

# New Views on the Plant Cytoskeleton

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The advance of modern approaches in cell research, including genomics, proteomics, molecular genetics, and new and improved imaging technologies, is changing our views on the form, the function, and the regulation of the plant cytoskeleton. Ever since their discovery in plant cells in the 1960s and 1970s, the function of microtubules and actin microfilaments has been analyzed largely by pharmacological strategies. The use of cytoskeleton-disrupting drugs provided broad insights into the participation of microtubule or actin microfilament arrays in specific cell functions. The shift to a more integrative approach in the last few years has revolutionized the way we look at the plant cytoskeleton. Our initial view of static images has shifted to dramatic motion pictures of live, dynamic networks, and descriptive views have been replaced by mechanistic insights. Indeed, we are now attempting to understand how the organization and dynamics of the cytoskeleton are integrated into the regulatory networks underlying complex plant processes, from sexual reproduction to organ morphogenesis and cellular differentiation. Investigating the mechanisms underlying cytoskeletal organization and dynamics has also revealed previously unknown cytoskeletal functions. Integrating this new knowledge is reflected by a large volume of recent reviews (Kost and Chua, 2002; Mathur and Hulskamp, 2002; Wasteneys, 2002, 2004; Deeks and Hussey, 2003; Hashimoto, 2003; Smith, 2003; Wasteneys and Galway, 2003; Gu et al., 2004; Lloyd and Chan, 2004; Sedbrook, 2004), including several *Update* articles in this focus issue (Bisgrove et al., 2004; Lee and Liu 2004; Takemoto and Hardham, 2004). Most of these reviews deal with specific aspects of the latest advances in our understanding of the plant cytoskeleton. In this *Update* article, we hope to provide an overarching but complementary view of this fast-growing field.

### NEW VIEWS ON FUNCTION

Multifaceted approaches integrating mutational strategies, improved technologies for high-resolution imaging of cytoskeletal arrays, and identification of cytoskeleton-regulatory proteins are revealing new roles for the cytoskeleton in fundamental and plant-

specific processes. The discovery of these previously unknown roles and an improved understanding of cytoskeletal remodeling are providing new ideas about how plant cells work.

A key example is the role of the cytoskeleton in cell shape determination. The predominant hypothesis that cortical microtubules modulate cell shape by directing cellulose synthase complex movement has always been marred because it neglected to explain how tip growth in pollen tubes and root hairs, or the complex shape of other diffusely expanding cells is achieved. In the past, these differences in form have generally been considered to be governed by fundamentally distinct mechanisms. In recognition of common cytoskeletal and wall-building mechanisms across the full range of plant cell morphologies, a concept has been put forward that positions tip growth and isotropic diffuse expansion at the extremes of a growth continuum, with other forms of growth, including anisotropic expansion, somewhere in between (Wasteneys and Galway, 2003). Recent studies that demonstrate a role for cortical microtubules in anisotropic growth that is independent of orienting cellulose microfibrils (Himmelspach et al., 2003; Sugimoto et al., 2003; Baskin et al., 2004; Wasteneys, 2004) provide an opportunity to rethink our ideas about how plant cells are formed. A clear way forward is to consider how the relative distribution and activity of actin microfilaments and microtubules control the mechanical properties of cell walls (Wasteneys and Galway, 2003). Consistent with the growth continuum concept, the cortical patches of actin microfilament networks in diffusely expanding cells may have a role in wall construction and modification that is analogous to the tip-localized actin filament networks of tip growing cells (Fu et al., 2001, 2002; Frank and Smith, 2002; Jones et al., 2002).

### LIVE VIEWS: ADVANTAGES AND LIMITATIONS OF GREEN FLUORESCENT PROTEIN-BASED PROBES

Recognition that cytoskeletal polymers are not static has helped drive the quest for new technology that allows microtubules and actin filaments to be observed in living cells (Table I). Live probes usually utilize fluorescent protein-tagged monomers of the cytoskeletal polymers (e.g. green fluorescent protein [GFP]-tubulin; Ueda et al., 1999) or a protein domain

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**Table 1.** *Fluorescent fusion protein probes used in plant cell studies*

Probe	Description	Expression Method	Arrays Labeled	Advantages	Disadvantages	References
GFP-TUA6	Arabidopsis $\alpha$ -tubulin 6	Stable transformants	Incorporates into microtubules as functional protein analog	Excellent signal levels	Can generate right-handed twisting; does not incorporate into microtubules in root tissues	Ueda et al. (1999)
GFP-TUB	Arabidopsis $\beta$ -tubulin 6	Stable transformants	Incorporates into microtubules as functional protein analog	No apparent organ twisting	Does not incorporate into microtubules in root tissues	Nakamura et al. (2004)
GFP-MBD	Microtubule-binding domain of MAP4 from mouse (or human; heterologous)	Stable transformants	Decorates microtubules	Labels microtubules in roots	May compete with endogenous MAPs; 35S-driven expression can generate severe dwarfing phenotypes; expression levels deplete in older seedlings	Granger and Cyr (2001)
YFP-CLIP170	Mammalian cytoplasmic linker protein (heterologous)	Transient expression and stable transformants (tissue culture cells only)		Plus-end tracking	Effects on seedling development not determined	Dhonukshe and Gadella (2003)
AtEB1a-GFP (1) GFP-AtEB1 (2)	Arabidopsis putative microtubule plus-end tracking protein	Stable transformants (tissue culture cells only)	Decorates microtubule ends; whole microtubule when overexpressed	Plus-end tracking	Effects on seedling development not determined	(1) Chan et al. (2003); (2) Mathur et al. (2003b)
GFP-Talin	Binding domain of animal actin-binding protein	Transient expression and stable transformants	Microfilament bundles; fine and dynamic microfilament networks when transiently expressed	Decorates prominent actin bundles in all cells; fine dynamic filaments in certain cells	Induces developmental anomalies and actin microfilament bundling in stable transformants	Kost et al. (1998); Ketelaar et al. (2004b)
AtFIM1-GFP (1) GFPABD2 (2)	Arabidopsis fimbrin 1, various fragments	Transient expression and stable transformants	Various microfilament arrays	Decorates dynamic microfilaments	Labeling pattern varies from cell to cell depending on expression level	(1) Wang et al. (2004); (2) Sheahan et al. (2004)

that binds specifically to the cytoskeletal polymers (e.g. GFP-tagged actin-binding domain from mouse talin; Kost et al., 1998). They can be used to label all arrays, a specific array, or specific part of an array to provide novel insights into cytoskeletal dynamics and organization. Use of GFP-tubulin has uncovered a modified treadmilling mechanism for the dynamics of cortical microtubules in plant cells (Shaw et al., 2003), and the heterologous fusion protein YFP-CLIP170, which labels microtubule ends, revealed a difference in microtubule dynamics between interphase and preprophase cortical microtubules (Dhonukshe and Gadella, 2003). Similarly, use of GFP-EB1 (Chan et al., 2003; Mathur et al., 2003b) has provided

support for the concept of dispersed microtubule organization centers in plant cells (Wasteneys, 2002). Live probes may also visualize the finer and more dynamic cytoskeletal arrays that are usually difficult to detect using conventional fixation-based methods. For example, GFP-mTalin has been used to label a dynamic form of F-actin in the tip of pollen tubes and root hairs and fine actin microfilaments in the cortex of Arabidopsis leaf pavement cells, which previously had not been visualized in fixed cells (Fu et al., 2001, 2002).

Innovations in fluorescence microscopy and increases in computing capacity have greatly improved the ability to record time-lapse images of fluorescently

**Table II.** *Players in plant cytoskeletal organization and dynamics*

Protein(s)	Biochemical Activities	Cellular Functions	Predicated MW	No. Genes in Arabidopsis	References
Subunits of the ARP2/3 Complex	Presumably nucleate actin and branched actin formation	Trichome and pavement cell morphogenesis	Various	1 except for p40 (2 genes)	Deeks and Hussey (2003)
Formins	Presumably nucleate unbranched actin filaments	Unknown		20	Deeks et al. (2002); Cheung and Wu (2004)
Profilins	G-actin sequestration, and possibly actin polymerization	Cell morphogenesis and cell growth	12–15	4	Kovar et al. (2000)
ADF/cofilin	Actin severing	Pollen tube growth	~15	10	Hussey et al. (2002)
Actin-interacting proteins	Cap actin and cooperate with ADFs	Cell growth/cell division		2	Ketelaar et al. (2004a)
Fimbrins	Actin bundling	Unknown	~76	3	McCurdy et al. (2001)
Villins	Actin bundling	Unknown	~115	4	Klahre et al. (2000)
Gelsolin	Capping barbed end and severing actin	Unknown	~80	None, found in poppy	Huang et al. (2004)
CAP	Capping barbed end	Unknown		9	Huang et al. (2003)
MAP65 family	Microtubule bundling and polymerization	Cytokinesis	65–68		Muller et al. (2004); Van Damme et al. (2004)
SPC98	Putative component of microtubule nucleation complex	Unknown	98	1	Erhardt et al. (2002)
$\gamma$ -Tubulin	Putative component of microtubule nucleation complex	Unknown	55	2	Drykova et al. (2003); Kumagai et al. (2003); Shimamura et al. (2004)
MOR1/MAP215	Possibly microtubule stabilization and/or promotion of microtubule elongation	Cell morphogenesis and cell division	217	1	Whittington et al. (2001); Twell et al. (2002); Hamada et al. (2004)
EB1	Plus-end binding, marking nucleation sites	Unknown	26–36	3	Chan et al. (2003); Mathur et al. (2003b)
PLD	Anchoring microtubules to the plasma membrane	Cell morphogenesis	90	12	Dhonukshe et al. (2003); Gardiner et al. (2001)
SPR1/SKU6	Unknown	Cell morphogenesis	12	1	Sedbrook et al. (2004)
SPR2/TOR1	Unknown	Cell morphogenesis	94	1	Buschmann et al. (2004); Shoji et al. (2004)
Katanin p60	Severing microtubules	Cell morphogenesis	60	1	Bouquin et al. (2003); Stoppin-Mellet et al. (2002)

tagged cytoskeletal elements. However, care still needs to be exercised when undertaking such work. Placing live samples on horizontal stages can elicit touch and gravity responses that are likely to alter cytoskeletal dynamics and organization. Incorporation into or decoration by live probes will potentially alter the organization or dynamics of the labeled cytoskeleton, especially if the probes are expressed to an excessive level. For example, GFP-mTalin, which is known to cause abnormal bundling of actin microfilaments, can alter the dynamic activity of microfilaments, generate defects in cell development, and may not faithfully report all microfilament arrays in stably formed plant cells (Ketelaar et al., 2004b). However, transient ex-

pression of GFP-mTalin has allowed visualization of both fine cortical actin networks and actin bundles in several cell types (Fu et al., 2001, 2002; Jones et al., 2002). A recent addition to the fluorescent fusion protein toolkit is GFP-fimbrin (Wang et al., 2004; Sheahan et al., 2004). Although this construct uses an Arabidopsis (*Arabidopsis thaliana*) gene as its starting point, potential microfilament bundling is avoided by trimming down the gene to encode only one of fimbrin's actin-binding domains (Sheahan et al., 2004). This probe appears to label the finer, more dynamic microfilaments missed by some GFP-Talin fusion proteins and promises to extend our understanding of actin microfilament arrays.

Another key issue is to evaluate the level of live probes that provides a faithful report of different cytoskeletal arrays. The adverse effects of GFP-mTalin (Ketelaar et al., 2004b) may be at least in part caused by high levels of expression. The usefulness of a specific live probe may also be tissue and cell specific. GFP-tubulin fusion proteins do not seem to incorporate into microtubules in roots, whereas GFP-MAP4 (and GFP-MBD) constructs (Granger and Cyr, 2001) work well in roots (Van Bruaene et al., 2004). There is no doubt that the use of live cytoskeleton probes has cast new views of the plant cytoskeleton arrays and their dynamics, yet we still need to fine-tune the existing probes and to generate new ones.

Important criteria to consider include:

- (1) Are the expression levels of the fusion protein interfering with its normal function?
- (2) If the fusion protein is being expressed in a heterologous system, is it competing with an endogenous protein for a tubulin- or actin-binding site?
- (3) Is the positioning of the seedling or explanted organ in its microscope chamber generating pressure, wound, or gravity responses?
- (4) How much repeated excitation with high-energy light can the cell or fusion protein tolerate without generating aberrant behavior?
- (5) Is specimen drift through the *z* axis generating an illusion of movement of the microtubule or actin filament bundle?

The bottom line is that these experiments need to be scrutinized carefully and, where possible, results backed up by other experimental approaches.

## DYNAMIC VIEWS: CYTOSKELETAL ORGANIZATION AND DYNAMICS

### Actin and Its Regulatory Proteins

The existence of actin nucleation mechanisms allows the cell to modulate the timing, rate, and location of actin filament formation. Two types of conserved actin nucleation mechanisms have been characterized (Table II). In fungi and animals, the actin-related protein (Arp) 2/3 complex initiates the polymerization of branched actin filaments to form an actin network, whereas formin nucleates unbranched filaments that can cross-link to form actin bundles. Orthologs for all seven subunits of the Arp2/3 complex appear to be present in plants, but surprisingly, knocking out any of the seven *Arabidopsis* genes only alters trichome shape, leaf pavement cell, and root hair morphology (Deeks and Hussey, 2003; Le et al., 2003; Li et al., 2003; Mathur et al., 2003a, 2003c; El-Din El-Assal et al., 2004). Although the biochemical activity of the putative *Arabidopsis* Arp2/3 complex has not been studied, these observations suggest that another actin nucleation mechanism may play a predominant role in the regulation of actin assembly in plants. Perhaps this is

consistent with the fact that 20 formin-homology genes are present in the *Arabidopsis* genome (Deeks et al., 2002). Indeed, overexpression in pollen suggests that formin may initiate the formation of bundled actin microfilaments (Cheung and Wu, 2004).

Remodeling the actin cytoskeleton and regulating actin microfilament assembly and dynamics is dependent on actin-binding proteins (ABPs; Table II), which include profilins, actin depolymerizing factors (ADFs), actin-interacting proteins, and gelsolins and other capping proteins (McCurdy et al., 2001; Hussey et al., 2002; Huang et al., 2003, 2004; Wasteneys and Galway, 2003; Ketelaar et al., 2004a). Profilin, a G-actin-binding protein, is likely to have a dual role, depending upon the physiological condition and the presence of profilin-associated proteins. Overexpression suggests a role for sequestering G-actin in plant cells, in which G-actin pool exceeds critical concentration for polymerization (McKenna et al., 2004). G-actin sequestration by profilins appears to be calcium mediated (Gibbon et al., 1998; Kovar et al., 2000). Profilin also binds formin, and a recent study shows that profilin participates in a rate-limiting step of formin-mediated actin polymerization (Romero et al., 2004). It is likely that profilins have a similar role in formin-mediated actin polymerization in plant cells. Suppression of profilin gene expression affects cell growth, morphogenesis, and seedling growth, highlighting the important physiological function of profilin-mediated actin dynamics (Ramachandran et al., 2000; McKinney et al., 2001).

Actin microfilaments are most prominent in plant cells when bundled, and these so-called actin bundles serve as tracks for the myosin-mediated movement of organelles. Two classes of actin cross-linking proteins, villins and fimbrins, may be involved in the formation of actin bundles (Wasteneys and Galway, 2003). It will be interesting to see if different villins and fimbrins have distinct roles in organizing different populations of bundled actins, as appears to be the case for MAP65 involvement in microtubule bundling, which is discussed below.

### Microtubule Dynamics and Organization

The lack of centriole-based microtubule organizing centers in plant cells presents considerable challenges for nucleating microtubules in the right place at the right time (Schmit, 2002; Wasteneys, 2002; Dixit and Cyr, 2004).  $\gamma$ -Tubulin, a critical factor in fungal and mammalian microtubule nucleation, has now been confirmed to be associated with putative sites of microtubule nucleation in plant cells (Drykova et al., 2003; Kumagai et al., 2003; Shimamura et al., 2004) and associated at these sites with the plant homolog of the SPC98 protein (Erhardt et al., 2002).  $\gamma$ -Tubulin's apparent distribution along the entire length of microtubules (Drykova et al., 2003; Kumagai et al., 2003) suggests either that additional functions for  $\gamma$ -tubulin exist, or that the dispersal of nucleation activity takes advantage of microtubule polymers as tracks (Wasteneys, 2002).

Organizing plant microtubules into functional arrays no doubt involves cross-linking mechanisms that substitute for the lack of centrosomes. The unique character and function of cortical microtubules in plant cells is to a large degree dictated by their close contact with the plasma membrane. Recent progress in understanding this relationship has come from the discovery that phospholipase D associates with microtubules (Marc et al., 1996; Gardiner et al., 2001) and that compounds activating phospholipase D result in microtubule detachment from the plasma membrane and loss of parallel order (Dhonukshe et al., 2003), inhibiting normal seedling development (Gardiner et al., 2003).

Cortical microtubule function depends on the presence of microtubule-associated proteins (MAPs) and their regulatory kinases and phosphatases (Sedbrook, 2004). The MOR1 homolog of the highly conserved MAP215 family (Gard et al., 2004) appears to be essential for microtubule function throughout plant development (Whittington et al., 2001; Twell et al., 2002; Wasteneys, 2002). This year, the identities of two proteins involved in the control of directional expansion and associated with microtubules were revealed. SPR1/SKU6 protein is a tiny (12-kD) protein originally identified from mutants that cause right-handed organ twisting (Nakajima et al., 2004; Sedbrook et al., 2004). Similar phenotypes are generated by mutations in the *SPIRAL2/TORTIFOLIA1/CONVOLUTA* gene, which encodes a 94-kD protein that colocalizes with microtubules (Buschmann et al., 2004; Shoji et al., 2004).

Transmission electron microscopy reveals just how common microtubule bundles are in plant cells, not just in preprophase bands but also apparent for many cortical microtubules. Bundles might generate stability but could also provide a means of bulking up proteins or other storage material on microtubule surfaces. Overlapping microtubules of opposite polarity, as occurs in some regions of the phragmoplast (Segui-Simarro et al., 2004), is also likely to involve many of the same mechanisms as more substantial bundles. Recent studies confirm that cortical microtubules also exist in arrays of mixed polarity. It has been suggested that the MOR1 protein may cross-link microtubules. The tobacco (*Nicotiana tabacum*) homolog of MOR1, TMBP200, was reported to have in vitro microtubule-bundling activity (Yasuhara et al., 2002), and an antibody raised against a C-terminal MOR1 fragment labeled the zone of microtubule overlap in phragmoplast arrays in *Arabidopsis* protoplasts (Twell et al., 2002). A study just published, however, concludes that the tobacco MAP200 homolog of MOR1 has no bundling activity, and the authors attribute the previously reported property to contamination by a MAP65 fraction (Hamada et al., 2004). Both in vitro biochemical assays and protein localization studies have implicated the MAP65 family proteins in microtubule bundling (Chan et al., 2003; Muller et al., 2004; Wicker-Planquart et al., 2004). In *Arabidopsis*, MAP61-1 is localized to a subpopulation

of the interphase cortical microtubules, the center of the preprophase band, and to antiparallel overlapping regions of spindle and phragmoplast microtubules. MAP65-3/PLE knockout mutants show a specific defect in cytokinesis (Muller et al., 2004). In tobacco BY-2 cells, GFP-tagged MAP65-1 and MAP65-5 are localized to different subpopulations of cortical microtubules, whereas GFP-MAP65-4 is localized to a specific array of microtubules that rearranged to form spindles (Van Damme et al., 2004). These observations raise the possibility that different members of the MAP65 family may have distinct functions in cross-linking specific subpopulations of microtubules.

#### A FAR-REACHING VIEW: SIGNALS AND PATHWAYS REGULATING THE CYTOSKELETON

Given the wide range of processes modulated by the cytoskeleton, it is not surprising that a variety of intracellular, extracellular, hormonal, and environmental signals are known to regulate the dynamics and organization of both microtubules and actin microfilaments. A specific cytoskeletal array itself (e.g. the preprophase band) might act as a signal to regulate other types of arrays. Identification of specific signals and dissection of signaling networks interfacing with cytoskeletal organization and dynamics are the ultimate goal of integrating the cytoskeleton with plant growth, development, and physiological responses.

One of the better-characterized signal-cytoskeleton response systems is the modulation of changes in the actin cytoskeleton in poppy pollen tube self-incompatibility (SI) responses by SI protein (Staiger and Franklin-Tong, 2003). An SI protein produced by self-pistil triggers rapid growth arrest and subsequent cell death of pollen tubes (Thomas and Franklin-Tong, 2004). These responses include rapid reorganization of actin microfilaments in the tip and subsequent massive depolymerization of the whole actin cytoskeleton system in pollen tubes (Snowman et al., 2002). Changes to the actin cytoskeleton seem to be regulated by calcium signaling (Huang et al., 2004). The most rapid SI response in poppy pollen detected so far is the disappearance of tip-focused calcium gradients followed by a massive influx of calcium in the shank (Franklin-Tong et al., 2002). Calcium-sensitive profilin and gelsolins appear to be involved in SI-induced changes to the actin cytoskeleton (Huang et al., 2004).

In any signal-cytoskeleton response system, the final signaling targets are most likely cytoskeleton-associated proteins that control organization and dynamics. How a specific extracellular or intracellular signal regulates MAPs or ABPs is a question beginning to attract significant attention. ROP/Rac family GTPases have emerged as a key signaling switch in the regulation of the cytoskeleton in plants (Fu and Yang, 2001; Fu et al., 2002; Jones et al., 2002; Yang, 2002; Gu

et al., 2004). ROPs (Rho-related GTPases from plants) have been shown to control tip growth in pollen tubes and root hairs and polar cell expansion in different cell types (Kost et al., 1999; Li et al., 1999; Fu et al., 2001, 2002; Molendijk et al., 2001; Jones et al., 2002; Yang, 2002). A common theme linking ROPs to polar growth in different cells is that localized ROP promotes localized organization of fine actin filaments (Yang, 2002), although ROPs appear to coordinate the actin-promoting pathway with distinct pathways in different forms of polar growth (Gu et al., 2004). ROPs are most likely activated by polarity signals in these cases, but the nature of polarity signals in plants and the mechanism by which polarity signals activate ROPs remain elusive. The *SPK1* gene, originally identified in screens for trichome morphology mutants, encodes a putative guanine nucleotide exchange factor for ROPs and thus could be involved in signaling to ROPs (Qiu et al., 2002).

How ROP GTPases regulate cytoskeletal organization and dynamics is also becoming a topic of intense scrutiny. A potential link between ROPs and the putative Arp2/3 complex is hinted at by several recent studies showing that knocking out homologs of subunits of the WAVE complex produces phenotypes similar to those of the Arp2/3 complex mutants (Basu et al., 2004; Brembu et al., 2004; Deeks et al., 2004; El-Assal et al., 2004). In mammalian cells, Rac GTPases modulate the WAVE complex, which in turn affects either the activity or localization of the Arp2/3 complex (Bompard and Caron, 2004). In addition to the WAVE complex, Rac/Cdc42 can activate another class of Arp2/3 complex-activating proteins, such as WASP, which are apparently absent from the Arabidopsis genome (Yang, 2002). However, disrupting the function of ROPs results in a much more dramatic defect on both fine cortical actin microfilaments and polar cell growth than knocking out subunits of the putative Arabidopsis Arp2/3 complex (Li et al., 1999, 2003; Fu et al., 2001, 2002). These observations support the notion that ROPs may use a mechanism for the regulation of the actin cytoskeleton that is independent of the conserved Arp2/3 complex. This notion is consistent with the existence of a class of plant-specific ROP-interacting proteins, known as RICs, that appear to act as ROP-signaling targets (Wu et al., 2001). ROP inactivation of ADF appears to be another means by which these GTPases modulate actin remodeling (Chen et al., 2003).

Another potentially important cytoskeletal signaling mechanism is the MAP kinase (MAPK) cascade. One such MAPK cascade in tobacco has been shown to be required for cytokinesis (Nishihama et al., 2002; Soyano et al., 2003). Signaling targets of this kinase have not been clearly identified but could be microtubule-associated proteins that regulate the dynamics of phragmoplast microtubules. Interestingly, the MAPK cascade is activated by a kinesin-like protein that is also localized to the phragmoplast, implicating microtubules as signals for feedback regulation of phragmo-

plast microtubules (Soyano et al., 2003). The idea that a MAPK cascade mediates microtubule organization is further supported by demonstrating the involvement of a MAPK phosphatase in the control of cortical microtubule organization (Naoui and Hashimoto, 2004).

The study of cytoskeletal signaling in plants is still in its infancy, but linking signals and pathways to the regulation of MAPs and ABPs is expected to be a major future thrust in the field of the plant cytoskeleton.

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