)	AT3G05030.1	1394654-AAAATAAAAGTCTCA-1394668	-	1393304–1396790 bp reverse

Table 5. (Resume)

	Protein	TAIR	Location of consensus (bp)	+/-	Gene location (bp)
Chromosome IV	ATDMC1 (RECA-LIKE GENE)	AT3G22880.1	8100076-AAAATAAAAGTCCCG-8100090	-	8097694–8100827 bp reverse
	Aspartyl-tRNA synthetase	AT4G31180.1	15159438-AGAGATTTTTACTTC-15159452	+	15155885–15159595 bp forward
	PIP3 (Plasma membrane Intrinsic Protein 3)	AT4G35100.1	16710088-AGTGACTTCTATTTC-16710102	+	16708603–16710253 bp forward
	F-box family protein (FBL21)	AT4G05470.1	2765506-GAAATAGAAGTCACG-2765520	-	2763253–2767954 bp reverse
	Phospholipase D gamma 2	AT4G11830.2	7116371-GAAGTAGAAGTCTCT-7116385	-	7115788–7121239 bp reverse
	PLDGAMMA3 (phospholipase D gamma 3)	AT4G11840.1	7122538-GAAGTAGAAGTCTCT-7122552	-	7121959–7125876 bp reverse
	MATE efflux family protein	AT4G21910.2	11628400-AAAATAAAAATCCCA-11628414	-	11625374–11629349 bp reverse
	AGL24 (AGAMOUS-LIKE 24); transcription factor	AT4G24540.1	12673358-AAAATAAAAATCTCA-12673358	-	2412670980–12674082 bp reverse
Chromosome V	Splicing factor, putative	AT4G38780.1	18103648-AAAATAAAAATCACA-18103662	-	18101290–18111023 bp reverse
	CER3 (ECERIFERUM 3)	AT5G02310.1	480344-TGGGATTTCTATTTT-480358	+	474277–482781 bp forward
	Basic helix-loop-helix (bHLH) family protein	AT5G48560.1	19702997-TGAGACTTTTATTTT-19703011	+	19701232–19704377 bp forward
	Zinc finger (C3HC4-type RING finger) family protein	AT5G62460.1	25092689-TGAGATTTTTATTTT-25092703	+	25092109–25094520 bp forward
	AtEB1b (Q9FJJ5_ARATH)[a]	AT5G62500	25111878-AGAGATTTTTACTTC-25111892	+	25109867–25112470 bp forward
	LRP1 (Lateral Root Primordium 1)	AT5G12330.2	3989374-AAAATAAAAGTCACA-3989388	-	3987376–3989755 bp reverse
	KAN (KANADI); transcription factor	AT5G16560.1	5407756-AAAATAAAAATCACT-5407770	-	5406866–5411427 bp reverse
	DCL4 (DICER-LIKE 4)	AT5G20320.1	6861135-AAAATAGAAATCTCT-6861149	-	6859220–6869268 bp reverse
	Nucleotide binding	AT5G50230.1	20466963-AAAATAAAAGTCCCT-20466977	-	20465596–20468107 bp reverse
	AtEB1c (Q9FGQ6_ARATH)[a]	AT5G67270	26857871-GAAATAGAAATCTCT-26857885	-	26857298–26859307 bp reverse

[a]Known homologues of animal MAP1: AtEB1a, AtEB1b, and AtEB1c.

TABLE 6. Regions of the *A. thaliana* genome with consensus to the HGNGAYTTYTAYTTY[a] – motif.

No.	Localization of consensus		Product description	Clone/gene name	GenBank accession	Comment
Chromosome I						
1.	523183-CGAGATTTTTATTTT-523197	(+)	Unknown product	T14P4	AC022521	Host - *Escherichia coli*
2.	699950-CGAGATTTTTATTTT-699965	(+)	-//-	F10O3	AC006550	-//-
3.	1057427-AGAGATTTTTATTTC-1057441	(+)	-//-	F20D22.17	GeneID: 839298	AT1G04090.1. Host - *Escherichia coli*
4.	1861198-CGTGACTTTTACTTC-1861198	(+)	-//-	T21E18	AC024174	Host - *Escherichia coli*
5.	4766372-TGAGATTTTTATTTT-4766386	(+)	Unknown product	F16A14	AC068197	-//-
6.	11023767-AGAGATTTCTACTTT-11023781	(+)	-//-	F17F8	AC000107	-//-
7.	12037927-AGCGATTTCTATTTT-12037941	(+)	-//- -//-	T9L6 T16O9	AC021045 AC027035	-//- -//-
8.	13265431-AGTGATTTTTACTTT-13265445	(+)	-//-	F14D7	AC021198	-//-
9.	16286591-TGTGACTTTTACTTT-16286605	(+)	Hypothetical protein	F1I21.20	NM_103471	AT1G43200.1. Host - *Escherichia coli*
10.	16425675-AGAGATTTTTATTTT-16425689	(+)	Unknown product	T10P12	AC007203	Host - *Escherichia coli*
11.	16448566-AGTGATTTTTATTTT-16448580	(+)	Unknown product	-	-	No data
12.	16503190-AGAGATTTTTATTTT-16503204	(+)	Unknown product	F2J6	AC009526	Host - *Escherichia coli*
13.	18247626-AGAGATTTCTATTTT-18247640	(+)	-//-	F13F21	AC007504	-//-
14.	19098150-TGGGATTTCTATTTT-19098164	(+)	-//-	F5D21	AC024261	-//-
15.	19308381-TGTGATTTTTACTTC-19308395	(+)	-//-	T14L22	AC015448	-//-
16.	20713719-AGCGATTTCTACTTT-20713733	(+)	-//-	-	-	No data
17.	20921679-AGTGATTTCTACTTC-20921693	(+)	Unknown protein	F14J16	NP_001052872.1	AT1G55928.1
18.	22882104-AGAGATTTTTACTTT-22882118	(+)	Unknown product	F8K4	AC004392	Host - *Escherichia coli*
19.	23482814-CGTGATTTTTATTTT-23482828	(+)	-//-	F9N12	AC022355	-//-
20.	23688674-TGTGATTTTTATTTT-23688688	(+)	-//-	T12P18	AC010852	(20921136–20922032 bp forward)
21.	25297389-AGCGACTTCTATTTC-25297403	(+)	-//-	T1F15 and F12B7	AC004393	Host - *Escherichia coli*
22.	25662846-TGCGATTTTTATTTT-25662860	(+)	Unknown protein	T2E12.1	NM_105515	AT1G68440.1
23.	1343822-AAAATAAAAGTCACA-1343836	(-)	Unknown product	F13M7	AC004809	Host - *Escherichia coli*
24.	1355568-AAAATAAAAATCTCA-1355582	(-)	-//-	-//-	-//-	-//-
25.	1849923-AAAATAAAAATCACA-1849937	(-)	-//-	T21E18	AC024174	-//-
26.	2203072-AAAATAAAAATCCCA-2203086	(-)	-//-	F10K1	AC067971	-//-
27.	2488172-GAAATAAAAATCTCT-2488186	(-)	-//-	T6D22	AC026875	-//-
28.	4669940-GAAGTAAAAGTCCCT-4669954	(-)	-//- -//-	F13B4 F21F23	AC027134 AC027656	-//- -//-
29.	5356886-AAAATAAAAATCTCT-5356900	(-)	-//-	T16N11	AC013453	-//-
30.	6682542-AAAATAAAAATCACT-6682556	(-)	Similar to unknown protein	F18O14.5	NM_101790 NM_001084092	AT1G19330.1 (TAIR:AT1G75060.1)
31.	9115920-GAAGTAAAAATCACA-9115934	(-)	Unknown product -//-	F28B23 T1K7	AC079829 AC013427	Host - *Escherichia coli* -//-
32.	10826603-AAAATAAAAATCACA-10826617	(-)	-//-	T5I8	AC007060	-//-
33.	12694512-AAAATAAAAATCACT-12694526	(-)	-//-	F12K21 F21H2	- -	-//- -//-
34.	15952376-AAAATAAAAATCACA-15952391	(-)	-//-	T8D8	AC025815	-//-
35.	16185539-GAAATAGAAATCGCT-16185553	(-)	-//-	F2H10	AC026757	-//-
36.	18247693-AAAATAGAAATCTCT-18247707	(-)	-//-	F13F21	AC007504	-//-
37.	18263799-AAAGTAAAAGTCGCT-18263813	(-)	-//-	-//-	-//-	-//-
38.	18987224-AAAATAAAAATCCCT-18987238	(-)	-//-	F11M15	AC006085	-//-
39.	21691952-AAAATAAAAGTCTCT-21691966	(-)	-//-	F19C14	AC008051	-//-
40.	22490330-AAAATAAAAATCTCT-22490344	(-)	-//-	T7P1	AC018908	-//-
41.	22544516-AAAATAAAAATCGCG-22544530	(-)	-//-	F11P17	AC002294	-//-
42.	23103959-AAAATAAAAATCTCT-23103973	(-)	-//-	NC_003070	NM_001084290	AT1G62422.1. Host - *Escherichia coli*
43.	23130489-AAAATAGAAATCTCA-23130503	(-)	-//-	T3P18	AC005698	Host - *Escherichia coli*
44.	25696987-GAAATAAAAATCTCA-25697001	(-)	-//-	T26J14	AC011915	-//-
45.	29494803-AAAGTAGAAATCGCA-29494817	(-)	-//-	F3F9	AC013430	-//-
Chromosome II						
1.	1412242-TGTGACTTCTATTTT-1412256	(+)	Unknown product	T16B23	AC007293	Host – *Escherichia coli*
2.	4311778-TGTGATTTTTATTTT-4311792	(+)	-//-	F16G22	AC007261	-//-
3.	5984630-TGAGATTTTTACTTT-5984644	(+)	-//-	-	-	No data
4.	6090877-AGAGATTTTTATTTT-6090891	(+)	-//-	T1O16	AC006304	-//-
5.	7197790-AGAGATTTCTATTTT-7197804	(+)	-//-	F1P15	AC007195	-//-
6.	7259771-AGAGATTTTTATTTC-7259785	(+)	-//-	T24I21	AC005825	-//-
7.	7444667-AGAGATTTTTATTTT-7444681	(+)	-//-	F6P23	AC002354	-//-
8.	7856717-AGAGACTTTTACTTT-7856731	(+)	-//-	T27K22	AC006201	-//-

Table 6. (Resume)

No.	Localization of consensus		Product description	Clone/gene name	GenBank accession	Comment
9.	11693949-AGTGATTTTTATTTT-11693983	(+)	Unknown product	F12K2	AC006233	No data
10.	12059934-AGCGATTTTTATTTC-12059948	(+)	-//-	T3B23	AC006202	-//-
11.	12366277-AGAGATTTTTATTTT-12366300	(+)	-//-	F8N16	AC005727	-//-
12.	13620829-TGTGATTTTTATTTC-13620843	(+)	-//-	F22D22	AC006223	-//-
13.	14798212-AGTGATTTCTACTTC-14798226	(+)	-//-	T4C15	AC004667	-//-
14.	16916825-TGTGACTTCTATTTT-16916839	(+)	-//-	T2P4	AC002336	-//-
15.	18333693-TGTGATTTCTATTTT-18333707	(+)	-//-	F4I1	AC004521	-//-
16.	18975599-AGAGATTTTTACTTT-18975613	(+)	-//-	T3F17	AC005397	-//-
17.	763140-AAAATAAAAATCTCT-763154	(-)	-//-	T20F6	AC002521	-//-
18.	2984444-AAAATAAAAATCTCT-2984458	(-)	-//-	T25N22	AC005693	-//-
19.	5011175-AAAATAGAAATCTCA-5011189	(-)	-//-	F24C20	AC007112	-//-
20.	6960785-AAAATAAAAATCTCA-6960799	(-)	-//-	F19G14 F7H1	AC006438 AC007134	-//- -//-
21.	7378124-AAAATAGAAATCCCT-7378138	(-)	-//-	F12A24	AC005167	-//-
22.	8690701-AAAATAAAAGTCACT-8690715	(-)	-//-	T2G17	AC006081	-//-
23.	10656416-AAAGTAAAAGTCACG-10656430	(-)	-//-	F27C12	AC006585	-//-
24.	10761831-AAAATAAAAATCTCA-10761845	(-)	-//-	T22F11	AC007070	Host – *Escherichia coli*
25.	11683592-GAAGTAGAAATCACT-11683606	(-)	-//-	AT2G27285.1	NM_128287	-
26.	13218168-AAAATAAAAATCTCA-13218182	(-)	-//-	F7F1 T16B12	AC004669 AC005311	-//- -//-
27.	13439579-GAAGTAGAAATCTCA-13439593	(-)	-//-	T9H9	AC007071	-//-
28.	13833500-AAAGTAGAAATCACA-13833514	(-)	-//-	T26B15	AC004681	-//-
29.	14013279-AAAGTAGAAGTCCCG-14013293	(-)	-//-	T21L14	AC003033	-//-
30.	18576375-AAAGTAAAAATCCCA-18576389	(-)	-//-	T14P1	AC007659	-//-
31.	18970109-AAAATAAAAATCTCT-18970123	(-)	-//-	T3F17	AC005397	-//-
	Chromosome III					
1.	496022-AGAGATTTTTATTTC-496036	(+)	Unknown product	F16B3	AC021640	Host – *Escherichia coli*
2.	3253167-TGAGATTTTTACTTT-3253181	(+)	-//-	F13M14	AC011560	-//-
3.	3285728-AGTGATTTTTATTTT-3285742	(+)	-//-	-//-	-//-	-//-
4.	3904541-AGAGATTTTTATTTT-3904555	(+)	-//-	F28J15	AC069472	-//-
5.	5293201-AGAGATTTCTATTTC-5293215	(+)	-//-	MSJ11	AB017071	-//-
6.	9435447-TGAGATTTCTATTTT-9435461	(+)	-//-	K13N2	AB028607	-//-
7.	11078763-AGAGATTTTTATTTT-11078777	(+)	-//-	MIXE2	AB018121	-//-
8.	11974183-AGAGATTTTTATTTT-11974197	(+)	-//-	T6J22	AP001314	-//-
9.	15450976-TGAGATTTTTATTTT-15450990	(+)	-//-	T18D12	AL138644	-//-
10.	15471961-AGTGATTTTTATTTT-15471975	(+)	-//-	F22J12	AL391734	-//-
11.	16948539-AGAGATTTTTATTTT-16948553	(+)	-//-	F12M12	AL355775	-//-
12.	21424405-AGAGATTTCTATTTT-21424419	(+)	-//-	T10K17	AL132977	-//-
13.	21953815-TGTGATTTTTATTTT-21953829	(+)	-//-	F25L23	AL356014	-//-
14.	853069-AAAATAGAAATCGCA-853083	(-)	Unknown protein	T21P5.2	NM_111227	AT3G03560.1
15.	2016060-AAAATAAAAATCTCG-2016074	(-)	Unknown product	F5E6	AC020580	Host – *Escherichia coli*
16.	2094796-AAAATAGAAATCCCT-2094810	(-)	-//-	T8E24	AC036106	-//-
17.	3598638-AAAATAAAAGTCCCG-3598652	(-)	-//-	F24K9	AC008153	-//-
18.	4580929-AAAGTAAAAGTCCCA-4580943	(-)	-//-	MDC16	AB019229	-//-
19.	6423326-GAAATAAAAATCCCA-6423340	(-)	-//-	MVE11	AB026654	-//-
20.	7634647-AAAATAAAAATCACA-7634661	(-)	-//-	MIL23	AB019232	-//-
21.	9408126-AAAGTAAAAGTCTCA-9408140	(-)	-//-	K13N2	AB028607	-//-
22.	11113236-AAAATAAAAATCTCT-11113250	(-)	-//-	MXE2	AB018121	-//-
23.	11819447-AAAATAAAAGTCTCA-11819461	(-)	-//-	T20F20	AP002060	-//-
24.	17337035-AAAATAAAAATCTCT-17337049	(-)	-//-	F13I12	NM_114571	AT3G47040.1
25.	18408551-GAAATAAAAATCACA-18408565	(-)	-//-	T9C5	AL132964	Host – *Escherichia coli*
26.	19698280-GAAGTAAAAATCTCT-19698294	(-)	-//-	T4D2	AL132958	-//-
27.	20775406-AAAATAGAAATCTCT-20775420	(-)	-//-	F27K19	AL163832	-//-
28.	21424438-AAAATAGAAATCTCT-21424452	(-)	-//-	T10K17	AL132977	-//-
29.	23141695-AAAATAAAAATCACT-23141679	(-)	-//-	T12C14	AL162507	-//-
	Chromosome IV					
1.	411616-TGCGACTTTTATTTT-411630	(+)	Unknown product	T18A10	AF013294	Host – *Escherichia coli*
2.	886175-AGAGATTTTTACTTT-886189	(+)	-//-	T10M13	AF001308	-//-
3.	3496105-AGAGATTTTTATTTC-3496119	(+)	-//-	T29P11	-	-//-
4.	5487696-TGTGACTTTTACTTT-5487710	(+)	Hypothetical protein	T3F12.9	GeneID: 3770175	AT4G08600.1. Host – *Escherichia coli*
5.	5565565-TGTGATTTCTATTTT-5565579	(+)	Unknown product	T32A17	AL161813	Host – *Escherichia coli*
6.	8303903-TGAGATTTTTATTTC-8303917	(+)	-//-	-	-	Record TAIR Not Found
7.	10161055-AGAGACTTTTATTTT-10161069	(+)	-//-	F28J12	AL021710	Host – *Escherichia coli*
8.	10171263-AGAGATTTTTATTTT-10171277	(+)	-//-	-//-	AL021710	-//-
9.	10747374-TGGGATTTTTATTTT-10747388	(+)	-//-	T16H5	AL024486	-//-
10.	11702125-TGCGATTTCTACTTT-11702139	(+)	-//-	-	-	No data
11.	12197236-TGAGATTTTTATTTT-12197250	(+)	-//-	F21P8 F16G20	AL022347 AL031326	Host - *Escherichia coli* -//-
12.	12494672-AGTGATTTTTACTTT-12494686	(+)	-//-	T19F6	AC002343	-//-
13.	12758805-TGAGACTTTTACTTT-12758819	(+)	-//-	F6I7	AL049657	-//-
14.	13578463-AGTGACTTCTATTTT-13578477	(+)	-//-	F10M23	AL035440	-//-

Table 6. (Resume)

No.	Localization of consensus		Product description	Clone/gene name	GenBank accession	Comment
15.	14672138-TGAGACTTTTATTTC-14672152	(+)	Unknown product	F6G3	AL078464	Host - *Escherichia coli*
16.	15959767-TGAGACTTCTATTTT-15959781	(+)	-//-	F26P21 F4I10	AL031804 AL035525	-//-
17.	17599858-TGAGATTTTTATTTT-17599872	(+)	-//-	F6G17	AL035601	-//-
18.	18076216-CGTGATTTTTATTTC-18076230	(+)	-//-	F20M13 T9A14	AL035540 AL035656	-//-
19.	1586031-AAAATAGAAATCTCT-1586045	(-)	-//-	F9H3 T5L23	AF071527 AC005142	-//-
20.	3528575-AAAGTAAAAGTCACA-3528589	(-)	Hypothetical protein	T24C1	GeneID: 3769930	Pseudogene: AT4G06571.1
21.	4342950-AAAGTAAAAGTCACA-4342964	(-)	Unknown protein	AT4G07520.1	NM_116825	4341583–4345234 bp reverse
22.	5357747-AAAATAAAAATCTCA-5357761	(-)	Unknown product	C18G5	AL110116	Host - *Escherichia coli*
23.	5562737-AAAATAAAAATCACA-5562751	(-)	-//-	T3F12 T32A17	AC002983 AL161813	-//- -//-
24.	6235094-AAAATAGAAATCTCT-6235108	(-)	-//-	-	-	No data
25.	6355873-AAAATAAAAATCACT-6355887	(-)	-//-	-	-	No data
26.	7230548-AAAGTAAAAATCTCT-7230562	(-)	-//-	F16J13	AL049638	-//-
27.	11176998-GAAATAAAAATCTCA-11177012	(-)	-//-	T13K14	AL080282	-//-
28.	14529900-AAAATAAAAGTCTCA-14529914	(-)	-//-	T16L4	AL079344	-//-
29.	15860451-AAAATAAAAATCTCT-15860465	(-)	-//-	T16I18	AL049915	-//-
30.	15891876-AAAATAGAAATCGCA-15891890	(-)	Glycine-rich protein	AT4G32920.1	NM_119446	15887578–15896009 bp reverse
	Chromosome V					
1.	675728-TGAGACTTTTATTTT-675742	(+)	Unknown product	F9G14	AL162973	Host - *Escherichia coli*
2.	841914-TGTGATTTTTATTTC-841928	(+)	-//-	F12E4	AL162751	-//-
3.	912111-TGAGATTTTTATTTT-912125	(+)	-//-	F17C15	AL162506	-//-
4.	7231365-TGAGATTTTTATTTT-7231379	(+)	-//-	F13M11	-	-//-
5.	7276681-AGAGATTTTTATTTT-7276695	(+)	-//-	T6G21	AL090689	-//-
6.	7453281-CGGGATTTCTATTTT-7453295	(+)	-//-	-	-	No data
7.	8556715-AGAGATTTTTATTTT-8556729	(+)	-//-	F6A4	AF069716	Host - *Escherichia coli*
8.	9564549-TGTGACTTTTACTTT-9564563	(+)	Unknown protein	AT5G27160.1	NM_122598	9562599–9565844 bp forward
9.	13679796-TGAGATTTTTACTTT-13679810	(+)	Unknown protein	AT5G35430.1	NM_122934	TPR domain's containing protein
10.	17011117-AGAGATTTCTATTTT-17011131	(+)	Unknown product	MDH9	AB016888	Host - *Escherichia coli*
11.	18743470-AGAGATTTTTACTTT-18743484	(+)	-//-	MCL19	AB006698	-//-
12.	19711095-CGTGATTTTTATTTT-19711109	(+)	-//-	K15N18	AB015468	-//-
13.	21105672-AGAGATTTTTATTTT-21105686	(+)	-//-	MIO24 MJM18	AB010074 AB025623	-//- -//-
14.	22517887-AGTGACTTTTATTTT-22517901	(+)	-//-	MTE17	AB015479	-//-
15.	22591040-TGTGATTTTTATTTT-22591054	(+)	-//-	MDF20	AB009050	-//-
16.	22595972-TGTGACTTTTATTTT-22595986	(+)	-//-	-	-	-//-
17.	24292016-AGAGACTTTTATTTT-24292030	(+)	-//-	K9B18	AB015471	-//-
18.	24721664-AGAGATTTCTATTTC-24721678	(+)	-//-	MFB13	AB010073	-//-
19.	24901767-AGAGATTTTTATTTT-24901781	(+)	-//-	-	-	No data
20.	3861664-AAAATAAAAATCTCT-3861678	(-)	-//-	F14F18	AL163812	Host - *Escherichia coli*
21.	4219700-AAAATAAAAATCACT-4219714	(-)	-//-	-	-	No data
22.	4493336-AAAATAAAAGTCTCA-4493350	(-)	-//-	MAC12	AB005230	Host - *Escherichia coli*
23.	5301071-GAAATAGAAATCTCA-5301085	(-)	-//-	T21H19	AL391148	-//-
24.	5674222-GAAATAAAAATCGCT-5674236	(-)	-//-	MKP11	AB005238	-//-
25.	6372927-AAAATAAAAATCGCA-6372941	(-)	-//-	-	-	No data
26.	7412019-AAAATAAAAATCTCG-7412033	(-)	-//-	MWD9	AB007651	Host - *Escherichia coli*
27.	7606034-AAAGTAGAAGTCTCT-7606048	(-)	-//-	K8E10 MRN17	AB025618 AB005243	-//- -//-
28.	8413734-AAAGTAGAAATCACG-8413748	(-)	-//-	K18P6	AB010068	-//-
29.	8934601-GAAGTAGAAATCCCA-8934615	(-)	-//-	T14C9	AC006601	-//-
30.	10067367-AAAATAGAAATCTCT-10067381	(-)	-//-	F15F15	AC007627	-//-
31.	11150650-AAAATAAAAATCCCA-11150664	(-)	-//-	F23C8	AC018928	-//-
32.	13032838-GAAATAAAAATCGCA-13032852	(-)	-//-	T3J11 F11P10	AC019012 AC018660	-//- -//-
33.	14418264-AAAATAAAAATCCCA-14418278	(-)	-//-	F24C7	AP002029	-//-
34.	14458474-AAAATAAAAATCACT-14458488	(-)	-//-	-//-	-//-	-//-
35.	14477563-AAAATAAAAATCCCA-14477577	(-)	-//-	F24C7 MPK17	AP002029 AP000418	-//- -//-
36.	14517774-AAAATAAAAATCACT-14517788	(-)	-//-	F5H8	AB025605	-//-
37.	15062396-GAAATAAAAATCTCT-15062410	(-)	-//-	K22F20	AB016873	-//-
38.	15396183-AAAATAAAAATCTCT-15396197	(-)	-//-	MXI10 RBCS-B	AB005248 -	Host - *Escherichia coli* RBCS-B (Genetic Marker)
39.	17942353-GAAGTAAAAATCTCA-17942367	(-)	-//-	-	-	No data
40.	18697236-GAAGTAAAAATCTCA-18697250	(-)	-//-	MCL19	AB006698	Host - *Escherichia coli*
41.	24703875-AAAATAAAAATCTCA-24703889	(-)	-//-	MFB13	AB010073	-//-
42.	25152219-AAAATAAAAATCTCA-25152233	(-)	-//-	K19B1 MRG21	AB015469 AB020751	-//- -//-
43.	26847777-AAAATAAAAATCACA-26847791	(-)	-//-	K21H1	AB020742	-//-

[a] R=A or G, Y=C or T, H=A, C or T, N=A, C, G or T

TABLE 7. Conserved motifs of animal MAP2 and tau proteins and their coding regions in chromosomes of *A. thaliana* located in regions of unknown products of translation.

No.	Conservative motive	Localization of motif in animal MAPs	Coding sequence[a]	Consensus sequence location	Clone/gene name	GenBank accession	Product description
Chromosome I							
1.	HGAEI	C-terminal (after tubulin binding domain)	CAYGGNGCNGARATH	8178690-AATTTCCGCCCCATG-8178704 (-)	T26J12	AC002311	Unknown product
2.				11468654-GATTTCTGCCCCGTG-11468668 (-)	T12O21	AC074309	-//-
3.	HVPGG	TBR4	CAYGTNCCNGGNGGN	11224493-GCCACCGGGCACGTG-11224507 (-)	T19E23	AC007654	-//-
4.				17290759-ACCTCCAGGAACGTG-17290773 (-)	F2G19 F8G22	AC083835 AC079677	-//- -//-
Chromosome II							
5.	AKQGL	C-terminal	GCNAARCARGGNCTN	2920426-CAGCCCCTGTTTCGC-2920440 (-)	T4E14	AC005171	Unknown product
6.	HGAEI	C-terminal (after tubulin binding domain)	CAYGGNGCNGARATH	345472-CACGGTGCTGAAATC-345499 (+)	T8O11	AC006069	-//-
7.				12996204-GATCTCTGCACCGTG-12996218 (-)	AT2G30480	NM_128600 NM_001084512 NM_201836	Unknown protein
8.				16022087-AATTTCGGCGCCGTG-16022101 (-)	F16M14	AC003028	Unknown product
Chromosome III							
9.	AKQGL	C-terminal	GCNAARCARGGNCTN	1951612-GCAAAACAGGGTCTA-1951626 (+)	F24P17	AC011623	Unknown product
10.				10793372-GAGACCTTGTTTAGC-10793386 (-)	AT3G28750	NM_113796	Unknown protein
Chromosome IV							
11.	AKQGL	C-terminal	GCNAARCARGGNCTN	12073199-TAGGCCTTGCTTTGC-12073213 (-)	-	-	Unknown product
12.				15708584-AAGCCCCTGTTTGGC-15708598 (-)	-	-	-//-
13.				16329496-CAGTCCCTGCTTAGC-16329510 (-)	AT4G34090	NM_202949 NM_119570	Unknown protein
14.	HVPGG	TBR4	CAYGTNCCNGGNGGN	11599184-ACCACCAGGGACATG-11599198 (-)	T8O5	AL021890	Unknown product
15.	HGAEI	C-terminal (after tubulin binding domain)	CAYGGNGCNGARATH	9390823-CACGGGGCTGAGATT-9390837 (+)	FCAALL	-	-//-
16.				5986315-TATTTCAGCACCGTG-5986329 (-)	T15G18	AC006567	Unknown protein
Chromosome V							
17.	AKQGL	C-terminal	GCNAARCARGGNCTN	11975335-AAGCCCTTGTTTAGC-11975349 (-)	F14C23	AF296828	Unknown product
18.				12073199-TAGGCCTTGCTTTGC-12073213 (-)	F18O9	AF296831	-//-
19.				15708584-AAGCCCCTGTTTGGC-15708598 (-)	K3K3	AB010694	-//-
20.				16329496-CAGTCCCTGCTTAGC-16329510 (-)	MNF13	AB009052	-//-
21.	HGAEI	C-terminal (after tubulin binding domain)	CAYGGNGCNGARATH	8716162-TATTTCAGCACCATG-8716176 (-)	F21J6	AC006259	-//-
22.				20475867-TATCTCAGCACCATG-20475881 (-)	-	-	Unknown product

[a]R=A or G, Y=C or T, H=A, C or T, N=A, C, G or T

unknown genes of structural MAPs. So, scanning the *A. thaliana* genome, with help of the TBLASTN programme, revealed only three previously known homologs of MAP1 (AtEB1a, AtEB1b, AtEB1c).

This leads to the assumption that if new homologs of animal MAP1 will be detected within the predicted genome regions, their sequences will differ considerably from that of typical animal and human MAPs. In that case, using SMART, Pfam, PROSITE, etc. running sequence comparison algorithms (with the databases of homological sequences, patterns and HMMs) created for animal homologs, may be ineffective. Nevertheless, slight sequence homology/identity does not exclude the existence strong of functional homology, which in turn may be supported exclusively by analytical methods.

In the case of Type II MAPs we assume the possibility of existence of some proteins, functionally similar to MAP2 (Table 7). At the same time, if plant homologues do exist, the sequences similarity will be very low (supported by the absence of positive results under TBLASTN search). In the case of MAP4, the absence of positive results using TBLASTN and in the search for conserved consensus motifs (substituting alternative IUPAC symbols) confirm the results of others[62] concerning the absence of MAP4 in plants. In any case, if plant homologues of Type II animal MAPs do exist, their similarity will be very weak. So, the divergence probably took place at a very early stage in their evolution.

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PART 7

APPLIED BIOLOGY/BIOTECHNOLOGY

SPATIAL DISTRIBUTION OF TUBULIN MUTATIONS CONFERRING RESISTANCE TO ANTIMICROTUBULAR COMPOUNDS

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Abstract. Resistance to antimicrotubular drugs results from single amino acid replacements in α- and β-tubulin subunits. Two possible mechanisms of action of these replacements are proposed based on analyses of their spatial distribution in the three-dimensional protein model. The main mechanism of action is typical for mutations that are localized in the immediate proximity of binding sites for antimicrotubular drugs. In this case, amino acid replacements can directly influence binding site spatial structure, and result in decreased protein affinity causing resistance only to compounds binding at this site. Mutations that cause multidrug resistance can have an alternative mechanism of action. Spatial distribution of these mutations does not correlate with the ligands' binding sites. One may assume that they effect global changes in the tubulin molecule (e.g., increasing or decreasing the general level of molecular oscillations). Therefore, theses mutations can determine either nonspecific resistance to a number of different microtubule depolymerising agents and, simultaneously, hypersensitivity to microtubule stabilizing compounds, or *vice versa*.

Keywords: Antimicrotubular compounds, tubulin, resistance, mutations, mechanisms of action

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1. Introduction

The ability of tubulin molecules to interact specifically with low molecular-weight compounds is one of their important features.[1–3] Such interactions can induce two different types of disturbances in microtubule systems. In the first case, low molecular-weight compounds bind to tubulin monomers or dimers and prevent their assemblage into microtubules or MTOCs. Microtubules are in a state of dynamic instability in living cells, such that they are in a constant assembling/disassembling processes. Because monomers and dimers of tubulin bound with low molecular-weight compounds are incapable of participating in polymerization, the process shifts to microtubules dissociation, which leads to the disruption of interphase microtubular arrays and/or the mitotic spindle. Compounds with depolymerizing mechanism of action include substances that interact with the colchicine binding site (e.g., colchicine, colcemid, podophyllotoxin, combretastatins, steganacin),[2] with the vinblastin binding site (e.g., vinblastine, vincristine, vineralbine, hemiasterlin),[2] and also with the binding sites for dinitroanilines and phosphoroamidates,[4] the benzimidazoles and phenylcarbamates,[5–7] and many others. In the second case, ligands bind to microtubules and cause their irreversible stabilization. Taxanes, epothilones, lolimalides, discodermolide and eleuterobin are compounds with stabilizing mechanism of action.[1–3]

Research efforts focussing on the mechanisms that explain the specific interaction between tubulins and low molecular-weight compounds has significant practical importance. In particular, α- and β-tubulin subunits are the cell targets for many commercially significant compounds used as herbicides or fungicides, as well as antitumor, antihelminth and antiprotozoan drugs.[3,4,8,9] Understanding these mechanisms of interactions make it possible to develop new chemical compounds possessing antimicrotubular mechanism of action. Conversely, this knowledge will allow the construction of modified tubulin molecules resistant to antimicrotubular compounds. Generally, resistance to antimicrotubular compounds is a result of point mutations that occur in α- or β-tubulin molecules.[4,8,9]

It's reasonable to suggest that the distribution of ligand binding sites and the location of mutations conferring resistance to antimicrotubular drugs are characterized by certain regularities. Our previous investigations showed that mutations, which result in increased resistance to tubulin effectors, had a non-random arrangement with regard to the amino acid sequence and spatial structure of tubulins.[10] Positions of amino acid substitutions, which cause resistance to microtubule depolymerising compounds, are located in protein sequences near the longitudinal inter- or intradimer contact surfaces of tubulin, but do not coincide with them. This phenomenon can be explained because all known binding sites for these compounds are located

directly on the longitudinal surfaces of α- or β-tubulins, and if mutations increasing resistance to these drugs occurred at those specific residues, they could negatively impact normal heterodimer formation or their polymerization. We also revealed that mutations conferring resistance to microtubule stabilizing agents are distributed on the lateral contact interfaces between the protofilaments, and the corresponding binding sites are located on the surface of the lateral contacts.[10] However, recent and important results on the mutations inducing resistance to antimicrotubular compounds led us to new investigations and additional conclusions.

2. General distribution pattern of "resistant" mutations

Accordingly to accumulated data, 61 positions of amino acid substitutions result in resistance of microtubules to low molecular-weight ligand effectors. Twenty-seven positions are located in the α-subunit, and 34 in the β-subunit (Tables 1–5, Figures 1 and 2). So, the two α-tubulin mutations demonstrated for goosegrass[11,12] can no longer be considered unique.

However, one should mention that the number of β-tubulin mutations is still higher than number of α-tubulin mutations. An explanation for this phenomenon can be related to the differential ability of α- and β-tubulins to bind different compounds. Among all antimicrotubular compounds, the ability to bind α-tubulin is proven only for dinitroanilines, phosphoramidates and cyanoacrylates (and assumed for hemiasterlin). However, practically all antimicrotubular compounds, including dinitroanilines and phosphoramidates, are able to interact with β-tubulin.

Some of the positions are characterized by having more than one amino acid substitutions. In particular, these are positions 198, 200, 215, 220, 292 and 350 in β-tubulin and position 165 in α-tubulin. For example, substitutions in position 220 of β-tubulin can induce resistance to microtubule depolymerising compounds as well as to microtubule stabilising compounds. Therefore, mutations at position 220 (i.e., $P_{220}\rightarrow$C or S or T) result in microtubule resistance to colcemid and vinblastine with simultaneous hypersensitivity to taxanes and epotilones. Conversely, the changes $P_{220}\rightarrow$L or V cause resistance to taxanes and epotilones and hypersensitivity to colcemid and vinblastine.[13] Additionally there is the mutation $A_{254}\rightarrow$V in β-tubulin that compensates for the mutation $D_{45}\rightarrow$Y and increases the tubulin subunit's affinity for colcemid.[14]

The most popular experimental objects for investigating mutations causing resistance to antimicrotubular compounds are various human tumour cell lines, plant pathogenic fungi, and certain model organisms. To date, 38 positions of "resistance" mutations are described for animal tubulins (nine in α- and 29 in β-tubulin), 10 mutable positions in fungal

tubulins (β-subunit only), six positions in plant tubulins (4 in α- and 2 in β-tubulins), and 17 positions in protozoan tubulins (α-subunit only). Not surprisingly, some of the amino acid substitution sites are located in identical positions for representatives of the different species investigated. Thus the total number of mutable positions is lower than the number of mutations.

The distribution of tubulin mutations generally correlates with our previous data.[10] However, we need to revise a postulate about the conserved nature of mutable positions. Among the 27 α-tubulin substitution positions only two positions (i.e., 28 and 239) contain the same amino acids and eight positions (i.e., 8, 52, 231, 252, 275, 378, 383, 384) contain similar amino acids. Among the 34 positions in β-tubulin, seven positions (i.e., 102, 134, 215, 220, 228, 238, 250) contain the same amino acids and nine positions (i.e., 167, 173, 234, 237, 240, 254, 288, 292, 364) exhibit a different degree of conservatism. It should be mentioned that the existence of resistance mutations to in nonconservative positions cannot be fully explained without additional information regarding the amino acid microenvironment of each mutant residue. It was also revealed that mutations can occur in the N-terminal region of both α- (position 12) and β-tubulins (positions 26, 45 and 55). These substitutions are not situated in sequences in or adjacent to monomer or dimer contact surfaces as with many other mutations. Rather, they are located near longitudinal contact surface (as mentioned earlier).

Among the identified mutation positions, 41 results in resistance to depolymerising compounds and 15 leads to resistance to stabilizing compounds. Interestingly, three positions can have substitutions causing resistance to both types of drugs (198, 220 and 350), testifying to the exceptional significance of these amino acids in tubulin functionality. Most of the mutations causing resistance to depolymerising compounds are located in sequences no further than six amino acids from longitudinal contact surfaces. Amino acids that directly form other longitudinal contact interfaces are very rarely subjected to substitutions (e.g., positions α221, β220, β224, β250). This could be explained because such substitution could not only block drug binding but also disturb tubulin subunit interactions, resulting in the formation of abnormal tubulin complexes. On the other hand, the proximity of replacement positions, conferring resistance to microtubule depolymerising compounds, to positions of amino acids forming longitudinal contact surfaces allows one to assume that the binding sites for these compounds are located either near-by these contact surfaces or directly on them.

Mutations causing resistance to microtubule stabilizing compounds don’t display strong arrangements of their localization in the primary structure, which also correlates with previous results.[10] However, one should note that some of these mutations coincide with positions of amino

TABLE 1. Mutations in animal tubulins causing resistance to microtubule depolymerising compounds.

Mutation	Tubulin subunit	Effects of mutation	Species	Reference
S_{12}→A	α	Resistance to hemiasterlin	*Homo sapiens*	15
D_{45}→Y	β	Resistance to colcemid and vinblastine	*Cricetulus griseus*	16
E_{55}→K	α	Resistance to colcemid and vinblastine	*C. griseus*	16
S_{165}→P	α	Resistance to hemiasterlin	*C. griseus*	17
F_{167}→Y	β	Resistance to benzimidazoles	*Cylicocyclus nassatus*	18
S_{172}→A	β	Resistance to hemiasterlin	*H. sapiens*	17
D_{197}→N	β	Resistance to 2-methoxyestradiol	*H. sapiens*	19
E_{198}→A	β	Resistance to benzimidazoles Hypersensitivity to phenylcarbamates	*Haemonchus contortus*	20, 21
F_{200}→Y	β	Resistance to benzimidazoles	*Teladorsagia circumcincta* *H. contortus* *C. nassatus*	6 20, 21 18
C_{211}→F	β	Resistance to colcemid and vinblastine	*C. griseus*	16
P_{220}→C /S /T	β	Resistance to colcemid and vinblastine Hypersensitivity to taxanes and epothilones	*C. griseus*	13
R_{221}→H	α	Resistance to hemiasterlin	*H. sapiens*	17
D_{224}→N	β	Resistance to colcemid and vinblastine	*C. griseus*	16
S_{234}→N	β	Resistance to colcemid and vinblastine	*C. griseus*	16
L_{240}→I	β	Resistance to Vinca alkaloids	*H. sapiens*	22
A_{254}→V	β	Reverse sensitivity to colcemid	*C. griseus*	14
H_{283}→Y	α	Resistance to colcemid and vinblastine	*C. griseus*	16
E_{288}→K	β	Resistance to benzimidazoles	*C. griseus*	23
K_{350}→N		Resistance to colcemid and vinblastine Resistance to 2-methoxyestradiol	*C. griseus* *H. sapiens*	16 19
A_{383}→V	α	Resistance to colcemid and vinblastine	*C. griseus*	16
I_{384}→V	α	Resistance to hemiasterlin	*H. sapiens*	17
R_{390}→C	α	Resistance to colcemid and vinblastine	*C. griseus*	16

TABLE 2. Mutations in plant tubulins causing resistance to microtubule depolymerising compounds.

Mutation	Tubulin subunit	Effects of mutation	Species	Reference
Y_{24}→H	α	Resistance to dinitroanilines	*Chlamydomonas reinhardtii*	22
L_{136}→F	α	Resistance to dinitroanilines	*Setaria viridis*	25
T_{239}→I	α	Resistance to dinitroanilines and phosphoramidates	*Eleusine indica*	11, 12
		Resistance to dinitroanilines	*S. viridis*	25
		Resistance to cyanoacrylates	*E. indica*	26
R_{241}→K	β	Resistance to dinitroanilines	*Poa annua*	27
M_{268}→T	α	Resistance to dinitoranilines and phosphoroamidates	*E. indica*	12
K_{350}→E /M	β	Cross-resistance to colchicine and dinitoranilines, hypersensitivity to taxanes	*C. reinhardtii*	28, 29

TABLE 3. Mutations in the α–tubulins of the protozoan *Toxoplasma gondii* conferring resistance to microtubule depolymerising compounds.[30]

Mutation	Effects of mutation on dinitroaniline resistance
H_8→Y	Resistance
H_{28}→G	Weak resistance
F_{52}→I	Weak resistance
/L	Weak resistance
L_{136}→F	Resistance
N_{139}→K	Weak resistance
S_{165}→P	Different resistance to dinitroanilines
/A	Different resistance to dinitroanilines
/T	Different resistance to dinitroanilines
I_{231}→T	Weak resistance
I_{235}→V	Weak resistance
L_{238}→V	Weak resistance
T_{239}-→I	Resistance
R_{243}→C	Resistance
/S	Resistance
V_{252}→L	Resistance
I_{275}→R	Weak resistance
A_{295}→V	Weak resistance
M_{301}→T	Resistance
I_{378}→M	Weak resistance
M_{391}→I	Weak resistance

TABLE 4. Mutations in fungal tubulins causing resistance to microtubule depolymerising compounds.

Mutation	Tubulin subunit	Effects of mutation	Species	Reference
N_{102}→V	β	Resistance to rhizoxin	*Saccharomyces cerevisiae*	31
/I			*Schizosaccharomyces pombe*	
			Candida albicans	
			Aspergillus nidulans	
Q_{134}→H	β	Resistance to benzimidazoles	*Beauveria bassiana*	32
A_{165}→V	β	Resistance to phenylcarbamates Hypersensitivity to benzimidazoles	*A. nidulans* *Neurospora crassa*	33– 35
F_{167}→Y	β	Resistance to benzimidazoles	*S. cerevisiae*	36
			N. crassa	37
/L			*B. bassiana*	32
E_{198}→A	β	Resistance to benzimidazoles Hypersensitivity to phenylcarbamates	*Botryotinia fuckeliana,* *Monilinia fructicola* *Helminthosporium solani*	38 39, 40 5
/Q			*Colletotrichum gloeosporioides* *N. crassa*	41
/K			*B. bassiana*	35
/G				32
F_{200}→Y	β	Resistance to benzimidazoles	*Botrytis cinerea*	42
T_{237}→A	β	Resistance to benzimidazoles and phenylcarbamates	*N. crassa*	35
L_{240}→F	β	Resistance to benzimidazoles	*Monilinia laxa*	43
R_{241}→H	β	Resistance to benzimidazoles	*S. cerevisiae*	44
L_{250}→F	β	Resistance to benzimidazoles and phenylcarbamates	*N. crassa*	35

acids forming lateral contact surfaces between protofilaments in microtubule (positions 217 and 292 of β-tubulin), or are located in close proximity to them (positions 215, 270, 274 of β-tubulin). A difference in distribution of mutations causing resistance to compounds with different mechanism of action can be correlated with the distribution of binding sites on the tubulin surface. Interdependence between spatial binding sites localization and mutations conferring resistance to the different compounds is considered below.

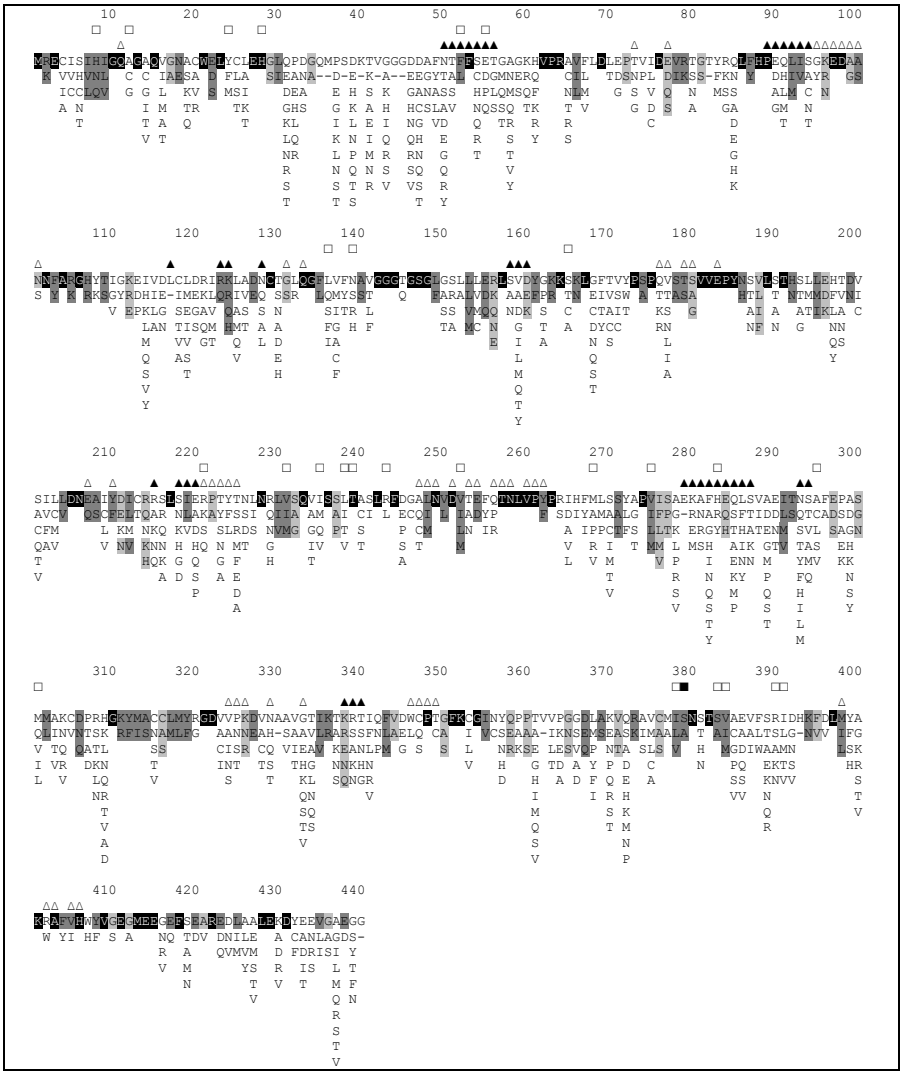

Figure 1. Localization of mutations conferring resistance to antimicrotubular compounds in α-tubulin protein sequences. □ – positions of microtubule depolymerising mutations, ■ – positions of microtubule stabilizing mutations, Δ – residues forming longitudinal (interdimer and intradimer) contacts, ▲ – residues forming lateral contacts, M – identical residues, R – high similar residues, G – low similar residues. Residues in variable positions are unhighlighted. Integral alignment of all full-length sequences of α-tubulin deposited in SwissProt databank[57] is computed with ClustalX software.[58] Image is prepared in text processor.

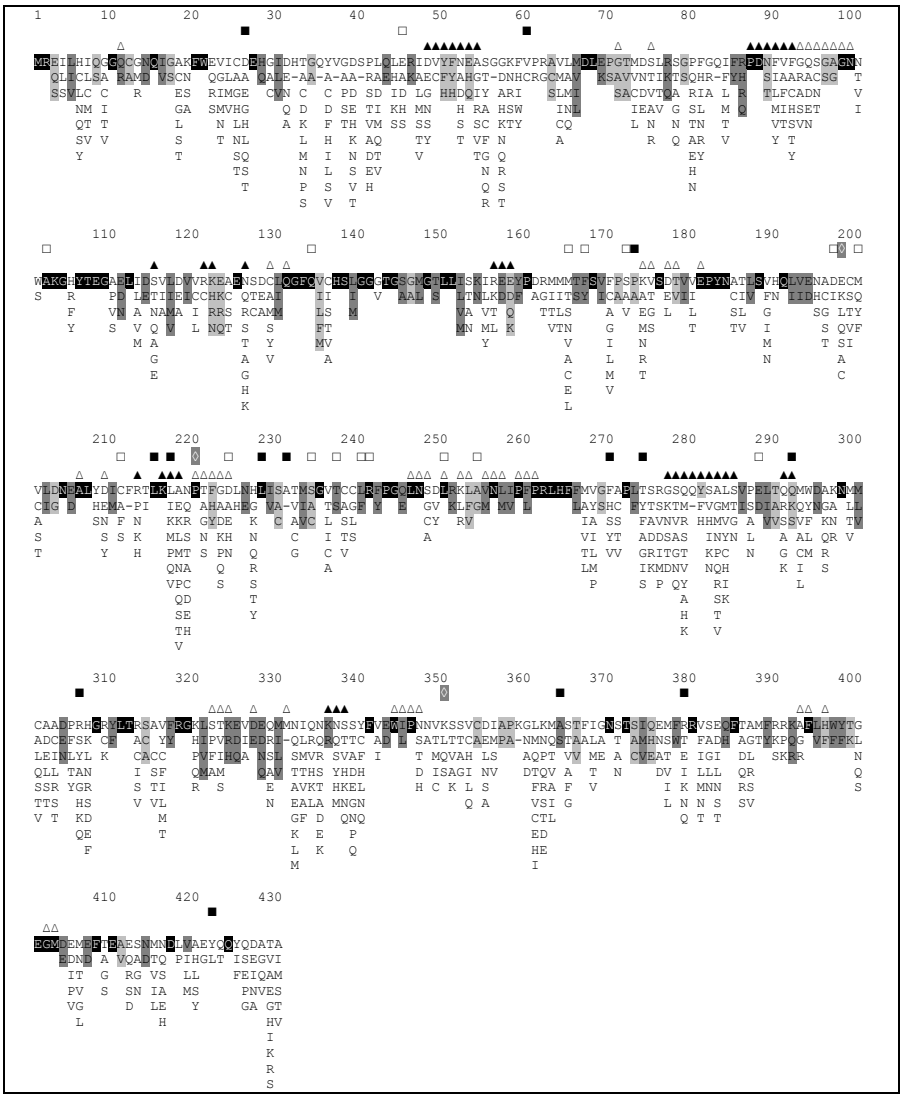

Figure 2. Localization of mutations conferring resistance to antimicrotubular compounds, in β-tubulin protein sequences: □ – positions of microtubule depolymerising mutations, ■ – positions of microtubule stabilizing mutations, ◊ – positions containing both type of mutations, Δ – residues forming longitudinal (interdimer and intradimer) contacts, ▲ – residues forming lateral contacts, M – identical residues, L – high similar residues, E – low similar residues. Residues in variable positions are unhighlighted. Integral alignment of all full-length sequences of β-tubulin deposited in SwissProt databank[57] is computed with ClustalX software.[58] Image is prepared in text processor.

TABLE 5. Mutations in animal tubulins conferring resistance to microtubule stabilizing compounds

Mutation	Tubulin subunit	Effects of mutation	Species	Reference
D_{26}→E	β	Resistance to taxanes	*H. sapiens*	45
V_{60}→A	β	Resistance to taxanes	*C. griseus*	46
		Resistance to epothilones	*H. sapiens*	47
P_{173}→A	β	Resistance to epothilones	*H. sapiens*	48
E_{198}→G	β	Cross-resistance to taxanes and epothilones little cross-resistance to discodermolide	*H. sapiens*	49
H_{215}→L	β	Resistance to taxanes	*H. sapiens*	50, 46
L_{215}→I		Sensitivity to taxanes and epothilones	*C. griseus*	
R_{217}→L	β	Resistance to taxanes	*H. sapiens*	50
P_{220}→L /V	β	Resistance to taxanes and epothilones Hypersensitivity to colcemid and vinblastine	*C. griseus*	16
F_{228}→L	β	Resistance to taxanes	*H. sapiens*	50
A_{231}→T	β	Resistance to epothilones	*C. griseus*	51
F_{270}→V	β	Resistance to taxanes	*H. sapiens*	52
T_{274}→I	β	Resistance to taxanes and epothilones	*H. sapiens*	53
R_{274}→G	β	Resistance to taxanes and epothilones	*H. sapiens*	53
G_{292}→E Q_{292}→E	β	Resistance to epothilones	*H. sapiens*	48, 51
Q_{292}→H	β	Resistance to taxanes	*C. griseus*	46
R_{306}→C	β	Resistance to taxanes	*H. sapiens*	54
K_{350}→N	β	Resistance to indanocine	*H. sapiens*	55
A_{364}→T	β	Resistance to taxanes	*H. sapiens*	52
S_{379}→R	α	Resistance to taxanes	*C. griseus*	56
Y_{422}→C	β	Resistance to epothilones	*H. sapiens*	48

3. Mutations resulting in resistance to microtubule depolymerizing drugs

The spatial distribution of amino acid substitutions conferring resistance to drugs, which prevent tubulin association, obeys definite regularities. With some exceptions (e.g., multidrug resistance), most of these mutations localize either immediately near the binding sites for these compounds or are components of the sites themselves. Colchicine/podophyllotoxin,[59] vinblastine,[60] benzimidazoles,[20] dinitroanilines and phosphoroamidates[61,62] binding sites have already been identified. All of them are localized on the

longitudinal intra- or interdimer contact surfaces, and binding of the ligands to these sites directly prevents tubulin association into protofilaments. Some particulars of the distribution of mutations causing resistance to the different groups of microtubule depolymerising compounds are considered below.

3.1. MUTATIONS CONFERRING RESISTANCE TO COLCHICINE

Despite the numerous studies on tubulin-colchicine interactions, only one amino acid replacement is rigorously proven to be responsible for increased resistance to this microtubule depolymerising compound. This is position 350 in β-tubulin of green alga *Chlamydomonas reinhardtii*, where lysine is substituted for methionine or glutamate.[28,29] This residue is exposed on the intradimer contact surface and is not widely conservative (e.g., position can contain lysine, aspartate, leucine or serine). Therefore, this amino acid is situated in close proximity to the known colchicines-binding site.[59] Thus the mechanism of action of this mutation can be easily explained. It is of interest that mutations in this position also cause cross-resistance to dinitroaniline and phosphoramidate compounds.[28,29]

3.2. MUTATIONS CONFERRING RESISTANCE TO DINITROANILINES

Dinitroanilines and phosphoramidates occupy a special place among microtubule depolymerising drugs. A characteristic feature is the ability to interact with plant and protozoan tubulin but not with animal or fungal tubulins[11,12] regardless of the observation that plant and animal tubulins share high levels of homology.

Many mutations conferring resistance to dinitroanilines and phosphoramidates are known in plant (six positions) and protozoan (17 position) tubulins. Four plant mutable positions are in α-tubulin and two in β-tubulin. All known mutable positions in protozoa locate to α-subunits. Somewhat surprisingly, only two substitutions (i.e., α-136 and α-239) coincide in plant and protozoan tubulins.

Among all "dinitroaniline-resistant" positions only four contain the same amino acids in the wild type tubulin. These are the α-8 (histidine), α-239 (threonine), α-243 (arginine except in the fungus *Histoplasma capsulatum*) and β-241 (arginine) positions. Seven other α-positions are conserved: position 52 can contain phenylalanine or leucine, position 231 can contain isoleucine, valine or methionine, position 238 – leucine, isoleucine, valine and methionine, position 252 – valine, isoleucine or leucine, position 275 – isoleucine, leucine, valine or methionine, and position 378 – isoleucine or leucine. Other positions contain different amino acids.

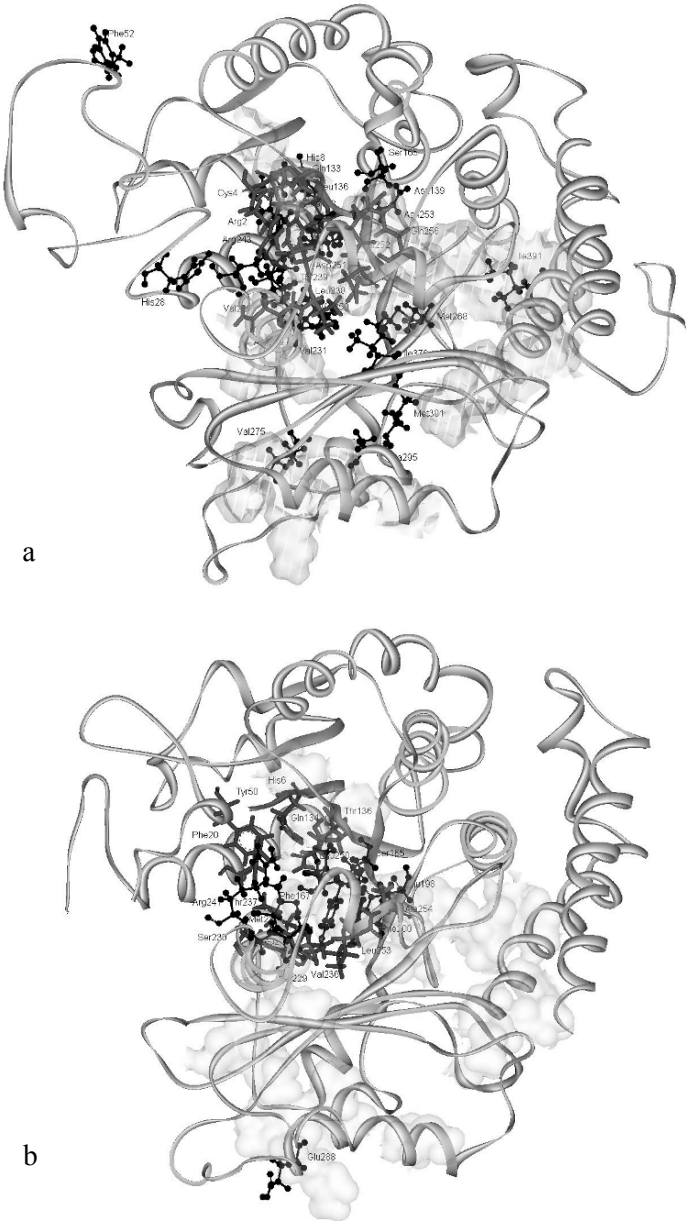

Figure 3. The arrangement of mutations conferring resistance to dinitroanilines/ phosphoramidates, in the spatial structure of α-tubulin (a) and mutations conferring resistance to benzimidazoles/phenylcarbamates in the spatial structure of β-tubulin (b). Residues of appropriate binding sites are represented by sticks. Residues in mutable positions are shown by balls and sticks. Residues forming interdimer (a) and intradimer (b) longitudinal contacts are represented as Van-der-Waals surfaces. Proteins are shown by ribbons.

Different opinions exist concerning the spatial localization of dinitroaniline binding sites on the surface of α-tubulin. Some scientists consider these sites to be identical in plant and protozoan tubulins.[64] On the other hand, others state that the sites are not the same in plants and protozoans, in spite of the substantial spatial overlap between their structures in tubulins from these two groups of organisms.[30,63]

Nonetheless, in both cases most of the mutations conferring dinitroanile-resistance are located on the α-tubulin subunit, very near to or in the dinitroaniline binding site (in particular, amino acid positions 8, 136, 252), and, thus, can immediately influence protein-ligand interaction (Figure 3a). Exceptions are mutations in positions 52, 139, 231, 275, 295, 301, 391, which are located in different areas of the protein. Among these replacements only mutations at position 301 of toxoplasma causes strong resistance to dinitroanilines.[30] Understanding the influence of this substitution, as well as mechanism of action of other "distant" mutations, is not yet clear. Suggested explanations will be considered in detail below.

Both "dinitroaniline-resistant" mutations in β-tubulin (i.e., 241 and 350 positions) are exposed on the molecule's surface near the intradimer contact interface. Position 241 locates in immediate proximity to dinitroaniline-binding site in β-tubulin, and position 350 is a component of this binding site itself. Thus, the likely mechanism of their action is obvious.

3.3. MUTATIONS CONFERRING RESISTANCE TO BENZIMIDAZOLES AND PHENYLCARBAMATES

Mutations causing resistance to benzimidazole and phenylcarbamate compounds have been characterized in fungal and animal tubulins. All of them are present in β-tubulins only. Nine mutable positions that can be divided into two groups have been described. Substitutions in positions of the first group (i.e., 165, 167, 198, 200) alter sensitivity/resistance to benzimidazoles in several species. Among this first group 167 is conserved and only phenylalanine or tyrosine are present here. The other positions are variable: 165 can contain alanine, asparagine, valine, leucine, glutamate, cysteine or serine, 198 – glutamate, glycine, alanine, cysteine or leucine, and 200 – phenylalanine, tyrosine, glutamine, methionine or isoleucine. The second group possess unique substitutions in positions 237, 240, 241, 250 and 288. The amino acids in these five positions of the second group are all conserved. These two groups differ by their structural environment. The changes of the first group are localized in β-sheet elements, but unique replacements of the second group are located in α-helical elements.

All mutant residues, except 288, are distributed in a specific region of the β-tubulin molecules (Figure 3b), such that they form a relatively compact group in the area of intradimer contact, in close proximity to the

benzimidazole-binding site. The amino acid residue in position 250 is a component of intradimer contact surface. The other residues (165,167, 198, 200, 237 and 250) are directly involved in forming the predicted benzimidazole-binding site.[20] In contrast, position 288 is located far from the other replacement positions, and its role in resistance and mechanism of action are uncertain.

3.4. MUTATIONS ASSOCIATED WITH MULTIDRUG-RESISTANCE

Some data concerning the location of mutations conferring resistance to microtubule depolymerising agents can appear dubious at first sight. For example, Hari and co-authors described multiple mutations causing simultaneous resistance to vinblastine and colcemid – drugs of different chemical nature and accompanied by hypersensitivity to taxol.[16] All these compounds have distinctly different binding sites. For example, the colcemid and vinblastine sites are located on opposite longitudinal contact surfaces of the β-tubulin molecule. Initially these data did not correlate with the results of other studies. New data has confirmed these "paradox" results. Similar multidrug resistance due to substitutions at position 220 of β-tubulin was shown by Yin and co-authors.[13] Mutations $P_{220}\rightarrow$C, S, or T results in resistance to colcemid and vinblastine with simultaneous hypersensitivity to taxanes and epotilones. Where as, substitutions $P_{220}\rightarrow$L or V cause the converse effects – resistance to taxanes and epotilones and hypersensitivity to colcemid and vinblastine.

Thus we can assume, in addition to "canonical" mechanism of action of mutation causing resistance to antimicrotubular compounds, the existence of some "alternative" mechanism of action for mutations conferring multidrug resistance. Indeed, such single amino acid replacements are unlikely to directly change the structure of all appropriate biding sites. It is quite possible that these mutations influence molecular oscillations of both α- and β-tubulins and, thus, can change the general level of microtubule stability. If this assumption is true, reducing tubulin oscillations will increase the stability of microtubules and, consequently, it will result in increased resistance to any depolymerising drugs and simultaneously increasing sensitivity to any microtubule stabilising drugs. This correlates well with data from the literature.[13,16] Conversely, intensification of tubulin oscillations will reduce the stability of microtubules and make them more sensitive to microtubule depolymerising agents and more resistant to stabilising compounds.

4. Mutations conferring resistance to microtubule stabilizing agents

Mutations conferring resistance to microtubule stabilizing agents are located on the surface of the tubulin subunits, and are distributed near or immediately on the lateral contact interface between protofilaments. These data correlate with information about the localization of binding sites for the well-known antimitotic compound taxol and its derivates,[65] and for epothilones.[66] These substances share the same binding site, which is located immediately near one of two lateral contact surfaces of the β-tubulin molecule. Thus, it is reasonable to consider mutations causing resistance to these microtubule stabilizing compounds simultaneously.

Among 18 positions with mutations known to cause increased resistance to microtubule stabilising compounds seven (β26, β217, β228, β270, β306, β364, α379) are responsible for resistance to taxanes, three (β173, β231, β422) for resistance to epothilones, and six (β60, β198, β215, β220, β274, β292) to both classes of drugs. Mutation in position 350 causes resistance to another stabilizing compound – indanocine. Positions 215, 220 and 228 contain only a single amino acids in all β-tubulins examined. Positions 173, 292 and 364 are conserved: position 173 can contain proline or alanine, 292 can contain asparagine, lysine or serine, and 364 contains either alanine or serine.

Spatial distribution of mutations causing resistance to taxanes and epothilones is a good illustration of our hypothesis regarding the interdependence between the location of binding sites for antimicrotubular compounds and the location of confirmed mutations. Practically all mutation sites (except 42, 60, 173 and 198) place near the corresponding ligand binding area (Figure 4). From this perspective, resistance to taxol caused by mutation at position 379 of α-tubulin is questionable. This mutation is located inside the globular α-tubulin, and is highly unlikely to influence both the taxol binding site and the lateral contact interface between tubulin protofilaments. Ability of four "distant" mutations to influence binding site structure appears also somewhat problematical. However, one must consider that these mutations can influence tubulin structure on the whole, similar to the mechanism of action proposed for substitutions conferring multidrug resistance described in 3.4 above.

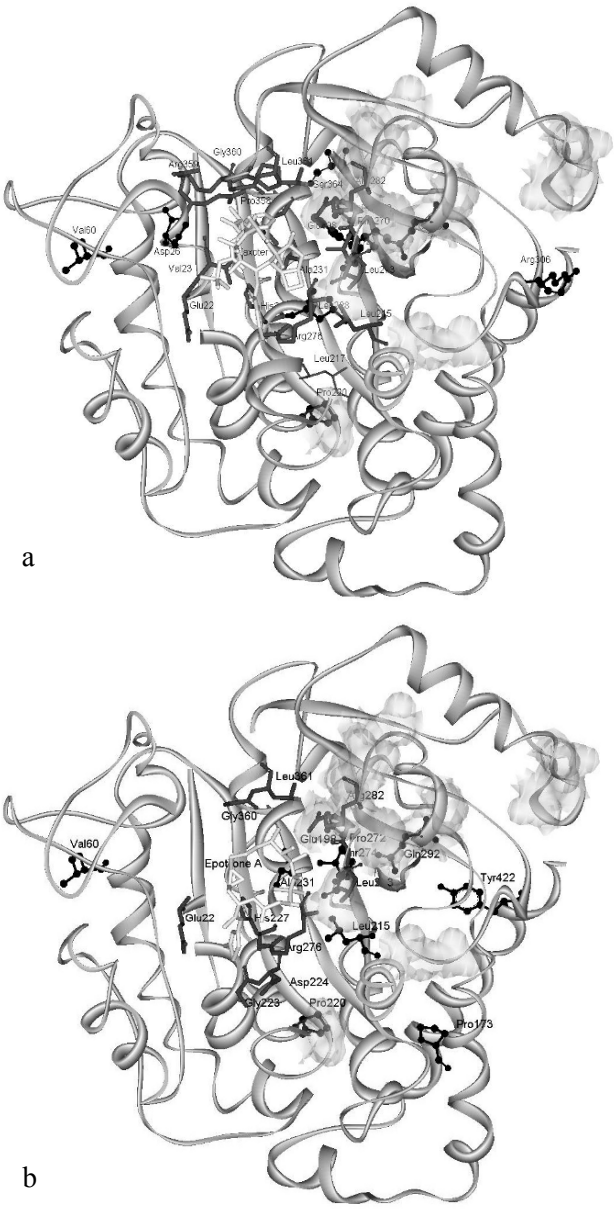

Figure 4. The arrangement of mutations conferring resistance to taxanes (a) and epothilones (b), in spatial structure of β-tubulin. Residues of appropriate binding sites are presented by sticks. Residues in mutate positions are shown by balls and sticks. Residues forming near lateral contacts are presented as Van-der-Waals surfaces. Proteins are shown by ribbon.

5. Conclusions

Through reviewing the material presented in this chapter, we can draw conclusions about the mechanisms of action of "microtubule-resistant" amino acid substitutions, and discuss possible criteria for predicting new mutable positions that will confer analogous properties. The main, "canonical" mechanism of action is conditioned by spatial proximity between the tubulin-drug interaction site and mutated amino acid residue. So, a mutation can immediately change the molecule's spatial structure, altering a specific binding site, and consequently causing a decrease in the protein's affinity for a specific chemical compound(s). From this point of view, the criteria for predicting new mutations, proposed by us earlier,[10] are straightforward. However, criteria on mutation-position conservatism needs correcting in accordance with newly discovered facts.

An alternative mechanism of action to explain "resistant" mutations is associated with the ability of a single amino acid substitution to influence protein behaviour on the whole. Possibly such substitution can cause global changes such as decreasing or increasing molecular oscillations and/or conformational energy of tubulin subunits, dimers or microtubules as a whole. In this case, the change in sensitivity to antimicrotubular compounds will be *less specific*. Furthermore, the general stabilisation of microtubular system in this case will cause hypersensitivity to any drugs that further stabilize microtubules and, simultaneously, cause resistance to any microtubule depolymerising compounds. The converse will hold true as well. Increasing of tubulin oscillations will be associated with general hypersensitivity to microtubule depolymerising drugs, and increase resistance to any microtubule stabilizing drugs. However, proof of alternative mechanism of resistance must include calculations of molecular dynamics for different tubulins having mutations conferring multidrug resistance. The methodical approaches put forwarded herein can be used not only for predicting new mutation possessing analogous properties, but for verification of data concerning newly described mutations and for rejection of mutated position that lack functional activity or which are experimental errors.

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ANTIMITOTIC DRUGS FOR MICROPROTOPLAST-MEDIATED CHROMOSOME TRANSFER IN PLANT GENOMICS, CELL ENGINEERING AND BREEDING

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Abstract. Achievements of the microprotoplast-mediated chromosome transfer (MMCT) technique are summarized. Overcoming technical barriers for large-scale gene transfer through fusion of isolated microprotoplasts, containing one or a few chromosomes, with recipient protoplasts are considered. The efficiency of antimicrotubular compounds – phosphorothioamides (amiprophos-methyl and cremart), dinitroanilines (oryzalin) and propyzamide – and disrupters of microtubule organizing centers (griseofulvin and phenylcarbamates) for the production of microprotoplasts is analysed. The combined use of antimicrotubular compounds with cytochalasin B, as an actin filament disrupter, for increasing the yield of microprotoplasts is also discussed. Results of experiments using microprotoplasts obtained from plant microspores are presented. The paper also discusses the advantages of MMCT as an effective technique for parasexual crossing compared with traditional somatic hybridization and as an important technique for plant genomics and breeding .

Keywords: Microprotoplasts, antimicrotubular compounds, chromosome transfer, micronuclei

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1. Introduction

Currently, only cloned genes may be transferred by means of DNA transformation. Polygenic traits or traits with unknown biochemical or molecular mechanisms (e.g., resistance to certain diseases or stresses and other economically important traits) are still recalcitrant to transfer using methods of genetic engineering. Simultaneously, the requirements of breeders with respect to the transfer of such characters as apomixis (production of seeds without sexual reproduction)[1,2] still cannot be implemented adequately by means of classic crossing technologies. These difficulties arise due to sexual incompatibility between donor wild polyploid species and cultivated species, manifestations of male sterility, weak seed production and low frequency of occurrence of desirable characters among backcross offspring.

Many attempts have been made to manipulate plant chromosomes ever since a specific chromosome containing a dominant resistance character for leaf rust was transferred from diploid *Aegilops umbellulata* to wheat.[3] Such work stimulated the development of methods for the transfer of chromosomes from wild species to cultivated ones and thus to manipulate meiotic crossing-over and recombination of chromosomes.[4–6] The transfer of a certain chromosome from one species to another and the direct selection of monosomic addition lines for different species of plants presents new opportunities for transferring economically important genes, introgressive crossing and analysis of the evolution of plant genomes among sexually incompatible plants.[7–9]

Somatic hybridization is a useful and practical solution to the problems of partial transfer of genomes, generation of new nucleus-cytoplasm interrelations, and overcoming the barriers of sexual incompatibility.[10,11] Asymmetric somatic hybridization was developed in an early attempt to accomplish the partial transfer of genomes from donor to recipient species. According to this technique, irradiated protoplasts of a donor species are fused with intact protoplasts of the recipient species.[12,13] However, taking into account that when using this method one can achieve successful transfer of one or two intact chromosomes,[14–16] asymmetric hybridization is still far from yielding consistent, predictable results. Asymmetric hybrids are often characterized by polyploidization, aneuploidy, weak regeneration and growth, reduced or lack of fertility due to genetic incompatibility of hybrid genomes, and/or genetic lesions induced by radiation.[12,13,17,18] In this context, devising conditions for the transfer of individual chromosomes, which contain genes of interest, likely shared between the related but sexually incompatible species, and obtaining monosome-supplemented lines (which would make it possible to accelerate gene introgression by

means of homologous pairing of chromosomes and recombination or other mechanisms of gene transfer[8,9,19, 20]) remains an important goal today.

For all intents and purposes, simultaneously with the development of methods of somatic hybridization, efforts were initiated to accomplish the transfer of genetic material on a larger scale than a single gene (or a small, specific group of genes), but less than a haploid genome (as is the case of sexual hybridization). As a result the technique known now as microcell-mediated chromosome transfer (MMCT) was developed in the 1970s for the fusion of microcells, containing single or a small number of chromosomes, with whole cells, and the subsequent selection of hybrids.[21,22] For example in mammalian cell lines, the mediated transfer of gene blocks has been achieved by the *in vitro* introduction of isolated metaphase chromosomes, on the condition that these chromosomes integrate in the recipient genome, although they very often are lost due to the absence of selection.[23] Despite all the shortcomings (e.g., loss of linked genes during chromosome fragmentation, emergence of incorrect linkage, etc.) the transfer of genes mediated by whole chromosomes in great measure made it possible to address issues of mapping the human genome, especially in the case of localizing important genes in immediate vicinity of genetic markers.[24,25]

Although, this methodology has not changed considerably since this time it is being used to great success in progressing several different fields in modern day biology.[21,22] MMCT is being employed by groups all over the world to hunt for tumour suppressor genes, DNA repair genes, senescence-inducing genes and telomerase suppressor genes. Some of these genomic discoveries are being investigated as potential treatments for specific cancers. Other fields have taken advantages of MMCT, and these include assessing genomic stability, genomic imprinting, chromatin modification and structure and spatial genome organisation.[22] Indeed, MMCT is used to transfer mainly fragmented mini-chromosome between cell types and into embryonic stem cells for the construction of transgenic animals.[22,26] MMCT has also been a very useful method in the construction and manipulation of artificial chromosomes for potential gene therapies.[26]

2. Microprotoplast-mediated chromosome transfer (MMCT)

Initially, in experiments with plants, this technique showed limited success[27,28] because there were substantial difficulties in terms of isolation and flow screening of individual plant chromosomes. The main problem arising in the course of such cytometric research is securing an effective halt to mitosis at metaphase using specific anti-mitotic drugs. In particular, complications emerging during the first attempts to transfer isolated plant chromosomes – singling out individual chromosomes for flow cytometry

sorting[29] and limited potential for efficient transfer of metaphase chromosomes[30] – in great measure are accounted for by a strong association of the chromosomes. To date these problems have continued to be significant despite constant progress in methodologies for the isolation and identification of individual plant chromosomes.[31–33]

The fusion of isolated microprotoplasts, which contain one or several chromosomes**,** with recipient protoplasts may be a promising alternative for restricted transfer of the genome in plants. Obtaining micronucleated cells in animal cell cultures following their incubation with colchicine was used effectively over the last quarter of the 20th century.[34,35] Following such treatment, metaphase chromosomes become scattered in the cell and decondense individually or in small groups. Subsequently, a nuclear envelope is formed arround the decondensed chromosomes creating micronuclei, which contain a different number of chromosomes per micronucleus. Centrifugation of the micronucleated cells is utilized to isolate microcells. MMCT eliminates the dependence on meiosis, thus reducing the recombination events, and allows the creation of partial hybrids between sexually incongruent species.[36] Such an approach made it possible to easily transfer a limited number of chromosomes to mammalian recipient cells.[23,37,38] Further on, MMCT was recognized as potentially useful not only for the transfer of individual chromosomes, but also for studying the expression of linked and resident genes, for isolating and identifying the sequences of a recombinant specific for chromosomes and manipulating specific chromosome fragments.[39–42] As a result, this method substantially facilitated the mapping of genes and arbitrary DNA sequences on certain chromosomes of the human genome[43] and proved to be a powerful approach to study the expression of genes in somatic human cells.[44]

The successful development and application of this method for manipulations with mammalian cells was accomplished by solving three fundamental problems, thus ensuring:

1. A high level of a mitotic block and mass induction of micronuclei by colchicine or colcemide in donor cell lines carrying selective markers
2. Isolation of small microcells, which contain one or several chromosomes and
3. The effective fusion of such microcells with cells of recipient lines

Success of the micronucleation procedure (e.g., the percent yield of micronucleated cells) depends on many factors among which are the mitotic index of the cell population subjected to micronucleation and the efficiency of anti-mitotic drug action, which is a derivative of its ability to block the assembly of microtubules in a cell. Even though synchronization of the cultured cells, preceding the actual micronucleation step, is an important

factor for increasing the percent yield of micronucleated cells, success of the procedure improves substantially through the use of potent microtubule inhibitors. Although colchicine has been used for polyploidization of plants,[45] and is widely used to block their cells at metaphase,[46,47] its affinity for tubulin of plant origin is much lower than that for animal tubulin,[48,49] At the same time, research has demonstrated that amiprophos methyl (APM), belonging to the phosphoroamide herbicides, and oryzalin, representing dinitroaniline herbicides, possess an extremely high affinity for plant tubulin and demonstrate, high depolymerizing activity with respect to microtubules.[48–52]

It is the use of APM that made it possible to induce the formation of micronuclei in suspension culture cells of *Nicotiana plumbaginifolia*.[53] Soon after there appeared a report describing the production of micronuclei using APM treatment of suspension cultures of potato (*Solanum tuberosum* L.), carrot (*Daucus carota* L.) and *Haplopappus gracilis* Nutt.[54] APM treatment of *N. plumbaginifolia* cells resulted in a scattering of metaphase chromosomes in the cytoplasm, which made it possible to isolate single chromosomes.[53] These findings resulted in the development of experimental conditions for the mass induction of micronuclei by means of prolonged APM treatment, and for the isolation of micronuclei from micronucleated cells followed by flow sorting. As a result of APM treatment of plant cells for 5–6 h, metaphase chromosomes are scattered in the cytoplasm, decondense and micronuclei emerge, while an increase in APM concentration to 36 μM together with extension of the treatment period to 48 h leads to an increase in the yield of micronuclei.[54,55] Furthermore, it was established that micronucleation efficiency under the influence of APM differs substantially for different genotypes of *N. Plumbaginifolia*.[53–56]

It turned out that the efficiency of APM for micronuclei formation in *N. plumbaginifolia* depended not only on the length of time of its action and its concentration, but also on the synchronization level of the suspension cell culture.[57] For example, the efficiency of micronucleation measured in percent (as the number of micronucleated cells to the total number of cells treated) was at a maximum (15%) when using 32 μM of APM, whereas using synchronization with oxyurea increased it to 30%.

During further experimentation to isolate microprotoplasts the same group of researchers investigated the addition of cytochalasin B to the incubation medium.[57] Because cytochalasin B is a very effective disrupter of actin filaments, early on it was proposed to use it for micronucleation of mammalian cells.[37] It was shown that further destabilization of the cytoskeleton, by the combined use of cytochalasin B and APM, leads to a substantial increase in the number of microprotoplasts due to increased fragmentation of treated protoplasts during high-speed centrifugation.[57] In

this case, after washing, the microprotoplasts retained the ability to form microcalli, which proved their resiliency. Approximately 40% of microprotoplasts contained the chromosome number corresponding to the subhaploid set of chromosomes, while some of them had a DNA content equivalent to the presence of just 1 or 2 chromosomes. The use of cytochalasin B to obtain microprotoplasts from *Helianthus* led to a two-fold increase of their yield and made it possible to maintain micronuclei in a stable state (without fusion and formation of restitution nuclei).[58] Although cytochalasin B possesses a pronounced negative impact on the regeneration of microprotoplasts,[59] its use in obtaining microprotoplasts should be accompanied by the utilization of an appropriate concentration, which would produce minimal effects on plant regeneration.[60]

Comparison of results on the micronucleating ability of APM and of oryzalin indicate that while oryzalin blocks plant cells in metaphase more effectively, APM is better at inducing the formation of micronuclei.[61] Another paper by the same authors presented results of studies on the impact of APM and of oryzalin compared with that of colchicine on blocking mitosis, inducing micronuclei and doubling chromosomes after treatment of a transformed fast-growing suspension line of potato.[62] While colchicine revealed its properties when used at concentrations of 0.5–5.0 mM, APM and oryzalin blocked mitosis at metaphase more efficiently at concentrations from 15–32 μM; inducing the emergence of micronucleated cells at a higher frequency and ensuring higher yield of micronuclei. As a result of analyzing DNA content of the nuclei by flow cytofluorometry and calculating the chromosome number in mitotic cells after the removal of these substances from subsequent subcultivation, it was established that oryzalin doubled chromosomes more effectively than APM or colchicine.

However, soon it was established that cremart (butamiphos, *O*-ethyl-*O*-(3-methyl-6-nitrophenyl)-*N*-sec-butylphosphorothioamidate), also a member of the phosphoroamides, is a much more effective inducer of micronucleation compared with APM and oryzalin.[63] After treatment of potato suspension culture cells with cremart (3.7–15 μM) for 48 h, followed by incubation in a mixture of cellulolytic enzymes in the presence of cremart and cytochalasin B, micronucleation frequency increased even more compared with the values obtained after treatment of the suspension cells with cremart alone. Efficiency of micronucleation with cremart (3.7 μM) was confirmed by us in experiments using suspension cultured *N. plumbaginifolia.* At the same time, experiments conducted on suspension-cultured cells *Hemerocallis hybrida* Hort. (Hemerocallis x hybrida Hort.) demonstrated the limited possibilities of using both APM and cremart in the induction of micronucleation in this liliaceous species.[64] In this case, treatment of *H. hybrida* cells synchronized with propyzamide (8 μM) for 60 h followed by the

addition of cytochalasin B (20 μM) 20 h after the start of the antimicrotubular agent treatment was effective.[65]

Because developing microspores are another potential source from which to obtain micronuclei,[66,67] micronucleation efficiency of various types of antimicrotubular substances recently has been characterized in detail during treatment of developing microspores from a *S. Tuberosum*.[66] Just as in the case of somatic cells, APM and oryzalin turned out to be the most powerful inducers of micronuclei (with a slightly greater effect when APM was used at an identical concentration of 25 μM). Colchicine and colcemide (concentration = 0.5 mM) induced the formation of gamete microprotoplasts 9–10 times weaker than APM. It is interesting that griseofulvin and chloroisopropyl-N-phenyl carbamate (CIPC)[66] – substances blocking mitosis by destroying microtubule organization centers – are very effective inducers of micronucleation.[48] In particular, CIPC (which also belongs to the phenyl carbamate class of herbicides) was shown to have pronounced activity (at a concentration of 100 μM). Later, CIPC was successfully used to obtain gamete microprotoplasts from developing microspores of three lily species – *Lilium regale* E. H. Wilson, *L. longiflorum* Thunb. and *L. speciosum* Thunb.[67] In this case, CIPC was used at a concentration of 10 μM, but the treatment time of microsporocytes was three to four days and its micronucleation properties turned out to be better than that of APM or propyzamide.

Earlier, griseofulvin was sporadically studied as a potential inductor of micronuclei in suspension cell cultures of *Medicago sativa* L.[68] whereas other phenyl carbamate compounds were typically not used for micronuclei formation in somatic cells. Such an attempt was made by us for the induction of micronuclei from a biotype of goosegrass, *Eleusine indica* (L.) Gaertn, resistant to dinitroanilines.[69] Because dinitroaniline-resistant plants are characterized by cross resistance to phosphoramides**,** treatment of *E. indica* cells with APM and oryzalin had little effect.[70] When treating cell suspensions of goosegrass with these compounds at concentrations of 5–200 μM, micronuclei were produced inconsistently and with low efficiency. More conspicuous formation of nuclei was noted when using colchicine at concentrations exceeding 0.5 mM; however, such high concentrations of this antimicrotubular agent are capable of bringing about the destruction of intracellular structures. Subsequent treatment of the suspension culture of the dinitroaniline-resistant line of *E. indica* with isopropyl-N-phenyl carbamate (IPC) at concentrations of 1–100 μM turned out to be most effective for inducing micronuclei.[70] When using IPC at concentrations exceeding 10 μM one observes the formation of 5–8 micronuclei per a cell, which made it possible to efficiently isolate microprotoplasts.

To date, sequential experiments helped establish that because of their high affinity for plant tubulin, dinitroanilines, phosphoroamides, propyzamide, and phenyl carbamate can effectively block metaphase and induce massive micronucleation in suspension-cultured cell[63,65,67,70–73] and in developing microspores[66,67] of various plant species. Together with the development of effective fusion procedures for microprotoplasts,[74] these approaches made it possible for the subsequent development of the microprotoplast-mediated chromosome transfer technique, whose first adaptation to plants was the production of hydrids containing one potato chromosome and an entire set of tobacco *(Nicotiana tabacum* L.)[74,75] or tomato (*Lycopersicon esculentum* P. Mill./*Solanum lycopersicum* L.) chromosomes,[74] or containing one chromosome of *N. plumbaginifolia* and an entire set of tomato chromosomes.[74] The same procedure was successfully used for the transfer of a single chromosome from a potato donor line to recipient cells of wild tomatoes, *Lycopersicon peruvianum* (L.) P. Mill./*Solanum peruvianum* L.[75,76]

In the experiments dealing with the transfer of a small portion of genetic material (one or two chromosomes) from donor microprotoplasts of *S. tuberosum* to mesophilic protoplasts of *N. tabacum* and *L. peruvianum*, the donor line used was triploid potato – a transgenic line containing marker genes of neomycintransferase II (*npt II*) and ß-glucoronidase (*uid*).[75] The induction of massive micronucleation in donor suspension cells was implemented by means of exposure to cremart (7.5 μm) for 48 h prior to enzyme treatment, but with the simultaneous addition of cytochalasin B (20 μM). The protoplasts were isolated from micronucleated cells by high speed centrifugation. Enrichment by multiple filtrations through nylon filters having successively smaller pore sizes made it possible to separate microprotoplasts, containing one or two chromosomes, from the larger subdiploid microprotoplasts. Then these microprotoplasts were used for fusion with recipient protoplasts. It was shown that introduction of a small portion of the genome (1–2 chromosomes) along with a very small quantity of cytoplasm (a thin layer surrounding the micronucleus) destabilized the acceptor cell to a lesser degree, and produced a rather small stress response. This approach positively affected the morphogenetic potential of the acceptor cell. This is an important finding because very often infertile plants, which typically lack practical value, are formed as a result of standard somatic hybridization procedures.

Subsequently, identification and molecular-genetic analysis of transgenic potato chromosomes, transferred to tomato by means of microprotoplast fusion, was made.[77] Transgenic chromosomes 3 and 5 of potato, containing the *npt II* and *uid* genes, respectively, were identified in the genome of hybrids with *L. peruvianum* by RFLP analysis using chromosome-specific markers. It was found that in the donor chromosomes of potato each of

three integration sites contained genes *uid* and two of them also contained the *npt II* gene. The results of the analysis of monosomic addition hybrid plants, obtained by fusion with microprotoplasts, showed that each of these three sites is located on different potato chromosomes. In this case the hybrid plants had only chromosomes carrying the selectable gene for resistance to kanamycin (*npt II*). By crossing monosomic addition hybrid plants with wild tomato (*L. peruvianum*) sexual transfer of a donor chromosome of potato, containing *npt II* and *uid*, was achieved. Although most of these plants contained the corresponding potato chromosome, in some cases (i.e., absence of a potato chromosome) the *npt II* and *uid* genes appeared to be integrated into the tomato genome.

Some time ago an alternative method of transferring portions of the genome using microprotoplasts obtained from gametes of *S. tuberosum* was proposed.[66] Though the approach, using APM as the most effective inductor for micronuclei, has not been worked out to perfection because a number of technical difficulties were encountered (for example, to the isolation of gamete protoplasts, with the increase of the synchronization level of microspores, etc.) this method is considered very promising for further development. Such a conclusion is based on the fact that the isolation of gamete microprotoplasts does not require centrifugation, because the micronuclei formed after treatment with APM immediately form microcells in the microspores (at the tetrad stage). Therefore, the isolation of protoplasts from microspores requires only enzymatic treatment.

3. Attempts of plant genetic improvement with MMCT

The method of microprotoplast-mediated chromosome transfer was successfully adapted to representatives of several families of the dicotyledons and monocotyledons. MMCT was used to obtain sunflower plants (*Helianthus annuus* L.) with extra chromosomes from giant sunflower (*H. giganteus* L.) or Maximilian's sunflower (*H. maximiliani* Schrad.).[58] As a result, MMCT is considered an effective tool for the development of sunflower plants with resistance to *Sclerotinia sclerotiorum* (Lib.) de Bary, which is the most important fungal pathogen of this crop worldwide.[78] Protoplasts of perennial sunflower, the source resistance, were used for micronuclei induction and isolation of microprotoplasts. Regenerable annual sunflower, genotype Florom-328, was used in these experiments as the recipient of the microprotoplasts. Regenerated hybrid plants produced viable seeds, and these results have been confirmed in preliminary field experiments.

There is a recent publication reporting the production of a large number of microprotoplasts from grapefruit (*Citrus* x*paradisi* Macfad.) var. Ruby Red, and also from *Swinglea glutinosa* (Blanco) Merr. (allied to the genus

Citrus L.) by treating suspension-cultured cells with APM.[60] In these cases, about 75.2% of the microprotoplasts contained one chromosome, 17.1% – two chromosome, 4.6% – three chromosomes, and 2.0% – four chromosomes. High levels of protoplast micronucleation were obtained from Satsuma mandarin (*Citrus unshiu*) when suspension cultures in early-log phase were pre-treated with 10 mM hydroxyurea for 24 h and then treated with 32 μM APM for 24 h.[79] Furthermore, 61.5% of the protoplasts isolated from such sequentially treated suspension cells were micronucleated. Ultracentrifugation of the micronucleated protoplasts generated microprotoplasts of different sizes, most of them below 5 μm in diameter, with one or a few chromosomes. Microprotoplasts obtained from grapefruit were fused with protoplasts of sweet orange [C. x *sinensis* (L.) Osbeck] of the variety Succari, while the microprotoplasts *S. glutinosa* were fused with the protoplasts of marmalade orange (*C.* x *aurantium* L.).[60] As a result, embyoids or cell suspension cultures of recipient species were obtained. Although the embryogenic potential of the fusion products was reduced or inhibited, practical solutions are being evaluated to use this approach for citrus genetic improvement.[80]

4. Conclusions

Summarizing the material presented in this review, one should note the progressive development of the technology of transferring a portion of the nuclear genome (isolated metaphase chromosomes) of plants using the phenomenon of micronucleation based on the formation of microprotoplasts containing genetic material at the subhaploid level. The use of antimicrotubular agents to enhance the process of protoplast micronucleation made it possible to develop the procedure of microprotoplast-mediated transfer of single chromosomes. In the very near future it is highly probable that new compounds with antimicrotubular activity will be developed, these compounds being even more effective compared with those discussed here.

The microprotoplast-mediated transfer of partial genomes is an effective technique for parasexual crossing and the transfer of intact chromosomes from one species to another. This method differs from traditional somatic hybridization in that only a small part of the donor's genetic material is present at the time of fusion. It makes it possible to avoid time-consuming steps and numerous backcrosses, which are necessary when obtaining such a line using the traditional method or by means of somatic hybridization. The potential advantages provide a basis to predict the extension of the technology of microprotoplast-mediated transfer of individual chromosomes and groups of chromosomes to a wide variety of plants having economic value, in particular, to annual plants. But it should be remembered that the

problem of partial transfer of genetic material by means of this technique lies not only in obtaining a large quantity of subhaploid microprotoplasts, but also in obtaining fertile monosomic addition plants regenerated from the fusion products of the protoplasts of the recipient with the donor microprotoplasts (i.e., complex approaches are necessary to spread the technique on a larger scope segment of the plant kingdom).

In addition to the transfer of economically important traits (e.g., resistance to stresses and diseases), which may be controlled as polygenic traits linked on certain chromosomes, or as yet unidentified genes (e.g., apomixis or nonspecific resistance), the technique of fusion through microprotoplasts has other experimental advantages.[81] Monosomic and disomic additions can have specific morphological characteristics, but more often they need additional confirmation of molecular marker analyses and assessment by fluorescence *in situ* hybridization with genomic and chromosome-specific DNA as probes. Their specific genetic and cytogenetic properties make them powerful tools for fundamental research into:

1. The design of DNA libraries for specific chromosomes by PCR cloning of the added chromosome fragments
2. Physical mapping with a high separability of added chromosomes by means of *in situ* hybridization
3. Investigation of distribution of chromosome-specific markers and repetitive DNA sequences
4. Investigation of three-dimensional organization of added chromosomes in connection with heterologous gene expression and transmission and
5. Elucidation of the regulation of homeologous recombination

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MODIFIED TUBULIN GENES AS SELECTABLE MARKERS FOR PLANT TRANSFORMATION

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Abstract. This review describes new approaches to the development and utilization of marker genes for the selection of transformed plant cells. These selectable markers are based on the use of mutant tubulin genes from natural biotypes or *in vitro* selected mutant plants. The results of numerous studies on plants resistant to herbicides with antimicrotubular modes of action are summarized. In addition, the transfer and expression of mutant tubulin genes, conferring resistance to amiprophosmethyl (phosphorothioamidate herbicide) and trifluralin (dinitroaniline herbicide), by somatic hybridization to related and remote species are discussed. The results of experiments on transformation of monocotyledonous and dicotyledonous plants with a mutant α-tubulin gene conferring resistance to dinitroanilines are described to test the possibility of its use as a selectable marker gene and, simultaneously, as an agronomic trait to obtain dinitroaniline-resistant plants.

Keywords: Tubulin, resistance, dinitroaniline herbicide, phosphorothioamidate herbicide, trifluralin, amiprophosmethyl, mutations, marker genes, plant transformation

1. Introduction

Over 50 marker genes used in transgenic or transplastomic studies and for the development of agricultural crops have been comprehensively studied in

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terms of their efficiency, biological safety and potential for commercial use.[1] Selective marker genes can be grouped into several categories depending on whether their use results in positive (transformed cell survival) or negative (transformed cell death) selection, and whether selection can be performed in the presence of exogenous substrates. Positive selective marker genes are used most widely. Their utilization is based on the use of toxic (e.g., antibiotics, herbicides, and other biologically active compounds) or, currently, non-toxic compounds as well. Positive selection marker genes, whose expression does not depend on exogenous substrates but that influence physiological processes regulating plant development, are also widely used.

Reporter genes, which make it possible to keep track of transformed cells in order to select them manually, are often used as selective marker genes. Selection pathways have been proposed in which selectable marker genes are eliminated by using inducible promoters that results in the excision of target sequences,[2] by expressing a recombinase through agroinfection [3] and others.

However, although a large number of marker genes have been proposed, only a very few are currently employed in plant genetic engineering. This limitation is determined by many factors, including high costs, difficulty of development, necessity to avoid side effects, and the complexity of their use. Despite the fact that no detrimental effects of marker genes, in terms of biological safety, have been revealed so far, still it is societal anxiety that limits the use of genes conferring resistance to antibiotics in practice. The use of genes conferring resistance to antibiotics is already prohibited for use in practical applications (the prohibition came into effect January 1, 2005), and in the future will be prohibited for research purposes (the prohibition will come into effect after January 1, 2009; EC Directive 2001.18.EC). Therefore, testing and introduction of new marker genes that carry genetic information of solely plant origin is of a great scientific and practical importance.

This problem can be solved by using a gene encoding a constitutive cellular protein, which can be regulated by a plant-specific selective agent. Tubulins, which are the primary building blocks of microtubules (a major structural component of the cytoskeleton of all eukaryotic cell), meet those requirements. Microtubules are involved in the regulation of many key processes, such as nuclear and cell division, cytoplasm organization, transport of vesicles or organelles, morphogenesis, and cell motility.[4] Microtubules are highly conserved filamentous structures consisting of α- and β-tubulin subunits, each with a molecular weight of ~50–55 kDa.[5] Although the amino acid sequences of tubulins isolated from various organisms are highly conserved, tubulins from fungi, plants, and animals are clearly distinguishable by some pharmacological and immunological

properties,[5,6] as well as by amino acid sequence.[7] As a result, certain compounds that bind to tubulins and thereby disrupting microtubule functions exhibit different affinities for fungal, plant, and animal tubulins. This further allows these compounds to be used as key tools for studying the structure and functions of microtubules on the whole, and of tubulin in particular.[4,8,9]

The group of chemical agents that can disrupt or stabilize microtubules includes compounds that are currently used as antiprotozoan, antihelminthic, and anticancer drugs, as well as fungicides and herbicides. Studying the genetic bases of acquired resistance to such compounds has also a practical aspect. In particular, genetic and molecular studies of microtubules have been performed, which included the isolation of mutant genes and the identification of changes in amino acid sequence of the encoded proteins that determine the level of resistance of microtubules to these compounds.[8,10]

A vast number of studies reporting the isolation of mutant strains of fungi, algae, higher plants and mammals that are resistant to agents with antimicrotubular mechanism of action have been performed.[8,11] On the basis of the study of the role of mutant tubulin in the development of resistance of *Neurospora crassa* cells to benomyl (a benzimidazole derivative), it was suggested that the gene encoding this tubulin can be used as a selective marker trait for fungal transformation.[12] The discovery of natural biotopes of plants with a mutant tubulin resistant to antimicrotubular herbicides and the selection of similar mutants *in vitro*[8,11] opened opportunities for using an analogous approach for the genetic engineering of plants.

Nearly one-fourth of herbicides used in the market are classified as mitotic or microtubular disrupters.[13,14] This group includes the widely used dinitroanilines (in particular, trifluralin, pendimethalin, and oryzalin), phosphorothioamidates (amiprophosmethyl, AMP), and phenylcarbamates (isopropyl-N-phenylcarbamate, IPC; ethyl-N-phenylcarbamate, EPC; and chloroisopropyl-N-phenylcarbamate, CIPC). In contrast to the alkaloid colchicine, a mitotic disrupter that ineffectively disrupts plant microtubules, the above-mentioned compounds are effective at considerably lower (micromolar) concentrations. Numerous studies identify the target of dinitroaniline and phosphorothioamidate herbicides and many of the N-phenylcarbamates as tubulin.[8,15,16] For phenylcarbamates, the data indicates that these compounds can directly destroy microtubules or disrupt microtubule organization centers (discussed in more detail below). The binding of mitotic-disrupter herbicides to tubulin decreases the polymerization capability of the latter, preventing the formation of microtubules and, hence, the mitotic spindle. Therefore, the effect of these herbicides on plant cells is a complete loss of microtubules not only during mitosis but also in interphase, and may ultimately lead to cell death. For this reason, the genes for mutant tubulin,

which confers resistance of microtubule to such herbicides, can be used as marker genes in positive selection.

Simultaneously with our development of the use of mutant tubulin genes as selectable markers in plant genetic engineering, similar studies were pursued by another research group.[17] This concept was based on mutant microtubules of rice that are resistant to EPC,[18] and the assumption that resistance is determined by an α-tubulin lacking the C-terminal moiety.[19] However, because evidence confirming the existence of such a mechanism for EPC resistance is insufficient, and owing to technological difficulties, this project was not developed further. Taking into account our experience with obtaining mutants resistant to dinitroanilines, phosphorothioamidates, and phenylcarbamates,[15] and the fact that the high selectivity of dinitroanilines and phosphorothioamidates is associated with their direct interaction with tubulin, we developed approaches to create new marker systems for plant transformation.. These selectable markers are based solely on the use of mutant tubulin genes from natural plant biotypes or from induced and *in vitro* selected mutant plants.

2. Natural plant biotypes resistant to herbicides with antimicrotubular activity

It is known that many plants exhibit an increased resistance or sensitivity to herbicides with antimicrotubular activity; however, the genetic nature of the variability of their sensitivity to these compounds has not been studied comprehensively.[14] The best example of a plant displaying natural resistance of microtubules to herbicides that specifically bind tubulins is carrot. It was shown that carrot seedlings are resistant to the dinitroanilines at concentrations that are 100-1000 times greater than the concentrations that cause microtubule disruption in tolerant (cotton, soybean) and sensitive (maze, sorghum) plant species.[20] It appeared that organized microtubular structures of carrot roots (i.e., cortical network, preprophase band, spindle, and phragmoplast) are not destroyed even after 24 h of treatment with various dinitroanilines. It was assumed that the resistance of carrot to dinitroaniline herbicides results from pre-existing resistance, rather than natural selection or accidentally breeding for resistance.

However, resistance to herbicides with antimicrotubular mechanism of action, as a spontaneously acquired trait, was detected in a number of monocotyledonous and dicotyledonous weeds. The characteristics of many of these plants, including natural biotypes of *Eleusine indica*, *Setaria viridis*, *Alopecurus myosuroides*, *Sorghum halepense*, *Lolium rigidum*, *Poa annua*, *Amaranthus palmeri*, and *Echinochloa crus-galli*, have been described in numerous papers and summarized in several recent

reviews.[11,14,15] Many plant biotypes with an acquired resistance to antimicrotubular herbicides also exhibit either cross-resistance to related compounds or multiple-resistance to herbicides with other mechanisms of action.[15] The latter fact is suggestive of the existence of either parallel resistance mechanisms that ensure microtubule functioning or mechanisms based on modification of other structural and functional cell components unrelated to microtubules.

Over many years, the genetic and molecular mechanisms of resistance to herbicides with antimicrotubular activity have been studied in the greatest detail in the dinitroaniline-resistance biotypes of *E. indica* and *S. viridis*.[11,14,15] In both cases, cross-resistance of these biotypes to some other structurally dissimilar mitotic-disrupter herbicides was observed. It appears that the highly resistant *E. indica* biotype is cross-resistant not only to all dinitroanilines but also to the phosphorothioamidate herbicide amiprophosmethyl (APM; N-isopropyl O-(2-nitro-p-tolyl) phosphoroamidothioate.[21,22] Both the resistant and susceptible *E. indica* biotypes exhibited the same sensitivity to antimicrotubular herbicides, such as pronamide (3,5-dichloro-(N-l,l-dimethyl-2-propynyl)benzamide), terbutol (a carbamate herbicide), and 2,3,5,6-tetrachloroterephthalic acid dimethyl ester (DCPA).[22] However, the dinitroaniline-resistant *S. viridis* biotype was equally resistant to APM, DCPA, and terbutol.[23] Similarly to the resistant *E. indica* biotype, the resistant *S. viridis* biotype displayed cross-resistance to the antimicrotubular herbicide dithiopyr (S,S-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-pyridinedicarbothioate). [23]

Because it is known that dinitroanilines prevent tubulin polymerization, thus disrupting microtubules, the ability of oryzalin to inhibit polymerization of the tubulin-enriched protein fraction isolated from resistant and sensitive *Eleusine* biotypes was investigated. Tubulin-enriched extracts from both biotypes were polymerized into microtubules in the absence of oryzalin. However, in the presence of oryzalin, microtubules were formed only with the protein fraction from the resistant biotype.[24] Preliminary biochemical analysis of *E. indica* revealed an additional peptide in the resistant biotype, which was initially suggested to be an altered β-tubulin.[24,25] However, analysis of an intermediately resistant biotype *E. indica* by two-dimensional electrophoresis did not reveal any differences in the properties of tubulin between this resistant and the susceptible biotype.[26] Later, while analyzing this highly resistant *E. indica* biotype (i.e., a 40-fold increase of resistance to trifluralin) a distinction in the electrophoretic properties of an α-tubulin isoform was detected. From this observation it was suggested that resistance to dinitroanilines in this biotype would correlate with a change of the tubulin-binding site.[27]

Later, it was demonstrated that the high level of resistance of *E. indica* to dinitroanilines is the result of a single nucleotide substitution (i.e., point mutation) in the nucleotide sequence of the αl-tubulin gene, leading to amino acid substitution of I1e for Thr239.[27–29] Because Thr239 is a highly conserved amino acid residue in α1-tubulin in many eukaryotes (plants in particular), Cronin et al.[27] assumed that its replacement with Ile in α-tubulin, as well as analogous substitutions of Thr237 in β-tubulin and Thr240 in γ-tubulin, could lead to the occurrence of an altered tubulin that determines the increased resistance of microtubules to dinitroaniline herbicides.[27]

Similar results were obtained independently from genetic and molecular analyses of dinitroaniline-resistant and dinitroaniline-susceptible *E. indica* biotypes.[30–33] Also, a "new" mutant α-tubulin isoform was detected in a more basic position.[30] While studying all three isotypes of α-tubulin cDNA (TUA1, TUA2, and TUA3), each isolated from the three biotypes of *E. indica* highly resistant (R), intermediate (I), and susceptible (S), Yamamoto et al.[32] found nucleotide differences between sensitive and resistant α-tubulins in TUAI and TUA2. Missense mutations in TUA1, which led to the substitution Thr239Ile in the R-biotype and Met268Thr in the I-biotype were the biologically significant differences. The importance of these mutations in conferring herbicide resistance in the R- and 1-biotypes was supported by allele inheritance and transcript sequence analysis.[32]

In the case of *S. viridis*, immunoblotting of tubulin, following one-dimensional[23,34] and two-dimensional SDS-PAGE,[26] with antibodies specific to α- and β-tubulin subunits[23,26,34] or to microtubule-associated proteins[34] revealed no qualitative or quantitative differences between the dinitroaniline-resistant and dinitroaniline-sensitive plants. However, recent analysis of tubulin sequences from the two lines revealed two amino acid substitutions in α2-tubulin that determine resistance to these compounds: Leu136Phe and Thr239Ile.[35] Both mutations resulted in cross-resistance to different dinitroanilines without changing the sensitivity to benzimidazoles and hypersensitivity to carbamates. Another mutation in the β-tubulin gene leading to the substitution Arg241Lys was identified in dinitroaniline-resistant *P. annua* plants.[36] Thus, mutant genes for each of the tubulin subunits can be used in experiments to develop new marker gene systems to obtain transgenic plants.

3. *In vitro* selection of dinitroaniline- and phosphorothioamidate-resistant plants

Herbicide-resistant plants can be effectively artificially created, because *in vitro* cell selection methods make it possible to detect and select mutant

cells with resistant genotypes and regenerate them into whole plants. This approach was first used to select mutant lines of higher plants resistant to dinitroanilines and phosphorothioamidates. As a result of using γ-irradiation mutagenesis, and direct cell selection, we obtained mutant *Nicotiana plumbaginifolia* lines resistant to APM[37–39] and trifluralin.[40–42]

The level of tolerance in the APM-resistant line was higher by an order of magnitude than in the original genotype, and the level of tolerance in the line selected for trifluralin-resistance was seven times higher than in the original, control line. It was found that the traits of resistance to APM and trifluralin are inherited in the generations of F_1 and F_2 mutants. The results of genetic analysis showed that, in the APM-selected line, resistance was inherited as a dominant nuclear trait[38,39]; whereas, in the trifluralin-selected line, resistance was inherited as a semidominant nuclear trait.[15,40] The APM-resistant mutants also exhibited cross-resistance to trifluralin, and the trifluralin-resistant mutants were cross-resistant to amiprophosmethyl. Stable expression of resistance to these herbicides was observed.

Because it was assumed earlier that both herbicides have similar binding sites on the tubulin molecule,[22,43–45] tubulin from both mutant lines was analyzed by two-dimensional gel electrophoresis and immunoblotting. It was found that both APM- and trifluralin-resistant plants contained an altered (ß-tubulin isoform. It was also established that the tubulin isolated from the APM-resistant plants exhibits a drastically decreased affinity for APM.[46] These findings strongly suggest that the presence of altered β-tubulin isoforms in the selected and regenerated *N. plumbaginifolia* lines may be the result of mutations in gene(s) associated with the acquisition of resistance to herbicides with antimicrotubular activity.[38–40]

With this in mind, we used the APM-resistant *N. plumbaginifolia* mutants to isolate and sequence the β-lubulin gene that confers resistance to this herbicide. It was found that the mutation results in the substitution of Pro for Ser in one β-tubulin gene.[47] Thus, along with the mutant α-tubulin genes that confer resistance to dinitroanilines/phosphorothioamidates, this gene also can be used as a marker-trait for transgenic plant selection and to obtain plants resistant to this class of herbicides.

4. *In vitro* selection of phenylcarbamate-resistant plants

Phenylcarbamate herbicides, the most widespread of which are IPC and CIPC, are antimitotic agents affecting microtubule organizing centers and/or solely microtubules. The mechanisms of action of these compounds is still debated because there is evidence that CIPC disrupts solely the functioning of MTOCs, leading to fragmentation of poles and formation of multipolar spindles with subsequent formation of abnormal (branched)

phragmoplasts, as was shown for onion root tip[48–50] and carrot suspension-culture[51] cells. Results obtained with oat cells, indicate that CIPC may disrupt both MTOCs and microtubules depending upon the concentration used.[52] Other data demonstrated that the target of CIPC is solely microtubules (the latter were destroyed by treatment with CIPC in wheat roots),[53] and that CIPC inhibits polymerization of plant microtubules *in vitro*.[6] Furthermore, experiments performed with tobacco suspension culture cells showed that CIPC most likely binds to β-tubulin at the benzamide binding site.[54] Attempts were made to clarify the mechanism of action of the less widely used agent EPC, in experiments with rice mutants resistant to this compound.[18] It was concluded that the C-terminal moiety of α-tubulin is for the site of EPC binding.[19]

The mechanism of action of IPC also remains to be clarified. To date, it is only known that it has no effect on the *in vitro* polymerization of bovine brain tubulin,[55,56] nor on the assembly of plant microtubules *in vivo*.[55] The most typical effect of IPC treatment is the formation of tri-polar or multipolar spindles, which was observed in phylogenetically remote organisms, such as the green alga *Oedogonium cardiacum*,[57] the higher plant *Haemanthus katherinae* Baker[58] and *N. sylvestris*,[59] as well as 3T3 fibroblasts.[60]

To study the mechanism of action of IPC on plant cells in more detail, *in vitro* selection of mutated cell lines of *N. plumbaginifolia*[61] and *N. sylvestris*[62,63] was performed using 30 μM IPC. The mutant plants obtained exhibited cross-resistance to EPC, but were sensitive to CIPC[61], which may be indicative of different mechanisms of action for IPC and CIPC (as was described above). IPC-resistant *N. sylvestris* plants also exhibited a decreased sensitivity to nocodazole (a benzimidazole that depolymerizes microtubules), whereas the percentage of aberrant metaphases, anaphases, and telophases in the control line increased two to three times after nocodazole treatment. In the cells of the control line, nocodazole at concentrations of 100–500 μM induced total DNA fragmentation in all cell-cycle phases, including interphase. Conversely in the mutant, DNA fragmentation was not observed at any nocodazole concentration.[64]

Immunofluorescence analysis using monoclonal antibodies specific to α- or β-tubulins showed that both microtubules and MTOCs in the cells of IPC-resistant lines are not destroyed in the presence of IPC, whereas cells in the control line retained intact cortical microtubules but the localization of MTOCs was disturbed. Following IPC treatment of the mutant lines, MTOCs were not destroyed, but remained associated with the perinuclear membrane and the nuclear periphery, which is characteristic for plant cells. However, in cells of the control line, the formation of multipolar spindles and branched phragmoplasts was observed.[59,63] These data once again

strongly support the conclusion that the target of IPC is the microtubule nucleation sites rather than the microtubules themselves.

Although the trait of IPC resistance was stably expressed in different lines of *Nicotiana*, it was impossible to perform genetic analysis and determine the mutation type because the majority of IPC-resistant mutants are infertile.[61] For example, one mutant *N. plumbaginifolia* line was characterized by growth delay and a complete absence of apical dominance. As a result the plant was unable to flower. The infertility of other lines resulted from the abnormal development of flowers or production of underdeveloped seeds.[61] Growth and fertility of mutant *N. sylvestris* plants was also depressed, which manifested itself in a reduced habit and size of vegetative and generative organs and semi-sterility of pollen, as determined by aberrant microsporogenesis.[65,66] Furthermore, it was established that the low seed productivity was caused by high pollen sterility (84.51±0.71%) and decreased viability of ovules.[65,66]

5. Modeling the three-dimensional structure of tubulin and herbicide docking into their binding sites

The relationship between changes in the amino acid sequence of mutant tubulins of plant origin (the mutant α1-tubulin genes from dinitroaniline-resistant *E. indica*, and the β-tubulin gene from APM-resistant *N. plumbaginifolia*) and their spatial interaction with antimicrotubular herbicides, was investigated. These results were obtained by modeling the spatial structure of higher plant tubulins, based upon the established three-dimensional structure of porcine tubulin (http://www.rcsb.org/pdb),[67] and the potential interaction sites of α- and β-tubulins with dinitroanilines and phosphorothioamidates. Residue Thr239 is highly conserved in all known fungal, plant, and animal α-tubulins. An analysis of the sequences flanking the mutation site showed that they also are conserved, though not identical in all plant α-tubulins.

To analyze the interaction specificity of dinitroanilines and phosphorothioamidates with tubulin from R- and S-biotypcs of *E. indica*, three-dimensional structures of both types of α-tubulins were constructed, including the electrostatic potential distribution on their surface.[21,68] A comparison of the spatial models of α-tubulins from R- and S-biotypes with regard for the position of Thr239 made it possible to identify the dinilroaniline binding site. It is located on the surface of the α-tubulin molecule in the interdimer contact zone.[68] The substitution of Leu for Thr at position 239 leads to considerable changes in the distribution of electrostatic charge on the surface of the α-tubulin molecule. This redistribution of surface energy, as a result of the C to T point mutation, is apparently due to

the rearrangement of side chains of neighbouring amino acid residues located on and near the surface of the tubulin molecule. This process is accompanied simultaneously by conformational changes in the predicted herbicide binding site on the α-tubulin molecule. Thus the substitution of Thr239, which is located in the vicinity of the seventh helix, can markedly alter the three-dimensional structure of the α-tubulin molecule.[68]

Modelling the Thr239Ile substitution revealed a drastic decrease in the positive electrostatic potential at the herbicide interaction site.[21,68] As a result., a redistribution of charge, accompanied by partial closing (narrowing) of the interaction cavity, takes place. Therefore, the affinity of this site for dinitroaniline and phosphorothioamidate herbicides appears to decrease dramatically.[21,68]

Analysis of the three-dimensional structure of trifluralin and APM revealed a similar spatial geometry for both types of molecules as well as similarly distributed surface potentials.[68] All active compounds from these herbicide groups are characterized by the presence of common structural elements – nitro groups that are linked to the benzene ring. This basic similarity implies that the polar (electronegative) moiety of their molecules should play a key role in interaction with tubulin. Therefore, we performed spatial docking of dinitroanilines and phosphorothioamidates into the binding sites on the surface of α-tubulin molecules from sensitive and resistant *E. indica* lines.

It was found that the hydrocarbon tails of the herbicides are oriented along amino acid residues Glyl33, Asn253, and Gly256. These amino acids are located on the surface of the α-tubulin molecule, and are involved in interaction with amino acid residues of the β-tubulin molecule of the next heterodimer. A clearly distinguishable and distinct cavity, with a high electrostatic charge, is formed in this area on the interdimer surface of the α-tubulin molecule.[68] Although amino acid residue Thr239 is not exposed on the surface of α-tubulin, it is located immediately under this cavity. Interactions of negatively charged NO_2-groups, which is a common structural feature of both dinitroanilines and phosphorothioamidates, with the identified binding site on the α-tubulin molecule can have significant consequences leading to the prevention of further microtubule polymerization. Furthermore, the position of the bound herbicide ligand will be stabilized as a result of additional interaction with negatively charged groups (such as $–CF_3$ or $–SO_2$) with the NH_2-group of Arg2.[68] Thus, these tubulin-ligand complexes acquire a sufficient level of stability so as not to dissociate under the influence of internal molecular oscillations.

Results from comparative analyses of the three-dimensional models of α-tubulins from the R- and S-biotypes of *E. indica* were confirmed by a subsequent investigation of the mutant α-tubulin from *S. viridis*.[35] In that

study it was concluded that the binding site for the dinitroanilines is located immediately adjacent to the contact zone between tubulin dimers, whereas modeling of the α-tubulin molecule from the protozoan *Toxoplasma gondii* assumed that dinitroaniline binding site is located in zone of lateral contacts between the microtubule protofilaments.[69] In the same time, the binding sites predicted for α-tubulins of higher plants and protozoans overlap partially. Therefore, it can be concluded that the binding patterns of dinitroanilines with tubulins in the two groups of eukaryotes are slightly different, likely as a result of their phylogenetic distance.

The results of constituting three-dimensional models of β- and γ-tubulins indicate that the Thr residues at positions 237 or 240, respectively, do not participate in the formation of the herbicide interaction cavity (i.e., it was absent in both cases).[70] The substitution of Thr with Ile at these positions does not lead to marked alterations or functional changes of the surface of β- and γ-tubulins. Hence, resistance of tubulin to dinitroanilines and phosphorothioamidates is determined not only by the position and nature of the substituted amino acid, but also by its spatial microenvironment formed with neighbouring amino acid residues. Thus, the earlier postulate that the substitution of Thr237 and Thr240 in the β- and γ-tubulin molecules, respectively, should result in the emergence of an altered tubulin with an increased resistance to dinitroaniline herbicides[27] is disputed by the results of spatial modelling.

A comparative analysis of three-dimensional structures of β-tubulin molecules from the APM-resistant and APM-sensitive *N. plumbaginifolia* lines, allowed us to identify the mutable residue Ser248, which is located in the interdimeric contact zone.[47] This amino acid residue, similarly to residues Lys252 and Lys350, is involved in the formation of the cavity that may be the interaction site with phosphorothioamidates and dinitroanilines, as well as in the creation of an altered microenvironment. The substitution of Ser248 with Pro nearly blocks the cavity capable of interacting with the herbicides. These results coincide with our data for an analysis of the structure of resistant and sensitive isoforms of β-tubulin from *Chlamydomonas reinhardtii*, for which the critical role of a missense mutation at Lys350 in the emergence of resistance to phosphorothioamidates and dinitroanilines was further demonstrated.[71]

The interaction site(s) of phenylcarbamates with plant tubulin remain to be determined precisely. However, it is known that some phenylcarbamates (CIPC in particular) compete for binding sites with benzimidazoles on the β-tubulin molecule.[54] It was also established that fungal resistance to benzimidazoles may be determined by point mutations in the β-tubulin sequence, leading to the substitution of Glul98 with Ala, Val, or Gly, or the substitution of Phe200 with Tyr.[72] The substitution of the amino acid

residue at position 198 in β-tubulin from *N. crassa* leads to negative cross-resistance to phenylcarbamates,[73] whereas the substitutions of Leu250 with Phe, Ala265 with Val, and Thr237 with Ala confer cross-resistance to phenylcarbamates.[74] The results of comparative analysis of the spatial structure of plant β-tubulin indicate that all these mutations are located in the same area. that is in the vicinity of the interdimeric contact zone. However, only residues Leu250 and Ala165 are exposed on the surface of the molecule.[75] Thus, it cannot be ruled out that at least some phenyl-carbamates whose structure is similar to the structure of benzimidazoles may have the same binding site on the surface of β-tubulin molecule, provided certain interaction conditions are met.

A key conclusion resulting from the analysis of the molecular genetic and structural biology of plant tubulins resistant to various types of herbicides with antimicrotubular activity is that mutant tubulin genes that determine resistance to these compounds can be used as selectable markers for the production of genetically modified plants. Furthermore, taking into account the fact that plant tubulin is a specific target for many widely used antimicrotubular agents[76] and for some recently discovered agents (e.g., pyrimidine derivatives[77] and cyanoacrylates[78]), the spectrum of mutant tubulin genes conferring resistance to antimicrotubular agents may be considerably broadened in the near future.

6. Transfer antimicrotubular herbicide resistance via somatic hybridization

Because construction of vector carrying a mutant tubulin gene for genetic transformation also required solving fundamental problems, including the identification of the mutant gene, cloning, characterization of the mutation, tests of the functional efficiency of such a gene, etc., and is highly time consuming, it was proposed to use somatic hybridization as the preliminary experimental system.[15] Today, somatic hybridization remains an alternative method of transfer of genes from one plant species to another. Initial efforts focused on transferring mutant β-tubulin genes from herbicide resistant *N. plumbaginifolia* lines to closely related and to distant species using symmetric and asymmetric somatic hybridization.[79–83]

The trait of resistance to APM or trifluralin was transferred to recipient *N. sylvestris* and *Atropa belladonna*. For targeted partial transfer of nuclear material (asymmetric hybridization), cells of mutant *N. plumbaginifolia* lines were inactivated by lethal radiation. Following cell fusion and selection in the presence of APM or trifluralin, a series of interspecific and intertribal hybrids of *N. plumbaginifolia* + *N. sylvestris* and *N. plumbaginifolia* + *A. belladonna* with resistance to APM or trifluralin, respectively, were obtained. The results

of cytogenetic analysis and inheritance of the resistance trait in the first generation of fertile somatic hybrids indicated that their resistance to APM and trifluralin is the direct result of the transfer of genetic material from the donor *N. plumbaginifolia* mutants.[79–83]

As with the initial *N. plumbaginifolia* mutants,[38–40] immunofluorescence analysis of microtubules showed that the cortical and mitotic microtubules of the protoplasts of *N. plumbaginifolia* + *N. sylvestris* and *N. plumbaginifolia* + *A. belladonna* hybrids are not destroyed after treatment with APM[79,80,82,83] and trifluralin,[81] whereas the microtubules in the protoplasts of the parental *N. sylvestris* and *A. belladonna* lines were completely depolymerized by the herbicide treatments. Therefore, the resistance of hybrid microtubules can be explained by the presence in them of the mutant β-tubulin isoform, which is expressed as a result of transfer of the mutant β-tubulin gene from APM- or trifluralin-resistant *N. plumbaginifolia*. The results of two-dimensional electrophoresis and immunoblotting of tubulin from hybrid and parental plants confirmed that *N. plumbaginifolia* + *N. sylvestris* and *N. plumbaginifolia* + *A. belladonna* hybrids express, along with α- and β-tubulin isoforms characteristic of the recipient, an additional β-tubulin isoform that is characteristic of the *N. plumbaginifolia* mutants.[79,81–83] This was the first proof of concept with somatic hybrids for the potential development of selectable markers based upon mutant tubulin genes conferring resistance to herbicides with antimicrotubule modes of action.

7. Creation of transformation constructs with mutant tubulin gene as a selectable marker

Considering the selective potential of dinitroanilines and phosphorothioamidates, and the success of using the resistance trait as a marker to obtain stable somatic hybrids, further development of the technology was based on designing vector constructs containing the mutant tubulin gene as a selectable marker for plant transformation. In principle, the use of tubulin genes in plant genetic engineering can help solve certain problems associated with the acquisition of resistance to various classes of antimicrotubular herbicides,[15,84–86] cold resistance,[86,87] cell elongation,[88] and even regulation of cellular architecture on the whole, including the gravitropic response, branching, rooting, plant height, and yield.[86] Various aspects of plant transformation with tubulin genes, including chimeric GFP-tubulin genes, have been described in earlier reviews.[15,89] Practical steps to obtain transgenic plants resistant to dinitroanilines and phosphorothioamidates were taken previously, however the authors could regenerated into plants only the model plant tobacco.[29,84,85]

To develop approaches for the use of mutant tubulin as a marker gene in transgenic plant selection, we also used a clone of the well-characterized mutant α1-tubulin gene from the R-biotype of *E. indica*.[32] The full-length β1-tubulin gene from barley was also used in these experiments. The latter was either inserted into the constructs carrying the mutant α-tubulin gene or used to construct separate vectors, because the presence of exogenous genes of both tubulin subunits makes it possible to achieve their required equimolar co-expression to function in native microtubular networks of transgenic plants.[85] The constructs for transformation of monocots and dicots were designed; the selective marker gene was transferred by biolistic or *Agrobacterium tumefaciens* mediated transformation.[90] For biolistic transformation of monocots, tubulin genes in individual vectors were inserted downstream of the constitutive ubiquitin maize promoter. For dicot transformation employing biolistics, the tubulin genes were used under the control of the constitutive 35S promoter. For *Agrobacterium*-mediated transformation, a construct in which both genes were located downstream of the 35S promoter was created.

The efficiency of the transformation systems with the *TuAm* mutant tubulin gene was tested using several monocot and dicot species, including barley (*Hordeum vulgare*), finger millet (*Eleusine coracana*), soybean (*Glycine max*), flax (*Linum usitatissimum*), and tobaccos (*N. plumbaginifolia* and *N. sylvestris*). For each of these species, protocols for transformation and selection of transgenic plants were developed. Trifluralin, the most efficient dinitroaniline herbicide, was used as a discriminating agent in experiments to select transgenic cells, tissues and plantlets. Effective trifluralin concentrations for each plant species were determined according to an established protocol[83,91] and the range of selective concentrations of this selective agent was determined (i.e., 10 μM for finger millet, soybean, *N. plumbaginifolia* and *N. sylvestris,* 3 μM for flax[90]). As a result of selection, dinitroaniline- and phosphorothioamidate-resistant transgenic lines of these species were obtained. The transgenic nature of the selected plants was confirmed using Southern and Northern blot hybridization and PCR analysis with a specific probes to the *nptII,* α-tubulin or β-tubulin genes. Band specific for the mutant α-tubulin gene was identified in transformed lines. Results confirmed the stable integration of the mutant tubulin gene into each species' nuclear genome. The present study clearly demonstrates the use of a mutant plant tubulin as a selectable gene for plant transformation as well as for producing dinitroaniline-resistant plants.

8. Conclusions

The results summarized in this review indicate that the understanding of tubulin gene structure and function, and the mechanisms of resistance of this protein to antimicrotubular drugs, together with application of functional genomics and bioinformatics, make it possible to develop efficient genetic engineering technologies to obtain genetically modified plants. This is possible through the design of transformation vector constructs containing new selectable markers – mutated tubulin genes conferring resistance to herbicides with antimicrotubular mechanism of action. This concept has considerable potential for further development, because tubulin is the key target for many compounds of various chemical nature. Simultaneously, the use of mutant tubulin genes conferring resistance to the dinitroanilines and phosphorothioamidates will allow the direct development of new varieties of cultivated plants resistant to these herbicides using genetic engineering methods.

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