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*Review*

# Plant tubulins: a melting pot for basic questions and promising applications

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## Why it is worth considering microtubules

Two recent results emerging from very different experiences exemplify the great importance of the plant cytoskeleton and microtubules (MTs). Attempts to isolate morphogenetic genes by screening for embryogenesis mutants in *Arabidopsis thaliana* did not uncover the expected homeotic genes but rather genes that are related to the formation of the cell plate or the symmetry of cell division (Mayer et al., 1993; Shevell et al., 1994; Lukowitz et al., 1996). Decades of continuos application of dinitroaniline herbicides used against weeds in cotton, soybean, wheat and oilseed crops has resulted in the selection of resistance against these compounds. In one of these weeds, goosegrass, the molecular cause of this resistance has been uncovered recently and shown to be linked to a mutation in the coding sequence of α-tubulin (Yamamoto et al., 1998; Anthony & Hussey, 1999a). So, these two apparently distant findings stress the same point – MTs are fundamental structures deeply involved in plant growth and development. Despite this, so far plant MTs have been substantially neglected as tools for applications in modern plant biotechnology. This nonchalance is both undeserved and astonishing since MTs represent versatile tools for the manipulation of plant morphogenesis and development:

1. They control numerous aspects of plant morphogenesis and adaptation to the environment.
2. Their major components, the tubulins, comprise a family of different members with specific patterns of regulation and different molecular properties. In other words, plant tubulins are ideal targets for approaches that are both subtle and specific.
3. The microtubular cytoskeleton of plants is functionally distinct from that of animals, and this allows for the design of chemical agents, such as herbicides or fungicides, that are toxic for plants but safe for animals.

The functions of plant MTs are numerous, and not confined to the establishment and movement of the division spindle. Plants have evolved specific microtubule arrays that serve to control cell shape in response to external signals such as light, gravity or mechanical strains, or to internal signals such as hormones or developmental state (for review see Nick, 1998). This microtubular response represents a key step in the flexibility of morphogenesis that is characteristic for plants. In interphase cells, the cortical microtubules control the direction that new cellulose microfibrils are deposited (Figure 1(a)) and, thus, the mechanic properties of the expanding cell wall (Giddings & Staehelin, 1991). It is possible to change cell shape merely by manipulation of cortical microtubules. In dividing plant cells it is a band of microtubules, the preprophase band, that marks the axis and symmetry of cell division (Figure 1(b)), and a different microtubule array, the phragmoplast, organizes the formation of the new cell plate following nuclear division (Figure 1(c)).

In addition to the functions essential for cellular morphogenesis, MTs participate in the response to abiotic and biotic stresses. They can disassemble in response to low temperature (Figure 1(d)) modulating the sensitivity of cold-sensitive calcium channels (Mazars et al., 1997). They are essential for an effective defence against fungal pathogens (Kobayashi et al., 1997), probably by guiding secretion towards the penetration site (Figure 1(e)). On the other hand,

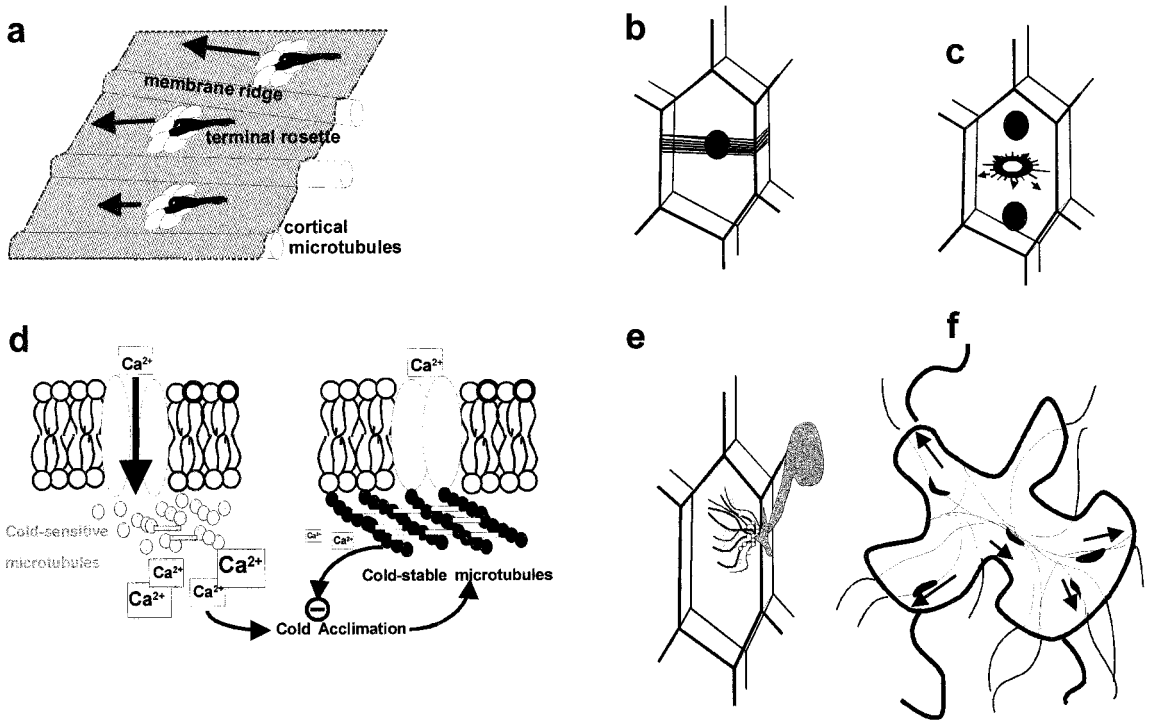


*Figure 1.* Different functions of microtubules in plant morphogenesis and during responses to abiotic and biotic stresses. (a) Control of cellulose deposition by cortical microtubules. The terminal rosettes, consisting of cellulose-synthetizing enzymes are guided by cortical microtubules through the plasma membrane resulting in the formation of microfibrils that are parallel to the microtubules. Transverse orientation of microtubules will result in a transverse orientation of microfibrils leading to cell elongation. (b) Formation of the microtubular preprophase band defines the axis of cell division by organization of the spindle in a direction perpendicular to the preprophase band. (c) The microtubular phragmoplast guides the transport of vesicles containing cell-wall matrix and thus controls the formation of the new cell plate following mitosis. (d) Microtubules disassemble in response to cold amplifying the activity of cold-sensitive calcium channels. The formation of cold-stable microtubules might modulate the activity of these channels allowing a balanced cold response. (e) Microtubules, together with actin microfilaments, prevent fungal attack in resistant cultivars by guiding vesicles containing callose and antifungal compounds towards the penetration site. (f) Microtubules are usurped by plant viruses as guiding tracks for cell–cell movement through the plasmodesmata.

microtubules are usurped by certain viruses (Heinlein et al., 1995) as guiding tracks for cell–cell movement (Figure 1(f)).

Similar to their animal counterparts, plant MTs are composed of heterodimers made up of one α-tubulin subunit and one β-tubulin subunit of similar molecular weight (between 50 and 55 kDa). These dimers assemble into hollow cylinders that are composed of 13 columns, the so-called protofilaments. The term cytoskeleton evokes the idea of a rigid framework that stabilizes the structure of the cell. Such associations are far from reality – the half-time of a plant microtubule has been calculated at about a minute (Moore et al., 1997)! Thus, it is more appropriate to figure the plant cytoskeleton as an everchanging river whose direction and width is defined by the relation between influx and efflux. The influx, in this image, is represented by the tubulin dimers that are assembled at the ends of the microtubule, the efflux by the heterodimers that dissociate from the microtubule ends. Interestingly, the relation between influx and efflux is practically never balanced – there is always one dominating over its antagonist. This statement is valid in both space and time. In space, because dimer addition and dispersal define a distinct polarity of each individual microtubule with dimer addition dominating at the plus end, dimer dissociation at the minus end. In time, because each microtubule can switch between a growing state when dimer addition at the plus end predominates over dimer dissociation at the minus end, and a shrinking state, when dimer dissociation at the minus end exceeds dimer addition at the plus end. The switch between both states is swift and dramatic, so dramatic that it has been termed microtubule catastrophe. The frequency of these conversions depends on associated proteins that can increase or

decrease the transition from one state to the other. With a few exceptions, these microtubule-associated proteins (MAPs) are neither identified in plants nor is it understood how they shift the frequency of microtubule catastrophes. Although a couple of microtubule binding proteins that have been discussed as structural MAPs have been recently isolated, their biological function remains rather unclear (Kumagai et al., 1999; Chan et al., 1999). In contrast some insight into the function of a second group of MAPs, microtubular motors of the kinesin-family, seems to emerge from the analysis of respective mutants in *Arabidopsis*, where mutations in the genes coding for these microtubular motors results in specific alterations of trichrome morphogenesis (reviewed in Oppenheimer, 1998). Plant MAPs seem to be fairly different from their animal counterparts, because several attempts to clone them via sequence homology have failed so far. The only exception seems to be γ-tubulin (Liu et al., 1994), a distant relative of α- and β-tubulins that seems to play a role in the nucleation of new MTs and initiates the ring of 13 dimers that are the primers of the protofilaments.

Although molecular access to plant MAPs is still limited at the moment, it is possible to manipulate microtubule regulation via the basic components of microtubules, the α- and β-tubulins. They exist in several forms that may conferr different properties to the microtubule (possibly by rendering it competent for interactions with specific MAPs). During the past few years, the molecular biology of plant tubulins has advanced rapidly. This review will, therefore, survey first the structural and pharmacological features of plant tubulins and then the regulatory aspects that control tubulin gene expression and accumulation. Ultimately, biotechnological applications that can exploit the uniqueness of the tubulin system will be described.

## Tubulin, the main constituent of microtubules: biochemical and pharmacological aspects

Tubulin is a heterodimeric protein resulting from the non-covalent association of an α and a β polypeptide (Fosket & Morejohn, 1992). These two related subunits are encoded by discrete genes that can be grouped into two corresponding small gene families: the α- and the β-tubulin gene families (Goddard et al., 1994). The α-tubulin gene family is further divided into subfamily I and II (Villemur et al., 1992). Studies of nucleotide and amino acid sequence composition conclude that these families separated early in the evolution from one ancestral β-tubulin gene and two different α-tubulin gene precursor. In the course of evolution, additional amino acid changes were progressively introduced within each of the different groups, and this eventually resulted in a range of individual α- and β tubulin isotypes. The complexity of the tubulin system is further enhanced by post-translational modifications, some of which interfere with microtubule stability (Smertenko et al., 1997; Huang & Lloyd, 1999). Thus, MTs can differ from each other depending on the presence of distinct α- and β-tubulin isotypes and the occurrence of specific modifications. This defines subpopulations of MTs that may have exclusive properties. These properties could originate either from the presence of unique tubulin isotypes that endow the MT structure with a specific function (functional hypothesis) or could simply result from the presence of a given tubulin isotype that, although functionally interchangeable, could modify some features of the MT array, that is, making it less dynamic or cold-stable. Since no evidence for functional specificity of distinct plant tubulin isotypes has been demonstrated yet, functional interchangeability of the different α- and β-tubulin isotypes is more widely accepted. As a consequence, tubulin polymorphism is considered the result of differential expression occurring in a given tissue or stage of development. A third, intermediate model is based on the co-evolution between MAPs and their target tubulin isoform. This debate about the significance of tubulin polymorphism is not just a matter of academic concern, but provides the basis for the design of potentially useful biotechnological strategies.

Many of the differences in the amino acid composition that define specific tubulin isotypes are confined to regions of the α- and β-tubulin proteins that are not involved in the basic functions of MT assembly and disassembly, dimer formation and GTP binding and hydrolysis that are absolutely indispensable. The amino acid differences are in the range of 10–15% of variability that represent the molecular basis for differential responses of MTs and their differential sensitivity to drugs, pathogens and stress conditions. This type of structure combines essential (and therefore highly conserved) with variable features and renders tubulin a key molecule for the manipulation of plant growth, development and related biotechnological applications. In fact, amino acid domains essential for cell growth and division represent the best target for herbicides and, in animals, for anti-cancer drugs. Plant tubulin domains not involved in basic functions can instead be

manipulated in attempts to produce MT arrays more resistant to cold, drugs, or herbicides (Figure 2).

Assembly and disassembly of MTs requires GTP binding and hydrolysis (Burns & Farrel, 1996). Conversely, plant α- and β-tubulin polypeptides contain in their amino acid structure domains that are able to bind GTP (exchangeable for β-tubulin, not exchangeable for α-tubulin) as well as a conserved motif putatively involved in GTPase activity (Davis et al., 1994; Figure 2). Mutational analysis with animal tubulins that maintain similar domains as plant tubulins have shown that GTP plays a structural role in tubulin folding (Tian et al., 1996; Zabala et al., 1996; Tian et al., 1999). GTP binding sites on α- and β-tubulin are also the targets for the oncoprotein 18/stathmin (Op18), highly expressed in leukemia cells (Belmond & Mitchinson, 1996; Moreno et al., 1999).

All plant β-tubulins and subfamily II of α-tubulins contain at their N-terminus the tetrapeptide motif MREI (Figure 2). This motif, which is also present in animal β-tubulins, has been shown to have a regulatory role in controlling tubulin accumulation when MTs are poisoned with anti-mitotic drugs such as colchicine (Cleveland et al., 1983; Cleveland, 1988). Increase of tubulin dimers caused by colchicine-mediated microtubule depolymerization or by microinjection of exogenous tubulin reduced the stability of tubulin-mRNA leading to a net decrease in the amount of tubulin transcript (Gay et al., 1989). The presence of a similar regulatory mechanism has not been shown in any plant system. In contrast, when rice MTs are depolymerized by oryzalin, unpolymerized α- and β-tubulin heterodimers are rapidly degraded with no significant changes in the amount of their corresponding mRNAs. This has been shown for rice coleoptiles, roots and, recently, in a cell culture system (Gianì et al., 1998; Breviario & Gianì unpublished observations).

$Ca^{2+}$ binds tubulin molecules with high affinity and causes MT destabilization. A similar destabilizing effect of $Ca^{2+}$ on plant microtubules was shown recently to be related to the modulation of cold-sensitivity (Bokros et al., 1996). Taxol-stabilized maize MTs containing a β-tubulin chain where the last 15 amino acids have been deleted were more resistant to cold-induced depolymerization than wild type MTs. Expression studies in *Arabidopsis thaliana* and rye roots have shown patterns of preferential expression of specific tubulin-isotypes in response to low temperatures (Kerr & Carter, 1990; Chu et al., 1993). In *Arabidopsis thaliana*, those tubulin isotypes characterized by a shorter C-terminus (*TUB9)* maintain or

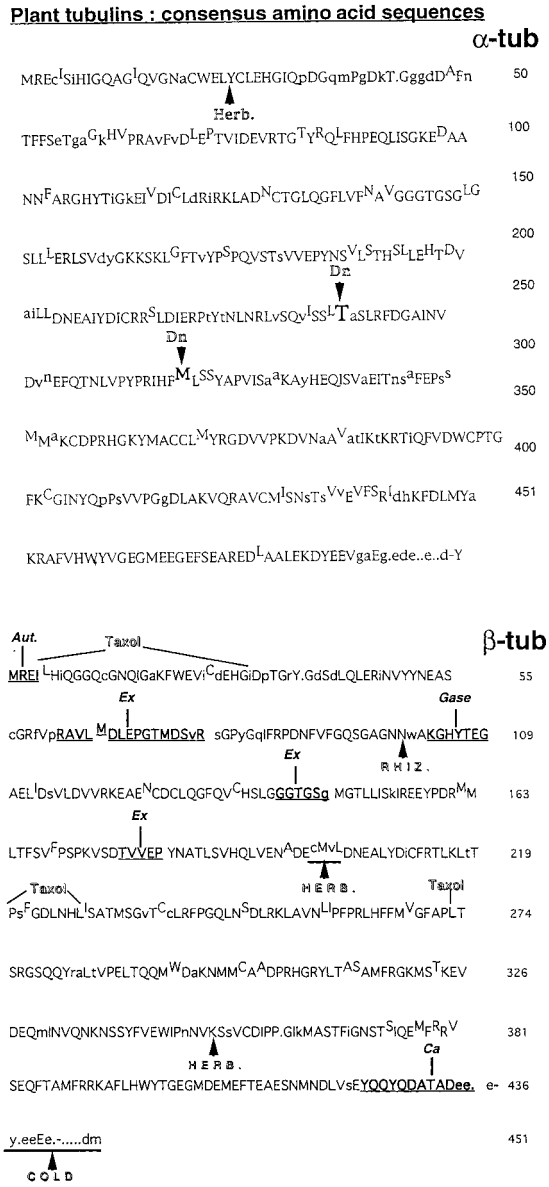


*Figure 2.* Consensus amino acid sequences were obtained by comparison among members of the α- and β-tubulin gene family of rice, maize, soybean and *Arabidopsis thaliana*. Amino acid residues typed in bold and underlined show the domains involved in putative mRNA autoregulatory expression (Aut.), GTP exchange (Ex), GTPase activity (Gase) and calcium binding ( Ca). Black arrows indicate those domains that are involved in the susceptible response to rhizoxin (Rhiz.), low temperatures (cold) and antimicrotubular herbicides (Herb) with a more specific reference to dinitroanilines (Dn) for α-tubulin. The presumed binding-sites for taxol are also shown. The consensus amino acid sequence is typed in capitals when the residue are fully conserved among the different plant tubulin members, in lower case when not all the compared sequences show the same amino acid residue. In that case, the amino acid more frequently present is shown. Amino acid residues in superscript refer to those plant residues that are different from those of their vertebrate tubulin counterparts. Dots indicate lack of consensus.

even increase their level of transcriptional expression. This led to the idea that plant MTs could withstand the effect of cold-mediated depolymerization better if they contain tubulin isotypes with shorter C-termini. In conclusion, correlations of cold resistance to the presence of specific tubulin isotypes in plant MTs have been uncovered.

Plant tubulin binds dinitroaniline herbicides (i.e. oryzalin) with high affinity in a rapid and reversible fashion. Dinitroanilines are antimicrotubular drugs that heavily damage plant MT structure with no apparent effect on animal MTs (Morejohn et al., 1987; Hugdahl & Morejohn, 1993; Hoffman & Vaughn, 1994). Amiprophosmethyl is equally effective as oryzalin in tubulin binding (Ellis et al., 1994; Murthy et al., 1994). Both herbicides are much more hydrophobic than colchicine and have much higher affinities for plant tubulin. Plant cells treated with oryzalin loose their anisotropy as a consequence of the loss of the cortical microtubule arrays (Gianì et al., 1998). It has been proposed that an additional effect of poisoning plant MTs with oryzalin is that of the activation of transmembrane calcium channels. This would lead to an increase in the level of the cytoplasmic concentration of $Ca^{2+}$ with an additional destabilizing effect on MT structure and assembly (Mazars et al., 1997; Thion et al., 1998). However, this is probably a secondary effect, since oryzalin can bind to tubulin directly.

The first evidence for a binding of dinitroanilines to tubulin came from the identification of three *Chlamydomonas* mutants that are resistant to colchicine and amiprophosmethyl (Schibler & Huang, 1991; James et al., 1993). These mutants were also resistant to dinitroanilines, but exhibited supersensitivity to taxol, a microtubule-stabilizing drug with a different binding site. Two of the mutations are located in the β-tubulin chain and seem to affect general MT stability rather than affecting the binding-sites for the herbicides (Schibler & Huang, 1991). In contrast, the third mutation (*upA12*) was shown to cause an exchange of the residue Tyr24 by an His residue in the α-1 tubulin polypeptide chain (James et al., 1993).

Further tubulin mutations have been identified in grasses. For a long time, dinitroanilines have been widely used for controlling monocotyledonous weeds in several crops including soybean, cotton, and wheat (Holt et al., 1993; Zeng & Baird, 1997; Anthony & Hussey, 1999a). This practice has caused the emergence of resistant biotypes, for instance in the weed *Eleusine indica* (goosegrass). These biotypes are capable of growing in the presence of dinitroanilines. Two laboratories recently analyzed the molecular basis of this resistance, and found that it is strictly associated with a single amino acid mutation occurring in the major α-tubulin isotype (*TUA1*) of goosegrass (Yamamoto et al., 1998, Anthony et al., 1998). A mutation converting Thr239 to Ile was found in the *TUA1* gene of the highly resistant (R) biotype. An intermediate degree of resistance (I biotype) was instead associated with the presence of a mutation in the *TUA1* gene that converts Met268 to Thr. These data were obtained both by classical genetics based on crosses between sensitive and resistant biotypes (Zeng & Baird, 1997; 1999; Yamamoto et al., 1998) as well as by transfecting the mutated form of *TUA1* into maize calli and tobacco plants that then became more resistant to the herbicide (Anthony et al., 1998; Anthony et al., 1999). It was also shown that the combined presence of both the I and R biotype mutations in a single α-tubulin isotype increased the herbicide tolerance of transgenic maize calli. This suggests that each mutation is likely to exert its effect by a different mechanism (Anthony & Hussey, 1999b).

These experiments prove that dinitroaniline resistance correlated with specific mutations occurring in the α-tubulin chain. No evidence for a similar mechanism of resistance, nor for any significant mutation, could be found in four β-tubulin genes isolated from the same R biotype of *Eleusine indica* (Yamamoto & Baird, 1999). Despite the fact that the resistant trait is clearly a function of the α-tubulin peptide, there exist two alternative explanations about the actual mechanism mediating the resistance. It could be that the mutation in the α-tubulin chain primarily reduces the dynamics of the microtubule (reducing its susceptibility to a block of dimer addition by the herbicide), or the presence of Ile at position 239 could actually reduce the affinity of the herbicide for its binding site. To this regard it is interesting to note that the vast majority of plant and vertebrate tubulins contain Thr at position 239 (Fosket & Morejohn, 1992). This would rule out the possibility that Thr239 defines the binding site for the herbicide since animal tubulins are substantially unaffected by treatment with dinitroanilines. Nevertheless, Thr239 could be involved in herbicide binding in a co-operative fashion with other amino acid residues. If so, the Thr239 to Ile mutation could cause a reduction in herbicide affinity. This possibility is suggested by data from animal tubulin electron crystallography (Nogales et al., 1998). The three dimensional structure of a tubulin dimer shows that residues

Tyr 24 and Thr239 (both involved in mutations that confer dinitroaniline resistance to plant tubulins) are very close to each other, and thus could participate in herbicide binding.

The transgenic studies performed with the mutated form of *TUA1* of *Eleusine indica* have also provided interesting data concerning the mechanisms that control the synthesis and accumulation of tubulin in plants (see below).

Taxol (paclitaxel), another chemical substance originally extracted from the bark of the pacific yew, *Taxus brevifolia*, as a very promising drug for the treatment of certain human cancers, binds to tubulin leading to a stabilization of MTs (Kingston, 1994; Wall, 1998; Vaishampayan et al., 1999). Taxol is actually commonly used in biochemical experiments where MTs structure must be reconstituted. Binding sites for taxol have been identified either by photoaffinity labelling or with the help of electron crystallography (Nogales et al., 1998; Rao et al., 1999). They are associated with the β-tubulin polypeptide. Further evidence for the binding of taxol to β-tubulin has also recently emerged from studies of mutated forms of β-tubulin in human cancers that have acquired resistance to taxol, in hamster ovary cell lines, and in taxol resistant mutants in fungi where several domains seem to be involved in conferring β-tubulin resistance (Mu et al., 1999; Gonzales-Garay et al., 1999; Monzo et al., 1999).

**Plant tubulin: regulatory issues**

As mentioned earlier, the variety of tubulin isotypes is produced by several genes encoding the α or the β chain that can be grouped into different families. Regulatory requirements, rather than functional specificities, have been the driving force of this evolutionary process. This means that a specific tubulin isotype has acquired preferential expression in a given tissue or developmental stage because its regulatory elements are better recognised in that specific context. Plant-tubulin gene families are made up by a variable number of expressed genes, which can be as high as nine for β-tubulin and six for α- tubulin (Goddard et al., 1994). Despite this moderately high and even unequal gene number, an important requirement has to be met by the regulatory network controlling tubulin expression – α- and β-tubulins must be produced in equimolar amounts to guarantee efficient assembly of tubulin dimers into the MTs. The achievement of this apparently simple condition is actually brought about through a large variety of regulatory steps.

A direct and clear demonstration that plant cells require an equal amount of both the α- and β-polypeptide comes from recent experiments where the mutated *TUA1* gene of goosegrass (*Eleusine indica*) was transfected into maize calli and tobacco plants. No transgenics could be obtained, when the expression of the α- and β-tubulin proteins was out of balance (Anthony et al., 1998; Anthony & Hussey, 1998), and no transformants were obtained when plant cells were solely transfected with either the α- or the β-tubulin transgene alone. Only the balanced combination of their expression in a two-gene vector allowed the recovery of transformants. This finding is reminiscent of that obtained in the yeast *Saccharomyces cerevisiae*, where it was shown that the unbalanced expression of the tubulin β2 gene caused loss of viability (Katz et al., 1990).

The simplest way to provide a cell with equimolar amounts of α- and β-tubulins would be to simultaneously express a single genomic copy of each gene. This was probably the ancestral condition that had to be modified later when more α- and β-tubulin genes appeared necessitating additional mechanisms of regulation.

The first of these regulatory mechanisms is located at the level of transcription. Within each of the plant-tubulin gene families that have been investigated in detail (i.e. maize, *Arabidopsis*, rice), tubulin genes have been identified where expression is constitutive while for other members of the gene family expression is regulated by tissue-specificity, developmental stage or external signals (Montoliu et al., 1989; Hussey et al., 1990; Joyce et al., 1992; Kopczack et al., 1992; Snustad et al., 1992; Villemur et al., 1994; KogaBan et al., 1995; Gianì and Breviario, 1996; Qin et al., 1997; Ludwig et al., 1998). This coexistence of different expression patterns is exemplified by the α-tubulin isotypes 1 and 2 in *Arabidopsis thaliana*, whereas the *AtTUA1* gene was shown by northern analysis and *AtTUA1*-promoter: GUS studies to be expressed exclusively in developing pollen (Ludwig et al., 1988; Carpenter et al., 1992), the *AtTUA2* gene exhibited a completely different pattern with expression in almost any tissue and stage of development (Carpenter et al., 1993). Under control of the *AtTUA2*-promoter, GUS became detectable in all tissues with the exception of very specific structures such as leaf trichomes, pollen grains and vascular tissues containing developing xylem elements. This type of study later uncovered

similar patterns in a range of plant species where, within each tubulin-gene family, members that are constitutively expressed coexist with genes that possess very specific patterns of expression that depend on tissue identity, stage of development or the response to external stimuli (Han et al., 1991; Dixon et al., 1994; Yoshimura et al., 1996; Whittaker & Triplett, 1999). Using both northern analyses and transgenic approaches, where the expression of reporter genes was placed under the control of specific tubulin promoter sequences, it was shown that the transcription of specific tubulin genes could be up- or down-regulated by a variety of signals such as light, cold, anoxia, hormones or symbiotic association (Bustos et al., 1989; Mendu & Silflow, 1993; Tonoike et al., 1994; Leu et al., 1995; Bonfante et al., 1996; Gianì & Breviario, 1996; Carnero-Diaz et al., 1996; Niini et al., 1996; Chu et al., 1998). The presence of such differentially regulated members within all plant-tubulin gene families opens the interesting possibility to use their promoter sequences to confer specific patterns of expression to a transgene of choice (see below).

In addition to transcriptional regulation, the tubulin system can be further controlled by posttranscriptional regulation; for instance, triggered by the accumulation of unpolymerized tubulin dimers in the cell. It has been shown for animal cells that this regulatory feedback can either affect directly mRNA stability or the efficiency of translation. When the endogenous pool of tubulin dimers was increased either by treatment with colchicine or by microinjection of tubulin (Cleveland et al., 1983; Cleveland, 1988) the unpolymerized tubulin was shown to bind via the MREI peptide motif to the nascent chain of β-tubulin activating a rapid degradation of β tubulin mRNA. Although all plant β-tubulins and all the members of subfamily II of α-tubulins contain the MREI-motif at their N-terminus, a similar regulatory mechanism has not been reported for plants. In contrast, in a rice experimental system, when MTs were depolymerized by oryzalin, the observed pattern of regulation was reversed. Whereas both α- and β-tubulin proteins decreased dramatically the level of the corresponding mRNAs was found to be essentially unaffected (Gianì et al., 1998). These observations indicate that the pool of unpolymerized tubulin dimers is controlled at the level of protein rather than mRNA stability. Even so, further experiments are required to understand if the observed decrease in tubulin protein could also result from mechanisms that affect the translation efficiency of tubulin mRNA.

To ensure a balance between α- and β-tubulin proteins, not only is there post-translational control of β-tubulin mRNA via a MREI-based feedback loop (as discussed above), but also a post-transcriptional control of α-tubulin. This control is exerted at the level of α-tubulin-mRNA translation and is complemented by degradation of excess α-tubulin protein as demonstrated (Gonzales-Garay & Cabral, 1996). By this combined mechanism a constant and fine tuning of the relative amounts of α- and β-tubulin polypeptides can be achieved. According to this model, excessive expression of β-tubulin would trigger a negative feedback on the amount of β-tubulin mRNA via a MREI-based mechanism, whereas excessive expression of α-tubulin would trap α-tubulin mRNA in the untranslated form until degradation of α-tubulin protein has reduced the amount to a level that is balanced with β-tubulin. Overexpression of both polypeptides would result in a default pathway that leads to protein degradation. It has been proposed that the mechanism controlling α-tubulin mRNA translation could operate by the recognition of negative regulatory elements present in the 5′-UTR region of the α-tubulin mRNA (Gonzales-Garay & Cabral, 1996).

Recently, the presence of a similar regulatory mechanism in plants has been evoked to explain results obtained in transgenic maize calli and tobacco plants transformed with the mutant *TUA1* from the R biotype of goosegrass. These data show that the products of the transgene (both mRNA and protein) progressively replaced the corresponding endogenous molecules via a mechanism that might involve translational control of tubulin mRNA (Anthony & Hussey, 1998; Anthony et al., 1999). In fact, to circumvent a potential negative feedback on the translation of the transgenic tubulin mRNA itself, the coding regions of the α- and the β-tubulin transgenes were placed within a transcriptional unit devoid of potentially interfering UTR regulatory elements. Even so, it still remains to be elucidated how this regulatory circuit would work and whether it acts at the transcriptional level or post-transcriptionally. The observed elimination of endogenous tubulin gene products by the transgenic gene products contradicts, at first glance, the results obtained after oryzalin treatment of rice cell culture (Gianì et al., 1998). However, this difference may just reflect different levels of regulation. Whereas MT assembly, as such, is not affected in the case of the transgenics, suggesting that the gene products of the transgene act mainly on mRNA stability and translation, in the case of the oryzalin treated cells the pool of

tubulin dimers is actually increased under conditions of net MT depolymerization with no assembly. This could eventually favor control via protein degradation.

The coexistence of such different control circuits, which are differentially triggered depending on the assembly state of MTs, was shown in rice when the responses to oryzalin and abscisic acid were compared. Whereas oryzalin causes an increase of unpolymerized tubulin heterodimers, abscisic acid induces a reorientation of MTs without affecting their integrity. While the amount of tubulin protein decreased in response to oryzalin, with only minor effects on the amount of the corresponding mRNAs, the opposite was true for cells treated with abscisic acid – a strong reduction in the amount of tubulin mRNA was observed, but the amounts of the α- and β-tubulin polypeptides were virtually unaltered (Gianì et al., 1998).

Whether 5′-UTR as putative regulatory elements upstream of plant tubulin genes generally affect mRNA translation may be questionable, since different laboratories have reported the successful use of tubulin promoter sequences, comprising the corresponding mRNA leader sequences, in transformation experiments (Leu et al., 1995; Uribe et al., 1998; Chu et al., 1998).

The identification of mechanisms that regulate tubulin synthesis and accumulation not only poses fascinating scientific questions, but influences the design and success of transformation strategies that make use of tubulin coding sequences and promoters. In this context an intriguing additional aspect should be mentioned, namely the presence of naturally occurring tubulin-antisense mRNA sequences of unknown function (Dolfini et al., 1993; Deng et al., 1996; Breviario, unpublished observations). Although the regulatory aspects concerning tubulin expression are still far from being understood, the MREI-mediated regulation of mRNA stablity and this natural antisense mRNA might represent a gold mine for novel and exclusive ways to control gene expression in plants.

**Plant tubulins: a tool for plant biotechnology**

Each plant species contains tubulin as the key component of the MTs that are essential for the control of plant growth, development and morphology. Any substance or signal that interferes with tubulin may alter the dynamics and the organization of the MTs, and this can have profound consequences on plant architecture, height and the responses to chemicals and stress. In the extreme situation where toxic substances, such as drugs or herbicides, bind to tubulin and block MT assembly this can lead eventually to cell death. This is the case for some anti-cancer drugs and anti-mitotic herbicides. Mutations that alter the binding site for these substances can produce resistant forms of tubulins and MTs as shown for resistance to dinitroanilines and taxol. This may be also the case for resistance to toxins of viral or fungal origin (Takahashi et al., 1990), as well as for cold-resistance. Other manipulations of the tubulin system may affect MT assembly not in such a drastic way but might simply modulate the rate constants of assembly and disassembly. This is likely to cause some consequences in general plant architecture such as changes in gravitropic setpoint angles, in branching, in rooting, plant height and fertility. This was shown in the rice mutant *ER31* (Nick et al., 1994) where a mutation in the coding region of isotype *TubA1* (Breviario & Nick, unpublished results) slows down MT dynamics. These plants show a marked change in the gravitropic response of the leaf sheaths that results in a fan-like appearance of the leaf system. By manipulating MT dynamics, the positioning of the leaves and thus photosynthetic efficiency, might be altered.

The regulatory features of the tubulin gene family can also be of help in designing strategies to be used in plant transformation. First, the isolation of promoters sequences that are specific for a given tissue, for a given stage of development or in response to a specific signal can be of help in driving the expression of a gene with a specific pattern. This was shown in tobacco plants with a maize tubulin promoter sequence that drove the expression of a GUS reporter-gene according to the pattern originally observed in maize (Uribe et al., 1998). Second, promoters of isotypes that are expressed constitutively may prove to be valuable tools for plant transformation. Their main feature is that they can be isolated from the same species that has to be transformed thus limiting horizontal DNA-transfer among different species and organisms. Third, the specific pattern of expression of a given tubulin isotype may help in designing strategies to arrest growth or differentiation of that specific organ. Attempts are made to knock out, by antisense strategies, the *Arabidopsis* tubulin isotype that is specifically expressed in pollen during late stages of flower development. This should allow the production of male sterile plants.

All these experimental strategies, actually, are derived from one central concept, that is, tubulin provides an essential function, but at the same time

V.M.T.K

**V**ersatile **M**olecular **T**ubulin **K**its

*Promotors*

| *Coding seq.* | Con. | R.s. | I.s. | P.s. | C.s. |
|---|---|---|---|---|---|
| Wt | | | | | |
| DnR | X1 | | | | |
| BrM | X2 | | | | |
| ToxR | | X5 | | | |
| HuT | X3 | | | | |
| Antis | | | X6 | X7 | |
| Cter-del | X4 | | | | X8 |

*Figure 3.* The Versatile Molecular Tubulin Kit box. Eight different combinations (X1-8) between plant tubulin promoters and coding sequences are shown. *Promoters* legend: Con – constitutive; R.s. – root specific; I.s. – internode specific; P.s. – pollen specific; C.s. – cold specific. *Coding sequence* legend: Wt – wild type, DnR – mutation conferring dinitroaniline resistance; Br.M. – mutation affecting branching-angle; Tox.R – mutation conferring resistance to fungal toxins; Hu.T. – human tubulin; Antis. – antisense-plant tubulin; Cter.d – tubulin with a C-terminal deletion. Each of these combinations can yield a plant with potentially advantageous features.

allows for some variation made possible by the intrinsic heterogeneity of its multiple isotypes. So, it becomes feasible, by recombining promoter and coding sequences, to produce 'new tubulin genes able to provide a function optimized for certain features and expressed in a neatly-tuned pattern (constitutive or time and tissue specific). This 'Versatile Molecular Tubulin Kit' (VMTK, Figure 3) could be further extended by mutagenesis and should lead, on the basis of what is currently known, to new advantageous tubulin isoforms that still maintain their basic functions, continuing the development that has actually occurred during evolution. At the beginning, one single β-tubulin gene and two α-tubulin genes were providing tubulin in each cell and for each microtubule array. Over time, plants have duplicated their α- and β-tubulin genes several times and the expression of some of them has been confined to specific tissues, stages of development or linked to the response to external signals. In principle, one could push this evolutionary strategy further by creating new combinations of promoters and modified tubulin coding sequences. In this way some aspects of plant growth and resistance to stress could possibly be addressed such as herbicide resistance, cold resistance, production of male-sterile plants, or the control of plant height and shape.

The VMTK-strategy could be entirely homologous since tubulin coding and promoter sequences can be isolated, manipulated and reintroduced within the same original species. In addition, the newly acquired features can be expressed either constitutively or in a regulated manner (tissue-specific or signal-triggered) depending on the tubulin regulatory element and the defined experimental task. Thus, this strategy based on transformation with engineered tubulin genes will meet with many of the ideal features that nowadays are called for by a new generation of recombinant biotechnological strategies less hazardous and more compatible with environment and consumer health.

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