The Americas Journal of Plant Science and Biotechnology ©2011 Global Science Books

Microtubules and Root Responses to Mechanical Impedance and Gravity

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ABSTRACT

Gravity and mechanical impedance represent two environmental cues that roots respond to as they grow through the soil. Roots generally grow down in response to gravity. However, rocks and other obstacles mechanically impede growth and roots need to maneuver around them as they penetrate into the soil. To do this roots change the direction in which they are growing by forming a bend that reorients the tip of the root. Microtubules are thought to play important roles in these root growth responses. By regulating the direction of cell expansion they are essential for ensuring that roots continue to elongate through the soil. In addition, microtubules are hypothesized to function in root responses to touch and gravity signals. These functions include signal detection, cytoplasmic organization in sensory columella cells, and the differential growth response that occurs during root bending. However, roles for microtubules in these responses are somewhat controversial since there are experiments that have produced conflicting results. We recently reported that Arabidopsis plants carrying mutations in the microtubule associated protein END BINDING 1 (EB1) have defects in their responses to touch/gravity stimuli (Bisgrove *et al.* 2008). These mutants open the door for analyses aimed at determining how and where in the response pathway EB1 and, by association, microtubules are involved. In animal and fungal cells EB1 appears to regulate the compliment of proteins that associate with microtubule plus ends. EB1 binding partners include proteins that function in signaling pathways, vesicle shuttling, and cross talk between the actin and microtubule cytoskeletons. Similar processes may occur during root responses to touch/gravity signals, although possible roles for EB1 are speculative. Further analyses of *eb1* mutants and the identification of EB1 interactors in plants should provide additional insights.

Keywords: cytoskeleton, End Binding 1, EB1, gravitropism, thigmotropism
Abbreviations: EB1, End Binding 1; **MAP**, Microtubule Associated Protein); **+TIPs**, Microtubule plus-end Tracking Proteins; **MscS**, Mechanosensitive channels of Small conductance; **MSL**, MscS-Like; **MLO**, Mildew resistance Locus O; **ER**, Endoplasmic Reticulum; **TOC**, Translocon of Outer membrane of Chloroplast; **PIN**, PIN-formed; **ABCB**, ATP Binding Cassette subfamily B; **MDR**, MultiDrug Resistance

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INTRODUCTION

Plants are exquisitely sensitive to changes in their surroundings. They are capable of detecting and responding to a number of different environmental cues. These include gravity, light, and mechanical stimulation as well as levels of moisture, chemicals, and temperature to name a few. Plants often respond to environmental stimuli by modifying their growth. Some alterations involve changes in organ size or shape. For example, plants that are repeatedly exposed to mechanical stimuli such as wind are shorter and have thicker stems than plants that are grown in the absence of these conditions (Jaffe 1973; McCormack *et al.* 2006; Chehab *et al.* 2009). Other growth modifications involve changing the direction of growth to place roots, leaves, stems, and flowers in the best possible locations. Roots for example, normally grow down in response to gravity but they will modify the direction in which they are growing to take advantage of areas of higher moisture or nutrient content in the soil. Changes in the direction of growth either towards or away from stimuli are called tropisms and they usually involve the bending of roots or stems to reorient growth in the most favorable direction.

Growth responses involve altering patterns of cell division or cell expansion. Cell divisions add more cells to a growing plant. The rates of cell divisions and the placement of cell plates at the time of division determine how quickly and where new cells are produced. After their production, many cells stop dividing and enter a period of expansion. Expanding cells undergo large turgor-driven increases in

Received: 22 January, 2010. Accepted: 10 December, 2010.

Invited Mini-Review

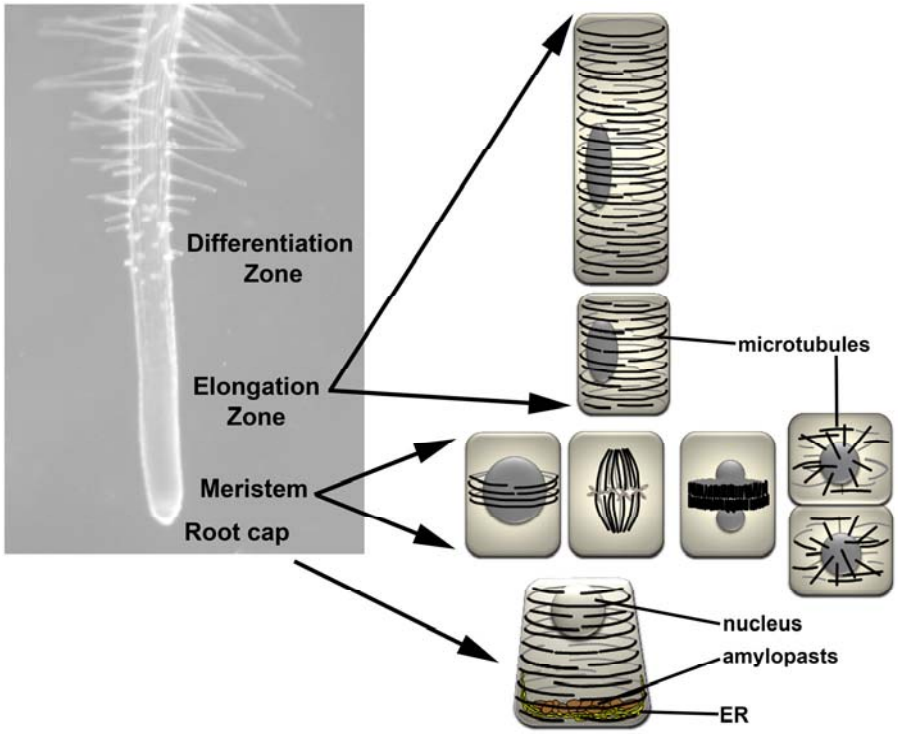

Fig. 1 Microtubule arrays in an elongating Arabidopsis root tip. The root shown on the left is divided into four developmentally distinct regions, the root cap, meristem, elongation zone and differentiation zone. Columella cells in the root cap (bottom right) contain amyloplasts and peripheral ER at the distal end of the cell. Cortical microtubules in the columella cells are organized into parallel arrays in the periphery of the cell. Dividing cells in the meristem have microtubules organized into preprophase bands, mitotic spindles, phragmoplasts, and interphase arrays (shown from left to right). Cells positioned further back from the meristem in the elongation zone have stopped dividing and are rapidly elongating. They have cortical microtubules organized into parallel arrays that are positioned perpendicular to the direction of cell elongation and the long axis of the root. In the differentiation zone cell elongation ceases and cells begin to adopt specific fates and form root hairs. Differentiating cells are not shown.

size. Some cells enlarge more than a hundred times their original size and these massive changes account for much of the increase in biomass in a growing plant (Le *et al.* 2001; Verbelen *et al.* 2006). In addition, the directional control of cell expansion largely determines organ shape. In roots for example, most of cell elongation is parallel with the long axis of the root and this results in a long and narrow root. Cell expansion also underlies the differential growth response that results in the bending of roots and stems during plant tropisms. Bending occurs when the cells on one flank of a stem or root elongate more than they do on the other side. Because the cells are held together and do not move apart from one another the difference in cell expansion rates across the organ cause it to form a bend.

Microtubules play key roles in both cell expansion and cell division. These cytoskeletal elements are long tubule-shaped filaments composed of α- and β-tubulin dimers. In cells they form dynamic sets of different arrays. Dividing cells have microtubules that become arranged into preprophase bands, mitotic spindles, and phragmoplasts as they transit through mitosis and cytokinesis (**Fig. 1**; Wasteneys 2002; Mineyuki 2007). The placement of these arrays in the cell and the speed at which they assemble and disassemble determine the positions and rates of new cell additions to a growing plant (Müller *et al.* 2009). Expanding cells that have exited the cell cycle no longer form the arrays associated with mitosis. Their microtubules are arranged in parallel hoops in the periphery of the cell closely associated with the plasma membrane. These cortical microtubules coordinate the direction of cell expansion; they are thought to regulate expansion by influencing the mechanical properties of the cell wall and the wall then constrains the direction of expansion (discussed in further detail below and reviewed in Mineyuki 2007; De Cnodder *et al.* 2007; Wasteneys and Collings 2007; Wasteneys and Ambrose 2009).

Microtubules are themselves dynamic structures and this feature underlies their ability to function and to form different arrays in the cell. Individual microtubules grow and shrink by the addition and loss of tubulin subunits at their ends. In a growing microtubule subunit addition occurs more rapidly at one end than it does at the other; the faster growing end is designated the "plus" end while the slower growing end is called the "minus" end. The ability of microtubules to polymerize and depolymerize provides them with the flexibility to rearrange into different structures or to position themselves in different locations within the cytoplasm. In cells microtubule function also depends on a large and diverse family of proteins called microtubule associated proteins or MAPs. When bound to microtubules these proteins alter polymerization/depolymerization rates and/or mediate interactions with other microtubules, proteins, organelles, or structures in the cell (Hamada 2007; Kaloriti *et al.* 2007; Wasteneys and Collings 2007; Wasteneys and Ambrose 2009).

To understand how microtubules and their associated proteins influence growth in plants we are analyzing a MAP called End Binding 1 (or EB1). EB1 belongs to a specialized group of MAPs known as microtubule plus end tracking proteins or +TIPs because they preferentially accumulate on the more rapidly growing plus-ends of microtubules (Akhmanova and Steinmetz 2008; Slep 2010; Jiang and Akhmanova 2010). We recently reported that Arabidopsis plants carrying large T-DNA insertions in *EB1* genes have roots that are defective in their responses to touch and/or gravity signals. When grown on the surface of vertically oriented agar plates mutant roots tend to grow in loops and deviate more to the left (as viewed from above the agar surface) than wild type plants. Mutant roots also exhibit delays in downward bending after tracking across an obstacle in their path. The phenotype suggests that *eb1* mutants have defects in their responses to touch and/or gravity signals, but where in the response pathway EB1 might function and how EB1 activities at the subcellular level might influence these responses are open questions. In this article root growth and responses to touch/gravity signals are discussed and hypotheses that describe roles for

microtubules are presented. The *eb1* mutants are discussed in terms of their utility as tools in the analysis of root responses to touch/gravity signals. Speculative roles for EB1 are postulated based mainly on extrapolations from studies in animal and fungal cells. It should be noted that root gravitropism, microtubule functions in plant cells, and the roles of EB1 proteins in eukaryotic cells are all areas of intense investigation and there are large bodies of literature on each of these topics. A comprehensive review of this work is outside the scope of this manuscript and readers are referred to other review articles for further details (Oliva and Dunand 2007; Bisgrove 2008; Lucas and Shaw 2008; Nick 2008; Sedbrook and Kaloriti 2008; Valster and Blancaflor 2008; Akhmanova *et al.* 2009; Guo *et al.* 2009; Monshausen and Gilroy 2009; Petrásek and Schwarzerová 2009; Szymanski and Cosgrove 2009; Wasteneys and Ambrose 2009; Morita 2010; Šlep 2010; Jiang and Akhmanova 2010).

MICROTUBULES AND ROOT ELONGATION

Roots grow by the division and expansion of cells located at the tip of the root. Dividing cells in the meristematic zone add more cells to the growing root. However, it is the expansion of cells positioned in the elongation zone behind the meristem that contribute the most to overall root elongation. These cells undergo large turgor-driven increases in length that effectively push the root tip forward through the soil (**Fig. 1**; Le *et al.* 2001; Clark *et al.* 2003; Blancaflor *et al.* 2006; Verbelen *et al.* 2006). Elongating cells have microtubules that are located in the cell periphery closely associated with the plasma membrane. These microtubules are arranged parallel to one another in helical arrays that encircle the cell. Mutations or treatments that shorten or depolymerize cortical microtubules disrupt cell elongation and induce radial swelling (see for example Baskin *et al.* 1994; Whittington *et al.* 2001; Sugimoto *et al.* 2003). Thus these cortical microtubules are important for ensuring that the cells in the elongation zone expand in the same direction as the long axis of the root (Szymanski and Cosgrove 2009).

Although the mechanism is not completely understood, cortical microtubules are thought to influence expansion by altering the mechanical properties of the cell wall and the wall then acts as a constraint for cell expansion. Cell expansion is a turgor-driven process; water entering the vacuole causes an increase in volume that exerts pressure on the cell wall. Expansion occurs when turgor exceeds the resistance imposed by the cell wall. The major load-bearing elements in the wall include cellulose microfibrils and the hemicelluloses, pectins, and proteins that make up the wall matrix between the cellulose microfibrils. In elongating cells cellulose microfibrils are deposited into the wall in parallel arrays that are oriented perpendicular to the direction of cell elongation. Cellulose microfibrils are resistant to stretching along their lengths; this means that when the wall yields to turgor adjacent microfibrils move apart from one another. Hence the cellulose microfibrils constrain cell elongation to a direction that is perpendicular to their orientation in the cell wall (Szymanski and Cosgrove 2009).

Cellulose microfibrils are deposited into the wall by large cellulose synthase complexes embedded in the plasma membrane. The synthases travel in the plane of the membrane depositing cellulose microfibrils behind them as they move (Mutwil *et al.* 2008). In elongating cells the microfibrils in the wall are co-aligned with the cortical microtubules that line the plasma membrane on the inside of the cell. This co-alignment led to the proposal that microtubules could serve as guides for the deposition of cellulose microfibrils into the wall (Green 1962; Ledbetter and Porter 1963; Heath 1974 and reviewed in Emons *et al.* 2007; Wasteneys and Collings 2007; Lloyd and Chan 2008; Szymanski and Cosgrove 2009). Recent work has linked cellulose synthase movements to microtubules. Synthase complexes were observed tracking along microtubules in live Arabidopsis plants expressing fluorescently tagged proteins. The synthases moved around curves in bent microtubules and when microtubules were reoriented the synthases followed the newly positioned microtubules (Paredez *et al.* 2006). Thus it seems that microtubules can serve as guides for cellulose synthase movements. However, it should be noted that aligned arrays of microfibrils can be deposited into the wall even after microtubules have been disassembled, suggesting that alternative guidance mechanisms also exist (Baskin 2001; Paredez *et al.* 2006; Wasteneys and Collings 2007; Lindeboom *et al.* 2008).

Aligning cellulose microfibrils may not be the only way that microtubules influence cell elongation. For example, when microtubules in Arabidopsis roots are disrupted cell elongation stops and radial swelling occurs even though cellulose microfibrils are deposited into the cell walls in ordered arrays (Sugimoto *et al.* 2003). This indicates that microfibril alignment is not sufficient to maintain cell elongation and suggests that microtubules may be influencing the wall in other ways. One hypothesis is that cells with compromised microtubules could be depositing shorter microfibrils into their walls. In theory, short microfibrils would be able to move apart from one another in both longitudinal and transverse directions leading to radial expansion in the affected cells (Wasteneys and Collings 2007).

In addition to guiding cellulose synthase movement in the plasma membrane, recent work suggests that microtubules have a role in the targeted delivery and removal of cellulose synthase complexes to and from the plasma membrane (Crowell *et al.* 2009). In this work Golgi bodies carrying cellulose synthase complexes were observed pausing on cortical microtubules at specific sites beneath the plasma membrane. These pauses often correlated with the insertion of synthases into the plasma membrane. In addition, internalized complexes were also associated with cortical microtubules in cells that were reducing their rates of cell expansion and wall synthesis, suggesting that microtubules are also involved in synthase removal from the membrane. Microtubules have also been implicated in the patterned deposition of two other proteins involved in cellulose synthesis into the plasma membrane, KORRIGAN (an endo-1,3-β-D-glucanase) and COBRA (a glycosylphosphatidyl inositol (GPI) containing protein (Robert *et al.* 2005; Roudier *et al.* 2005). The idea that microtubules mediate the insertion and removal of wall biosynthetic enzymes into and out of the plasma membrane suggests that, in addition to guiding cellulose synthase movements, microtubules also influence the number and spatial arrangement of enzymes that are actively involved in cell wall synthesis (Crowell *et al.* 2009).

ROOT RESPONSES TO GRAVITY AND MECHANICAL IMPEDENCE

The ability of roots to penetrate and exploit the soil depends not only on turgor-driven cell elongation, but also on an effective system for sensing and responding to the environment that the roots are growing through. Gravity and mechanical impedance represent two cues that roots frequently respond to as they wind their way around rocks and other debris in the soil. Roots normally grow down in response to gravity. However, when the tip of the root encounters an impenetrable obstacle in its path the direction of growth must be reoriented so that the root can maneuver around the object. Time-lapse videos reveal that these directional changes in growth involve bend formation in the elongation zone (Massa and Gilroy 2003; Thompson and Holbrook 2004; Monshausen *et al.* 2009). The first bend that forms appears to be a buckling of the root that occurs as cells in the elongation zone continue to push the tip against the obstacle. If the root cannot push through the barrier the forces that are generated by cell expansion cause the root to buckle (Thompson and Holbrook 2004; Monshausen *et al.* 2009). Buckling occurs in the basal portion of the elongation zone and it orients the root tip away from the down-

ward trajectory. This displacement of the root tip within the gravitational field triggers a gravitropic response in which differential rates of cell elongation across the root cause it to form another bend. The second bend forms in the elongation zone closer to the meristem than the first bend. It realigns the tip of the root with the gravity vector and brings the root cap back into contact with the barrier. Cell elongation between the bends pushes the second bend forward and slides the root tip across the barrier. This growth habit appears to involve a combination of touch and gravity sensing and it allows the root cap to maintain contact with the barrier as the root grows. Once the root has grown past the obstacle and the cap is no longer in contact with it, downward growth resumes (Massa and Gilroy 2003).

The mechanisms that underlie root responses to touch and gravity signals are not fully understood. Gravitropism has received a considerable amount of attention in the literature and there is a large body of information in this area. Although there are still gaps in our knowledge, we are beginning to understand many aspects of this response (Valster and Blancaflor 2008; Molas and Kiss 2009). Plant responses to mechanical stimuli, on the other hand, are not as well understood although recent studies have begun to shed some light on this process as well (Monshausen and Gilroy 2009). The first step in either response involves the perception and conversion of physical cues into biochemical signals. In roots the root cap is the first part of the root to encounter an obstacle and it also perceives gravity. Signals detected in the root cap are then transmitted to the responding cells in the elongation zone where the root mounts a response by forming a bend. Models describing how roots could perceive and respond to touch/gravity signals are summarized below.

PERCEPTION OF MECHANICAL STIMULI

Current hypotheses propose that touch is sensed by receptors in the plasma membrane that are activated when the membrane is deformed. Stretching or bending the plasma membrane would activate the receptors by structurally altering them or disrupting their interactions with ligands (Monshausen and Gilroy 2009). In plants, the relevant sensors are unknown although there are a few candidates. Transient changes in cytoplasmic Ca^{2+} levels have been measured in response to mechanical stimulation in many organisms and cell types including roots and these observations have led to the suggestion that mechanosensitive Ca^{2+} channels are involved in sensing touch in plants (Monshausen and Gilroy 2009). One candidate for a component of a Ca^{2+}-based mechanosensory system in plants is a plasma membrane protein designated Mca1 (Nakagawa *et al.* 2007). Mca1 can functionally complement a yeast mutant that lacks a putative component of a Ca^{2+}-permeable stretch activated channel. Furthermore, Arabidopsis loss-of-function *mca1* mutants have roots that are defective in penetrating a hard agar medium, suggesting that they have defects responding to touch stimuli. Other candidates include proteins related to mechanosensitive channels of small conductance in bacteria (MscS-like or MSL in plants). MSL proteins localize to the plasma membrane in roots and they are components of an active chloride channel in protoplasts derived from root cells (Haswell *et al.* 2008). Bacterial MscS channels are activated by structural changes in the membrane that occur during osmotic shock (Vasquez *et al.* 2008). However, roles for MSL channels in root responses to osmotic or mechanical stimulation are unclear since plants carrying mutations in the relevant genes have no apparent defects in their response to these stimuli (Haswell *et al.* 2008). A third category of possible mechanosensory pathway components includes the Mildew Resistance Locus O (MLO) proteins. These proteins have structural features resembling seven transmembrane G-protein-coupled receptors from animals, although they do not appear to function in a traditional heterotrimeric G protein signaling complex. Plants carrying mutations in two *MLO* genes have roots that form tight curls upon contacting solid surfaces, indicative of a possible defect in touch responses (Chen *et al.* 2009).

GRAVITY DETECTION

Columella cells in the root cap function as gravity-sensing statocytes; they contain specialized starch-filled amyloplasts (or statoliths) that sediment to the bottom of the cell in response to gravity. When a root tip is reoriented within a gravitational field the amyloplasts fall to the new bottom of the columella cells. According to the starch-statolith hypothesis, amyloplast sedimentation is somehow converted into a chemical signal that leads to differential growth and organ bending. A role for amyloplasts in gravity detection is supported by analyses of mutants that produce less starch than wild type plants. In starch-deficient mutants amyloplasts do not efficiently sediment and this correlates with reduced responses to gravity (Caspar and Pickard 1989; Saether and Iversen 1991; Kiss *et al.* 1996). In addition to amyloplast sedimentation the protoplast-pressure model postulates that the cell detects the settling of the protoplast within the cell wall. If this is true amyloplast sedimentation could enhance gravity signals by increasing the mass of the protoplast on the lower side of the cell (Morita and Tasaka 2004; Valster and Blancaflor 2008).

The mechanisms by which amyloplast sedimentation or protoplast settling are detected and converted into biochemical signals are unknown. One hypothesis is that mechanoreceptors in the endoplasmic reticulum (ER) and/or the plasma membrane are involved (Leitz *et al.* 2009). Columella cells have a polarized cytoplasm; the nucleus is closer to the proximal end of the cell while peripherally-located ER cisternae are more abundant at the distal end. (**Fig. 1**; Hensel and Sievers 1981; Sack 1997; Zheng and Staehelin 2001; Driss-Ecole *et al.* 2003). In a root tip that is oriented vertically the amyloplasts lie on top of and compress the ER at the distal end of the cell. Reorienting the root tip causes the amyloplasts to sediment onto and distort the ER/plasma membrane on the new bottom of the cell. Since the ER is more abundant on the distal end of the cell than it is along the sides, amyloplast settling onto the lateral membranes in a tilted root tip might also distort the plasma membrane. The compression and decompression of these membranes as the amyloplasts relocate within the cell has been postulated to activate mechanosensitive ion channels that are responsive to changes in membrane curvature (Leitz *et al.* 2009). Membrane distortion may not be the only mechanism by which amyloplast position is sensed. Sedimenting amyloplasts could contain ligands that contact receptors embedded in the ER and/or plasma membranes thereby triggering signal transduction (Perrin *et al.* 2005). Recently the complex that imports proteins across the outer chloroplast membrane (the Translocon of Outer Membrane of Chloroplast or TOC complex) has been linked to early events in gravity signal transduction. This finding raises the possibility that a protein in the amyloplast membrane could play a role in gravity sensing (Stanga *et al.* 2009).

Some models of gravity sensing include the microtubule and actin cytoskeletons (Blancaflor 2002; Nick 2008; Valster and Blancaflor 2008). The idea that the cytoskeleton might be involved in gravity sensing arose from a series of studies that described the polarized nature of the gravity-sensing columella cells and investigated possible roles for the cytoskeleton in the organization of these cells (Valster and Blancaflor 2008). In columella cells most of the microtubules are cortical and the interior of the cell appears to be depleted of them (**Fig. 1**; Hensel 1984; Baluška *et al.* 1997). In some species, *Lepidium sativum* for example, there is a criss-cross pattern of microtubules that lines the distal plasma membrane underneath the ER (Hensel 1984). Actin filaments surround the amyloplasts, the ER, and the nucleus and they are also located in the cell cortex adjacent to the plasma membrane. In addition, an actin-based network appears to exist in the inner, endoplasmic regions of the cell (Hensel 1988; White and Sack 1990; Baluška *et al.* 1997;

Staehelin *et al.* 2000; Collings *et al.* 2001; Yoder *et al.* 2001; Driss-Ecole *et al.* 2003). Both actin and microtubules appear to be involved in setting up and maintaining cell polarity in columella cells. In particular, the asymmetrically localized ER membranes require both microtubules and actin for their transport and anchoring to the distal end of the cell suggesting there could be an indirect role for the cytoskeleton in gravity perception (Hensel 1984, 1985, 1986; Wendt and Sievers 1986; Perbal *et al.* 1997).

Direct roles for actin and microtubules in gravity sensing have also been proposed. One idea suggests that falling statoliths could trigger signaling pathways by interacting with or pulling on actin microfilaments as they fall. This hypothesis, however, has been controversial, mainly because chemical treatments that disrupt the actin cytoskeleton do not inhibit gravitropism (Leitz *et al.* 2009). Instead these treatments increase the rate of amyloplast sedimentation and enhance the response to gravity (Yoder *et al.* 2001; Blancaflor *et al.* 2003; Hou *et al.* 2004). Another model postulates that microtubules interact with mechanosensitive transmembrane ion channels. In this scenario the membrane distortions that result from amyloplast sedimentation would physically displace or depolymerize the microtubules associated with an ion channel causing the channel to open (Nick 2008). This idea is based on models for the regulation of mechanosensitive ion channels in the nematode worm *Caenorhabditis elegans* in which microtubule interactions with either the channels or the plasma membrane modulate channel activity (Bounoutas *et al.* 2009). Support for a similar mechanism in plants awaits the identification of the relevant gravity receptors.

AUXIN REDISTRIBUTION AND THE DIFFERENTIAL GROWTH RESPONSE

Gravitropic signals perceived in the root cap are transmitted to the elongation zone where a bend forms that realigns the root tip with the gravity vector. The plant hormone auxin plays a key role in the transportation of these signals from the site of perception in the root cap to the responding cells in the elongation zone (Swarup *et al.* 2005). In plants auxin is transported in a directional manner from cell to cell in a process known as polar auxin transport (Muday and Rahman 2008; Petrasek and Friml 2009). Auxin flows through the root in a pattern that resembles a reverse-fountain stream. It moves down from the shoot towards the root apex through the central cylinder of the root. In the root apex it is directed laterally and then flows back towards the base of the root through the outer cell layers. According to the Cholodny-Went hypothesis gravitropic bend formation is driven by a lateral redistribution of auxin across the root that leads to a higher auxin concentration on the bottom side of the root. The difference in auxin concentration across the root induces differential growth and root bending by altering cell expansion rates (Muday and Rahman 2008; Petrasek and Friml 2009).

Current models propose that polar auxin transport through tissues and organs is driven by the asymmetric localization of auxin transporters located in the plasma membrane. AUX1 is a well-characterized auxin influx protein. It couples the inward movement of auxin with the outward flow of protons down a concentration gradient. Auxin that has accumulated inside the cell is deprotonated and this negatively charged form of auxin does not readily diffuse across the plasma membrane; it leaves the cell via efflux carriers. Two families of efflux carriers have been characterized, the PIN-FORMED (PIN) proteins and the plant orthologs of the mammalian ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance (MDR) protein family (Petrasek and Friml 2009). During polar auxin transport, the PIN proteins localize to one end of the cell and auxin flows out of the cell at this end. It is thought that the coordinated polar localization of PIN proteins across cells is responsible for directing the flow of auxin through a tissue (Muday and Rahman 2008; Petrasek and Friml 2009).

When a root is reoriented with respect to gravity, auxin flows preferentially to the lower side of the root leading to a higher concentration of auxin and an increase in cell elongation on that side. Altering the pattern of auxin flow in response to gravity appears to involve a redistribution of PIN proteins to the lower sides of the columella cells in the root cap thereby increasing auxin flow to the lower side of the root. Efflux carriers in the epidermal and cortical cells then direct the additional auxin on the lower side back towards the elongation zone. Auxin accumulates to higher levels in the elongating cells on the lower flank of the root and this leads to differences in cell elongation rates and organ bending (Friml *et al.* 2002; Muday and Rahman 2008; Kleine-Vehn and Friml 2008).

How PIN proteins in the columella cells re-localize in response to gravity is not known. The proteins have been observed cycling between the plasma membrane and internal cellular compartments and this endocytic cycling appears to be important for altering their distribution (Dhonukshe *et al.* 2008; Friml 2010). One hypothesis proposes that the cytoskeleton could play a role in PIN recycling, since PIN localizations are altered by the application of inhibitors that depolymerize actin or microtubules (Geldner *et al.* 2001; Kleine-Vehn *et al.* 2008). However, it is not clear whether this effect is due to a direct role for microtubules in vesicle transport or whether it reflects an indirect role, in cell elongation or cell wall biosynthesis for example. Alterations in PIN distribution and/or activity may also involve synthesis and degradation or the reversible phosphorylation of the transporters (Muday and Rahman 2008).

The mechanisms underlying auxin-mediated changes in cell elongation rates are not well understood, although alterations in cell wall extensibility are probably involved. Wall extensibility depends on the type of polysaccharide polymers present in the wall matrix and the activities of enzymes that modify these polymers. Some enzymes, pectin methyl esterases for example, stiffen the wall and reduce cell elongation rates. Expansins, on the other hand, have the opposite effect; they increase wall extensibility and elongation rates (Szymanski and Cosgrove 2009). Auxin alters gene transcription and genes encoding enzymes and other proteins known to modify cell wall extensibility are among those whose expression levels change in response to auxin (Muday and Rahman 2008). One hypothesis is that auxin-mediated changes in gene transcription could influence cell elongation by altering the complement of wall-modifying enzymes that are present in elongating cells.

MICROTUBULE ORGANIZATION, CELL ELONGATION, AND BEND FORMATION

Changes in the orientation of cortical microtubules also coincide with altered cell elongation rates in bending roots. When a root is reoriented in a gravitational field, microtubules on the lower flank reorient from perpendicular to parallel with the long axis of the root while the microtubules in the more rapidly expanding cells on the upper flank remain transversely oriented (Blancaflor 2002; Bisgrove 2008). This behavior led to the suggestion that microtubules could influence cell elongation rates during organ bending by altering cellulose deposition into the wall. In theory, cells with longitudinal microtubules would deposit longitudinal cellulose microfibrils and elongate less than cells with transversely oriented microfibrils (reviewed in Blancaflor 2002; Bisgrove 2008). This idea, however, is not well supported by the evidence. In some cases, roots treated with chemicals that either depolymerize or stabilize microtubules were still able to form gravitropic bends (see for example Baluska *et al.* 1996; Hasenstein *et al.* 1999). There are also issues regarding the timing of the microtubule reorientations with respect to bend formation. Microtubule reorientations sometimes occur after root bending has already begun, suggesting that a microtubule reorientation is not required to initiate a bend. However, these reports do

not rule out the possibility that microtubule orientation is important later in the response perhaps to reinforce root curvature once bending has started (Blancaflor and Hasenstein 1995).

There are additional ways in which microtubules could influence cell wall properties and hence cell elongation rates. As described above, microtubules have been linked to the insertion and removal of Golgi-derived vesicles containing cell wall synthesizing enzymes to and from the plasma membrane (Robert *et al.* 2005; Roudier *et al.* 2005; Crowell *et al.* 2009). These observations raise the possibility that microtubules could affect cell wall synthesis by influencing the number or spatial arrangement of enzymes that are active in the plasma membrane or cell wall (Bisgrove 2008; Crowell *et al.* 2009).

MICROTUBULES, EB1, AND ROOT RESPONSES TO TOUCH/GRAVITY SIGNALS

As it stands today, the question of how microtubules might influence plant responses to touch/gravity signals remains unanswered. Hypotheses have implicated microtubules in signal detection/transduction and auxin transport as well as the cell wall modifications that affect cell elongation during differential growth and root bending. In addition, both actin and microtubules are presumably needed to set up and maintain polarity in columella cells, raising the possibility that microtubules could indirectly influence root responses by ensuring the proper organization of receptive surfaces in the sensory cells. One way to assess the contributions of microtubules and their associated proteins to these processes involves analyzing plants carrying mutations in relevant genes. The availability of viable *eb1* mutants with defects in touch/gravity responses provides a tool for these analyses.

There are three *EB1* genes encoded in the *A. thaliana* genome, designated *EB1a*, *EB1b*, and *EB1c*. All three family members localize to microtubules in plant cells and exhibit plus-end tracking ability, although EB1b and EB1c are also found associated with endomembranes and in the nucleus respectively (Chan *et al.* 2003; Mathur *et al.* 2003; Van Damme *et al.* 2004; Van Damme *et al.* 2004; Abe and Hashimoto 2005; Chan *et al.* 2005; Dhonukshe *et al.* 2005; Dixit *et al.* 2006; Bisgrove *et al.* 2008; Komaki *et al.* 2010). The phenotypes of plants carrying T-DNA insertions in each gene as well as double and triple mutant combinations have been described (Bisgrove *et al.* 2008). These plants have roots that are defective in their responses to touch/gravity signals. When mutant roots encounter an obstacle in their path they are able to grow around it, but they do not immediately bend down at the edge of the barrier. Instead, mutant roots exhibit longer horizontal extensions before they bend down. In addition, mutant roots grown on vertically oriented agar plates tend to form loops and deviate more from a downward growth trajectory. These growth defects are most pronounced in *eb1b* and triple mutants, and the phenotype resembles that of plants with disruptions in auxin homestasis and/or the ability to respond to touch/gravity signals. However, it is not yet known whether *eb1* mutants are defective in signal perception, transduction, or the differential growth response. Double mutant phenotypes can provide information about the genetic pathways in which EB1 might function. One line of investigation involves analyzing offspring from crosses between *eb1* and plants with mutations in genes thought to function in touch/gravity responses. In addition, comparing the sensitivities of mutant and wild type plants to exogenously applied auxins or chemicals that disrupt auxin transport could reveal roles for EB1 in either auxin transport or root growth responses to altered auxin levels. Assessing the architecture of mutant columella cells by confocal and electron microscopy could unveil possible roles for EB1 in the cytoplasmic organization of these cells.

How might EB1, a protein that associates with the growing plus ends of microtubules, influence plant responses to touch/gravity signals? EB1 is the subject of numerous studies in animal and fungal cells and these analyses are providing information on the activities of the proteins at the subcellular level. From this work EB1 is emerging as a key regulator of microtubule plus ends. It interacts with a diverse array of proteins including other +TIPs, signaling molecules, and proteins associated with the actin cytoskeleton. Because it has so many binding partners, EB1 has been deemed a "master regulator" of complex formation on microtubule ends (**Fig. 2**; Akhmanova and Steinmetz 2008; Skube *et al.* 2009; Slep 2010). EB1 proteins appear to accumulate on the microtubule plus end by preferentially binding to structural features unique to the growing end. However, they do not move forward through the cytoplasm with the microtubule end as it grows. Instead, EB1 proteins bind to the end of the microtubule and remain in place. As additional tubulin subunits are added to the end, EB1 molecules that bound earlier become located on the tubule wall and they are released from the microtubule as the growing end extends forward through the cytoplasm. Because EB1 does not travel on the plus end, it is unlikely to have a role in microtubule-based transport in cells. Instead, EB1 is proposed to act as a diffusional sink for its interacting partners, concentrating them in the cytoplasm in the vicinity of the microtubule plus end (Akhmanova *et al.* 2009). While bound to microtubules, EB1 family members can also influence microtubule dynamics, although the effects on microtubule growth and shrinkage varies with cell type, the EB1 family member under consideration, its concentration, and the presence or absence of other +TIPs (Manna *et al.* 2008; Vitre *et al.* 2008; Coquelle *et al.* 2009; Komarova *et al.* 2009). Plant EB1 proteins also appear to affect microtubule dynamics (Van Damme *et al.* 2004; Komaki *et al.* 2010). However, the stability or integrity of the microtubule array is not correlated with the touch/gravity defects in *eb1* mutants since *eb1b* and wild type roots respond to chemicals that depolymerize or stabilize microtubules in the same dose-dependant manner (Bisgrove *et al.* 2008).

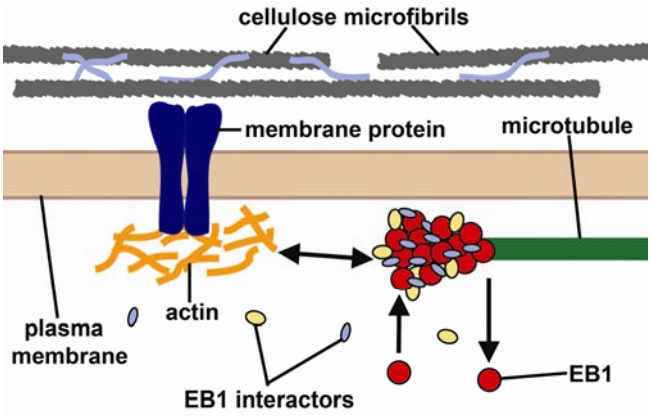

Fig. 2 A model of EB1 activities on microtubule plus ends. EB1 binds to microtubules at the plus end and falls off when it becomes associated with the side wall (single-headed arrows). In animal and fungal cells EB1 is known to bind to several other proteins (EB1 interactors), recruiting them to the microtubule end. One model proposes that this activity would raise the concentration of certain regulatory proteins for interactions with appropriate sites in the cell as the microtubule plus end grows past (double-headed arrow; see text for further details).

By controlling the network of proteins that accumulate at microtubule plus ends EB1 plays a role in several microtubule-dependent processes in animal and yeast cells. In some cases EB1 is involved in the cross-talk between actin and microtubules. For example, EB1 recruits polarity factors that induce actin assembly at new sites of cell growth in fission yeast (Minc *et al.* 2009). In migrating animal cells EB1 facilitates the accumulation of molecules involved in the cytoskeletal remodeling that is required for cell motility (Schober *et al.* 2009; Takahashi *et al.* 2010). EB1 has also

been linked to the shuttling of vesicles between the actin and microtubule cytoskeletons by associating with molecules that link vesicles to cytoskeletal motor proteins like myosin (Wu *et al.* 2005). In other cases EB1 interacts with signaling molecules such as kinases and phosphatases or effectors for GTPases (Rogers *et al.* 2004). EB1 is postulated to either concentrate these molecules in the vicinity of other components of the signaling pathway or to sequester them away from their signaling partners (Liu *et al.* 2008; Sun *et al.* 2008; Zhang *et al.* 2009).

Given that EB1 is involved in multiple processes in animal and fungal cells, several possibilities can be envisioned to explain how EB1 might participate in root responses to touch and/or gravity at the cellular level. Signaling is a key element in any response and it most likely involves coordinating the relevant pathway components in space and time. Some of the receptors are thought to be membrane bound as are the auxin transporters and endocytic recycling appears to be involved in coordinating the locations of these proteins in cells. Microtubules have been linked to the insertion/removal of proteins into and out of the plasma membrane. These proteins include enzymes involved in cell wall biosynthesis as well as the PIN auxin transporters (Geldner *et al.* 2001; Robert *et al.* 2005; Roudier *et al.* 2005; Kleine-Vehn *et al.* 2008; Crowell *et al.* 2009). EB1 proteins have also been linked to vesicle shuttling events in animal cells (Wu *et al.* 2005) and the idea that they might have similar functions in plants is a possibility. Links have also been suggested between EB1b and endomembrane organization in leaf epidermal cells (Mathur *et al.* 2003). EB1 is known to interact with an ER membrane protein called STIM1 in animal cells (Grigoriev *et al.* 2008). Given the models for ER function during gravity perception, it is tempting to speculate that the ER, EB1, and signal perception might somehow be linked in columella cells. EB1 is also a good candidate for a protein that could mediate cross-talk between the actin and microtubule cytoskeletons, since it is known to interact with proteins involved in actin remodeling in animal and fungal cells (Minc *et al.* 2009) (Schober *et al.* 2009; Takahashi *et al.* 2010). Both actin and microtubules have roles in the cytoplasmic organization of columella cells, and it is likely that the architecture of the columella cells depends on the coordinated activites of these two cytoskeleton systems (Hensel 1984, 1985, 1986; Wendt and Sievers 1986; Perbal *et al.* 1997; Blancaflor 2002). Whether EB1 has a role in any of these processes in plant cells awaits further analyses. In particular the identification of plant proteins that interact with EB1, either genetically or physically, would provide much needed information on the network of +TIPs associated with microtubule ends and their potential activities in plant cells.

ACKNOWLEDGEMENTS

Funding is from a Natural Sciences and Engineering Research Council of Canada Discovery Grant awarded to S.R.B.

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