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ORIGINAL ARTICLE

**Christine Andème-Onzighi · Mayandi Sivaguru
Jan Judy-March · Tobias I. Baskin · Azeddine Driouich**

The *reb1-1* mutation of *Arabidopsis* alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules

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Abstract The *root epidermal bulger 1* (*reb1*) mutant of *Arabidopsis thaliana* (L.) Heynh. is characterized by a reduced elongation rate of the primary root and by the bulging of many, but not all, root epidermal cells. In this study, we investigated cell wall structure of root epidermal cells in *reb1-1* by using serial sectioning, and light and electron microscopy in combination with immunocytochemistry and polysaccharide staining. We found that: (i) Cell bulging in the mutant was initiated in the zone of elongation of the root, and occurred exclusively in trichoblasts. (ii) *reb1-1* and wild-type root cells stained identically with anti-pectin antibodies, such as JIM5. In contrast, the anti-arabinogalactan-protein antibodies, JIM14 and LM2, stained all epidermal cells in the wild type and trichoblasts preferentially, but in *reb1-1* they stained the atrichoblasts only. (iii) Compared to the wild type, mutant trichoblasts had a thinner outer epidermal cell wall, which presented abnormal periodic acid–thio carbohydrazide silver proteinate (PATAg) staining. In addition, we investigated the organization of cortical microtubules in a *reb1-1* mutant line expressing a green-fluorescent protein fused to a microtubule-binding domain from human microtubule-associated protein 4. Microtubules in the swollen trichoblasts of *reb1-1* were either disordered or absent entirely. Together our findings indicate that the *reb1-1* mutation results in an abnormal trichoblast cell wall, and suggest that cell surface arabinogalactan-proteins are required for anisotropic expansion and for orienting cortical microtubules.

C. Andème-Onzighi · A. Driouich (✉)
UMR CNRS 6037,
Centre Commun de Microscopie Electronique,
IFRMP23, Université de Rouen,
76821 Mont Saint Aignan, Cedex, France
E-mail: azeddine.driouich@crihan.fr

M. Sivaguru · J. Judy-March · T.I. Baskin
Division of Biological Sciences,
University of Missouri Columbia,
Columbia, MO 65211, USA

Keywords *Arabidopsis* · Arabinogalactan-protein · Cell wall · Immuno-cytochemistry · Microtubule

Abbreviations GFP: green-fluorescent protein · MBD: microtubule-binding domain · MAP: microtubule-associated protein · PATAg: periodic acid–thio carbohydrazide silver proteinate · *reb1-1*: *root epidermal bulger 1-1*

Introduction

For all organisms, morphology is essential for function. The morphology of plant organs is a consequence of cell division and cell expansion. To build organs with the correct morphology, the plant must carefully control the extent and orientation of expansion among its cells. Cell expansion is driven by turgor pressure, which is generated by the osmotic potential of the contents of expanding cells. Turgor pressure is isotropic, and so for orientating expansion we must consider the cell wall, known to be the major determinant of morphogenesis (Roberts 2001). The cell wall comprises cellulose microfibrils embedded in a matrix of polysaccharides and proteins. Cellulose microfibrils have been considered for many years to be fundamental in controlling the direction of expansion (e.g. Green 1962). The primacy of microfibrils for controlling oriented cell expansion can be seen perhaps from the first crop of mutants harvested to understand this process: *rsw1*, *rsw2*/*kor*, and *rsw3*, isolated on the basis of a morphological, root swelling phenotype (Baskin et al. 1992), have defective cellulose synthesis (Arioli et al. 1998; Peng et al. 2000; Lane et al. 2001).

Nevertheless, cellulose microfibrils are unlikely to tell the whole story and the roles of other components of the cell wall in morphogenesis have been less well studied. In this context, our attention has been drawn to arabinogalactan-proteins. These proteoglycans were first implicated in cell expansion through the use of the beta-glucosyl Yariv reagent (henceforth "Yariv reagent"),

which binds and precipitates arabinogalactan-proteins (Yariv et al. 1967; Fincher et al. 1983) although it can bind cellulose under some conditions (Triplett and Timpa 1997). Cell expansion was inhibited when Yariv reagent was added to the growth media of suspension-cultured cells (Serpe and Nothnagel 1994; Willats and Knox 1996) and pollen tubes (Jauh and Lord 1996). Similarly, *Arabidopsis* seedlings grown on an agar medium containing Yariv reagent had reduced root elongation accompanied by appreciable bulging of epidermal cells (Willats and Knox 1996; Ding and Zhu 1997). Further supporting a connection between expansion and arabinogalactan-proteins, the mutant, *root epidermal bulger* (*reb1*, Baskin et al. 1992), partially phenocopies wild-type roots treated with Yariv reagent (Willats and Knox 1996) and has about 30% less total arabinogalactan-protein, as assessed by rocket gel electrophoresis (Ding and Zhu 1997).

The *reb1-1* mutant offers the possibility of extending the analysis of arabinogalactan-proteins in morphogenesis to the molecular level. The main objective of our study was to determine whether the observed alteration of these proteoglycans in *reb1-1* can be considered to cause the epidermal bulging as opposed to happening merely as a consequence. Here, using serial sectioning and immuno-cytochemistry, we show that the swelling phenotype of the mutant is restricted to trichoblasts and that, unlike pectin, certain arabinogalactan-protein epitopes are absent from the swollen cells, and absent or reduced prior to detectable bulging. These findings provide fresh evidence to say that arabinogalactan-proteins help to control oriented cell expansion.

An additional objective of this study was to examine the status of the cortical microtubules in the swelling epidermal cells. Like cellulose microfibrils, cortical microtubules play a fundamental role in plant morphogenesis (Shibaoka 1994). To the best of our knowledge, there are no examples where alterations in microtubules in growing cells fail to affect the direction of cell expansion. While it is widely believed that the function of microtubules is to orient cellulose microfibrils, this is not universally true (Baskin 2001), and other roles for microtubules such as directing vesicle secretion need to be considered. Using a construct that permits cortical microtubules to be imaged in living root epidermal cells (Marc et al. 1998; Granger and Cyr 2001), we report here that microtubules in the swollen trichoblasts of *reb1-1* are significantly disrupted and, in many cells, absent entirely. These findings link, apparently for the first time, the organization of cortical microtubules to the deposition of arabinogalactan-proteins.

Material and methods

Plant growth conditions

Lines of *Arabidopsis thaliana* L. (Heynh.) were as follows: wild-type Columbia, *reb1-1* (Baskin et al. 1992), and a transgenic line (in the Columbia background) that harbors a construct fusing green-fluorescent protein (GFP) to the microtubule-binding domain (MBD) of human microtubule-associated protein, MAP 4, with the expression driven by the 35-S promoter (Marc et al. 1998; Granger and Cyr 2001). The GFP::MBD plants were made, characterized, and generously supplied by Dr. Richard Cyr (Pennsylvania. State University). Additionally, we crossed this line onto *reb1-1* and selfed the resulting F1 plants. In the F2, mutant seedlings with brightly fluorescent microtubules were present at roughly 1 out of 16, and were selected for confocal microscopy. Because *reb1-1* is known to segregate as a recessive locus (Baskin et al. 1992), the 1/16 segregation ratio suggests that two copies of the GPF::MBD locus are required for full expression.

Seedlings were grown on vertical Petri plates containing nutrient-solidified agar under constant light (ca. 100 μmol photons m^{-2} s^{-1}) and temperature (19 °C) for 6–10 days, as described in Baskin and Wilson (1997). For plants grown to observe microtubules, the medium composition was modified slightly, as follows: 1% sucrose, 0.5% agar, 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 5 μM H_3BO_3, 0.1 μM $MnCl_2$, 0.2 μM $ZnSO_4$, 0.05 μM $CuSO_4$, 0.02 μM MoO_3 (adjusted to pH 4.5 with 0.1 N HCl).

Fixation and embedding

Seven-day-old seedlings were fixed for 1–2 h at room temperature in 4% (v/v) formaldehyde with 0.2% (v/v) glutaraldehyde in 50 mM 1,4-piperazinediethanesulfonic acid (Pipes) pH 7.0 buffer, with 1 mM $CaCl_2$. Fixative was added directly to the plates. Some samples were post-fixed in 1% osmium tetroxide for 1 h and all samples were dehydrated in a graded aqueous ethanol series, and embedded in either butyl-methyl methacrylate or LR White resin, as previously described (Baskin and Wilson 1997; Andème-Onzighi et al. 2000). Some root tips were fixed using high-pressure freezing and freeze-substitution and embedded in Epon resin as previously described (Zhang et al. 1993).

Light microscopy and histochemical staining

Roots of 7-day-old seedlings were separated from the shoots at the base of the hypocotyl with a razor. Short roots were mounted on glass microscope slides and directly examined using differential interference contrast optics on a compound microscope, and images were acquired with commercial software (Aquacosmos, image Acquisition and Analysis System for videomicroscopy; Hamamatsu). Staining with methylene blue–basic fuchsin was performed according to the protocol of Humphrey and Pittman (1974). Briefly, semi-thin sections (2.5 μm) from LR-White-embedded roots were prepared and mounted on glass microscope slides. They were then incubated with 0.15% (w/v) methylene blue for 20 min at 60 °C. After several washes with distilled water, the sections were incubated with 0.15% (w/v) ethanolic basic fuchsin for 5 min and washed thoroughly before observation. Calcofluor staining was done as described by Mori and Bellani (1996) and by His et al. (2001) for *Arabidopsis* sections. Fresh roots or sections (0.5 μm in thickness) from LR-White-embedded samples, mounted on glass slides, were incubated with the fluorescent probe (1 mg/l) for 30 min in the dark. After several washes, the sections were observed with ultraviolet excitation.

Cryo-scanning electron microscopy

Roots were placed on a stub and rapidly plunged into liquid nitrogen slush at –230 °C. The stub was transferred to the cryostage of a scanning electron microscope (S360; Cambridge Instruments, Cambridge, UK), warmed slowly to –80 °C to remove ice crystals from the surface, transferred to the cryo-preparation chamber (CT1500 Cryo Preparation System; Oxford Instruments, Oxford, UK) for coating with 10 nm of gold at –185 °C, and returned to the cryostage for observation at –185 °C and 20 kV.

Immunofluorescence labeling

The primary antibodies used in this study were: (i) JIM14 and LM2, specific for arabinogalactan-proteins (Knox et al. 1991; Smallwood et al. 1996); (ii) JIM5, specific for homogalacturonans (Willats et al. 2000). All of these are monoclonal antibodies raised in rat. In addition, we used an anti-blupleuran antibody that is also specific for pectins (Sakurai et al. 1998; Andème-Onzighi et al. 2000). The secondary antibodies used were either CY-3 conjugated goat anti-rat IgG (Jackson Immuno Research, West Chester, Pa., USA) or fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (BioCell).

Sections (1–2 μm) were cut and placed on small drops of distilled water on glass microscope slides. Methacrylate-embedded-sections were incubated in acetone for 10 min to remove the resin, then washed and hydrated immediately in phosphate-buffered saline [PBS: 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.3)] containing 0.05% (w/v) Tween 20, also named 0.05PBST. Sections were then incubated for 30 min in blocking solution (0.2% milk in PBS), and followed by primary antibodies JIM14, LM2 or JIM5 (diluted 1:30, 1:5 and 1:1, respectively, in 0.1PBST (PBS containing 0.1% Tween 20) for 2 h at 37 °C. After washing with 0.05PBST, the sections were incubated with the appropriate secondary antibody (diluted 1:50 in 0.1PBST) for 2 h at 37 °C. The sections were rinsed in 0.05PBST and mounted in a commercial anti-fade reagent. Sections were examined, either on a Zeiss Axioplan or on a Nikon Optiphot microscope, equipped with epifluorescence optics and standard filter sets for either rhodamine or FITC.

Periodic acid–thio carbohydrazide silver proteinate (PATAg) staining and immunogold labeling

Polysaccharides were detected by PATAg staining as previously described (Roland and Vian 1991). The thickness of the cell walls was measured on ultrathin-embedded sections using commercial software (Image analySIS, version 5; Soft Imaging System, Münster, Germany). Immunogold labeling with the mAb JIM14 antibody was performed on ultrathin LR-White-embedded sections as described for other JIM antibodies (His et al. 2001), with primary antisera diluted as above. Observations were made on a transmission electron microscope (model Tecnai 12, Bio-Twin; Philips).

Imaging cortical microtubules with confocal microscopy

Fluorescence from GFP::MBD was examined in living roots by imaging them with confocal fluorescence microscopy. Seedlings were removed from the plate immediately prior to observation and mounted in distilled water. Slides were placed on an inverted stand (Olympus IX70) interfaced to a confocal microscope (Radiance Laser Sharp 2000; BioRad, Hercules, Calif., USA). Fluorescence from GFP was excited with the 488-nm line of an argon laser, and images were captured through a 60× (NA 1.2), U-Plan apochromat, water-immersion objective. Consecutive Kalman scans were obtained starting from the surface and every 0.25/0.5 μm, comprising a stack of usually between 21 and 24 optical slices. For presentation, the stack was projected into a single stack using the instrument's software. Observations were repeated at least twice with 6–10 individual roots per session.

Results

Bulging of epidermal cells in *reb1-1*

As previously described (Baskin et al. 1992), in *reb1-1* some but not all epidermal cells bulged (Fig. 1). Bulging

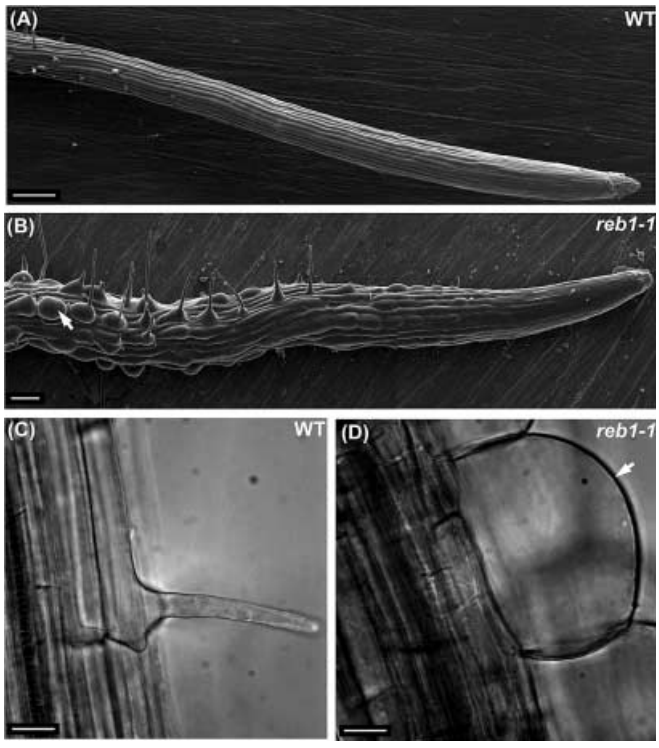

Fig. 1A–D. Morphological phenotype of wild type and *reb1-1* mutant roots of *Arabidopsis thaliana*. This and all subsequent figures show 7-day-old primary roots. **A, B** Cryo-scanning electron micrographs surveying the overall appearance of the root for the wild type (**A**) and *reb1-1* (**B**). **C, D** Light micrographs of root epidermal cells in the wild type (**C**) and *reb1-1* (**D**). *Arrowheads* point to highly swollen trichoblasts. Bars = 100 μm (**A**), 50 μm (**B**), 20 μm (**C, D**)

appeared to happen only in trichoblast files, and the degree of swelling among cells in even a single trichoblast file was variable, with some cells not bulging at all and others extremely swollen (Fig. 1B). Bulging appeared to be absent close to the root tip and became more pronounced with distance, reaching a maximum at a distance of ca. 1.5 mm from the tip, where root hairs matured. Some swollen cells produced a root hair whereas others did not (Fig. 1D).

To identify precisely the developmental stage at which bulging was initiated, we examined serial transverse sections taken at different distances from the root tip. Sections within the root cap were indistinguishable between *reb1-1* and wild type (not shown). In sections made within the first 250 μm of the meristem, no swollen epidermal cells were seen (Fig. 2A–D). In this region, trichoblasts are already distinguishable by virtue of their densely staining cytoplasm (Schiefelbein et al. 1997) and this distinction appeared similar in both genotypes.

The first sign of swelling was seen within the apical-most portion of the elongation zone (Fig. 2F, H). This region is where the epidermis emerges to become the outermost layer of the root, that is, where the remaining layer of lateral root cap is lost. Here, trichoblast cells, identified from their position over two underlying cortex

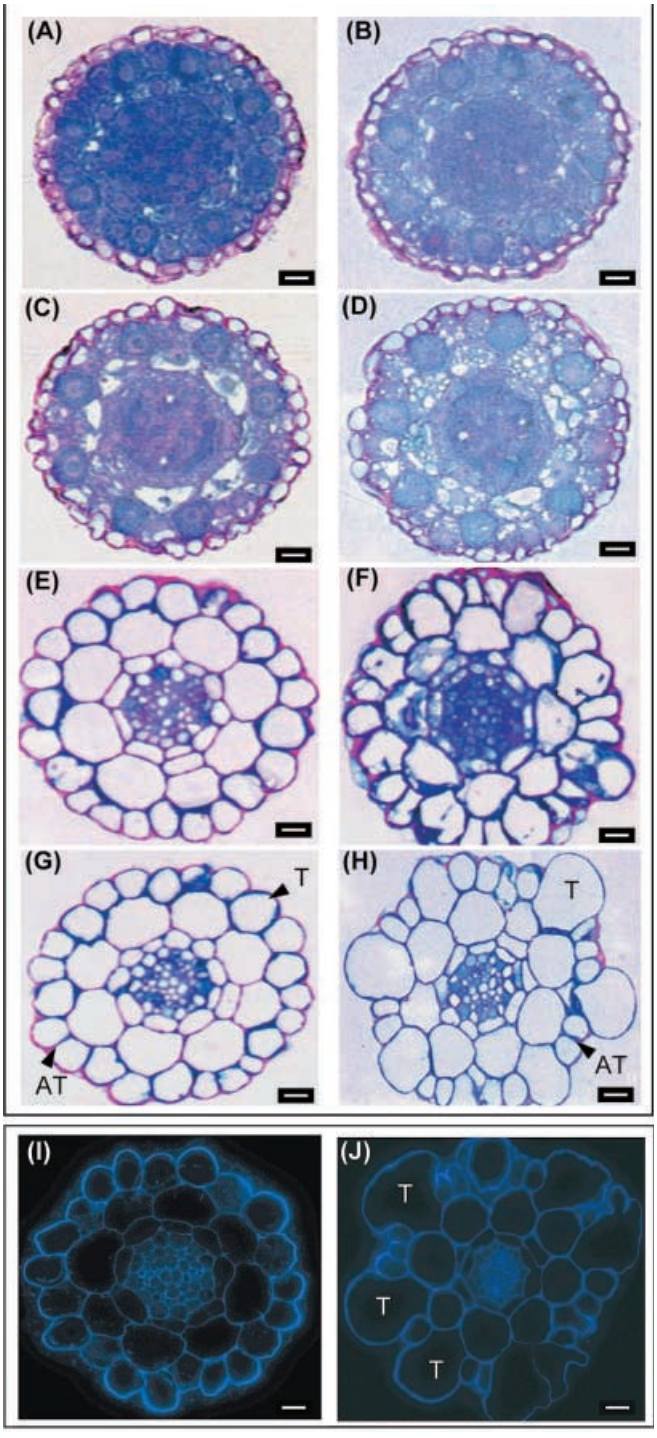

Fig. 2. Light micrographs of transverse cross-sections showing cell shape for wild type (**A, C, E, G, I**) and *reb1-1* (**B, D, F, H, J**). Sections **A–D** were made in the meristem, whereas sections **E–J** were made in the elongation zone. Sections were stained with methylene blue–basic fuchsin, except for sections **I** and **J**, which were stained with calcofluor. Note the swelling of trichoblasts in **H** and **J**. *AT* Atrichoblasts, *T* trichoblasts. Bars = 15 μm

cells, appeared notably larger in cross-sectional area than the neighboring atrichoblasts and the wild-type trichoblasts. With greater distance from the tip (e.g. 500 μm to 1 mm, Fig. 2D, H), the swelling in trichoblasts became pronounced. In contrast, swelling was noticeable neither in atrichoblasts nor in other tissues of the root. In Fig. 2, images of the two genotypes are matched by distance from the tip; when the wild type was examined at greater distance, where its root hairs were forming, trichoblasts still did not bulge appreciably (see Fig. 4C).

Together, these data demonstrate that the root epidermal bulging of *reb1-1* is specific to trichoblasts and is initiated within the zone of elongation.

Localization of cell wall epitopes in *reb1-1*

Cell wall components are known to play a role in determining the morphology of plant cells. Because of the previous observation that *reb1-1* has reduced amounts of arabinogalactan-protein (Ding and Zhu 1997), we examined the spatial distribution of this class of cell wall component. To this end, we probed longitudinal sections with the monoclonal antibody JIM14, which recognizes an arabinogalactan-protein epitope (Knox et al. 1991). JIM14 stained cell walls of the wild type ubiquitously, except for a small region in the root cap adjacent to the quiescent center (Fig. 3A). In contrast, in *reb1-1*, JIM14 stained epidermal cells in the elongation zone weakly or not at all (arrowheads on Fig. 3B).

To investigate the developmental stage at which the loss of arabinogalactan-protein staining occurred, we examined serial transverse sections taken from different positions of the root. In the elongation zone, JIM14 stained essentially all cells in the wild type but in *reb1-1*, JIM14 stained some atrichoblasts but rarely stained trichoblasts (Fig. 4C, F). The significance of this pattern was supported by following staining back into the meristem. In the wild-type meristem, JIM14 stained trichoblasts more strongly than atrichoblasts (Fig. 4A, B) whereas in *reb1-1* this difference between the cell types was largely eliminated (Fig. 4D, E).

The loss of arabinogalactan-protein staining from *reb1-1* trichoblasts was confirmed by examining immunogold label with the electron microscope. Immunogold labeling with the JIM14 antibody was found over the plasmalemma as well as over a thin layer close to the plasmalemma. In the wild type, the antibody labeled atrichoblasts and trichoblasts, whereas in the mutant, while the atrichoblasts were labeled as usual, the trichoblasts were not labeled to any appreciable extent (Fig. 5A, B). To confirm the results with JIM14, sections were stained for light and electron microscopy with another anti-arabinogalactan-protein antibody, LM2. Like JIM14, LM 2 stained all epidermal cells in the wild type with a preference for trichoblasts in the meristem (data not shown), while in *reb1-1* it stained trichoblasts in the elongation zone weakly or not at all (Fig. 5C, D).

To determine whether the loss of arabinogalactan-protein epitopes in the elongation zone of *reb1-1* was specific or instead resulted from some generalized cell

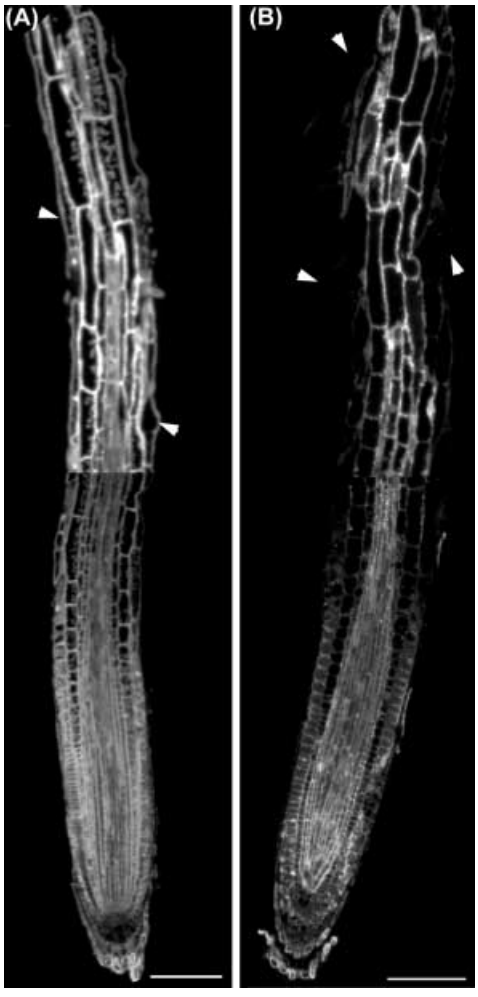

Fig. 3. Fluorescence micrographs of longitudinal sections showing staining with the mAb JIM14 for wild type (**A**) and *reb1-1* (**B**). *Arrowheads* point to root epidermal cells. Note that in *reb1-1* some epidermal cells are weakly labeled or not labeled. Bars = 100 μm

wall disruption in the swollen cells, sections were stained with anti-pectin antibodies. Cell walls throughout the growth zones in both genotypes stained intensely with JIM5 (Fig. 6) and with an anti-bupleuran antibody (data not shown), which is also specific for pectin (Sakurai et al. 1998; Andème-Onzighi et al. 2000).

Ultrastructural modifications of the epidermal cell wall in *reb1-1*

Staining of cross-sections with calcofluor, a specific dye for β-glucans such as cellulose, showed no major difference in the staining pattern, except that epidermal walls of trichoblasts in *reb1-1* were sometimes stained only weakly (Fig. 2I, J). This may reflect a difference in the thickness of the outer walls of trichoblasts in the mutant as compared to the wild type. Therefore, we quantified the thickness of the outer epidermal cell walls from electron micrographs of cross-sectioned cell walls (Table 1). In the wild type, atrichoblasts had outer epidermal cell walls that were about 150% thicker than the walls of the trichoblasts; however, in the mutant, the atrichoblast outer epidermal cell walls were about 400% thicker than those of trichoblasts, a difference in thickness caused by an appreciable thinning specifically of the trichoblast cell wall.

To determine whether the mutation disrupted the overall architecture of the outer epidermal cell wall, we examined ultrathin sections by PATAg staining. As typical of root epidermal cells, two major zones were discernable within the epidermal outer wall. In both trichoblasts and atrichoblasts of the wild type, the half-layer of the wall nearest the plasma membrane had a granular structure and was densely stained, while the outer half-layer was fibrillar and less reactive to PATAg (Fig. 7A, C). In *reb1-1*, although the outer cell wall of

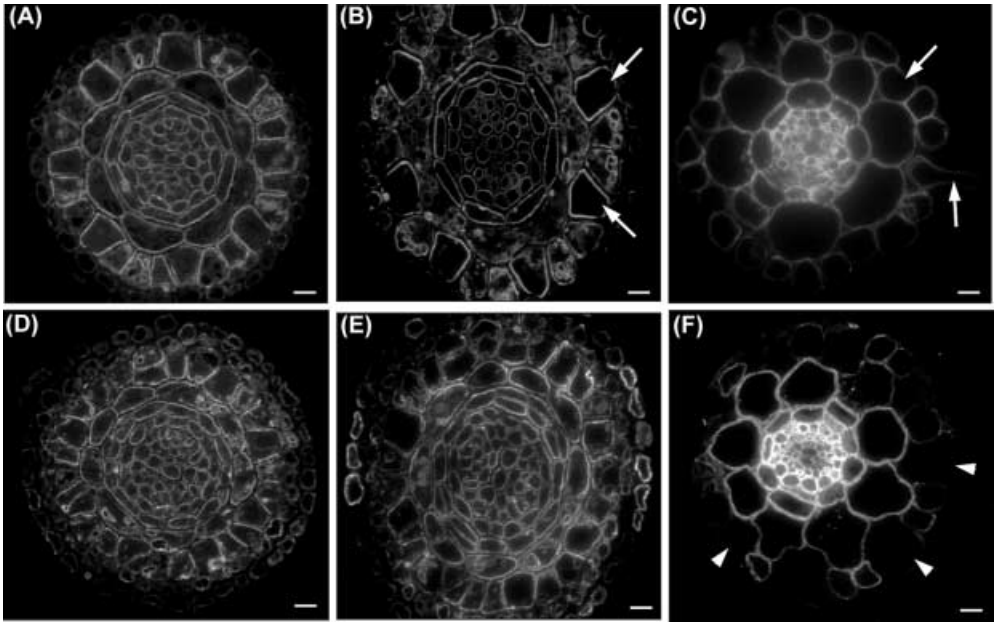

Fig. 4. Fluorescence micrographs of root cross-sections showing staining with JIM14 for wild type (**A–C**) and *reb1-1* (**D–F**). Sections **A**, **B**, **D** and **E** show the meristem, whereas sections **C** and **F** show the elongation zone. Note that in *reb1-1*, swollen trichoblasts are weakly labeled or not labeled at all (*arrowheads*), whereas, in the wild type, they tend to be labeled preferentially in the meristem (*arrows*). Bars = 15 μm

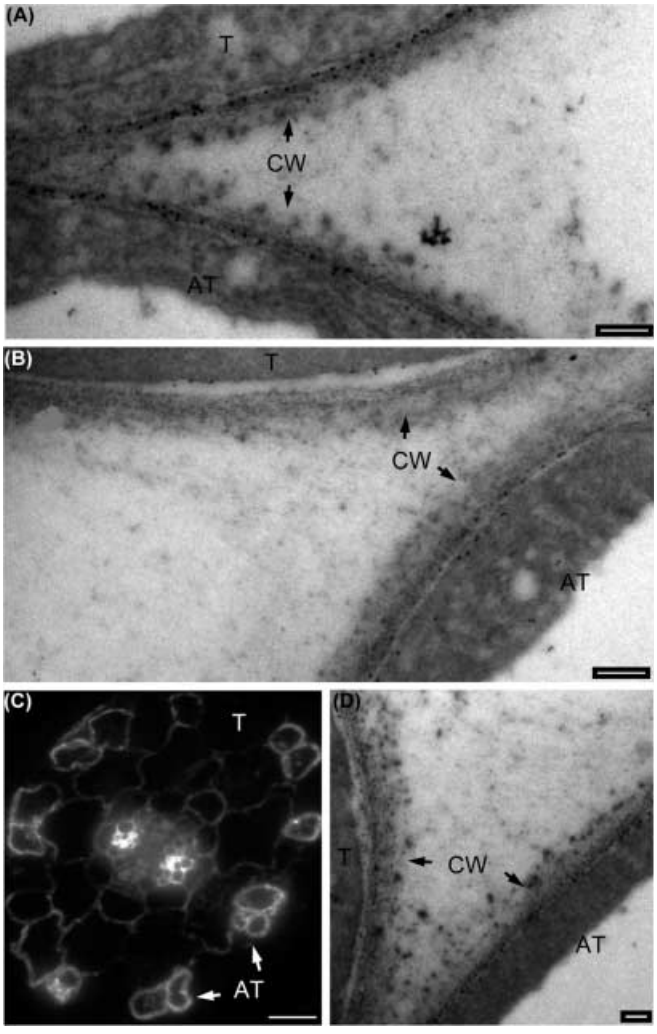

Fig. 5. **A, B** Transmission electron micrographs showing immunogold labeling with JIM14 for epidermal cell walls of wild type (**A**) and *reb1-1* (**B**). **C, D** Fluorescence (**C**) and electron (**D**) micrographs showing LM2 staining for *reb1-1*. *Black arrowheads* point to the cell wall in **A**, **B** and **D**. *White arrowheads* point to stained atrichoblasts in **C**. *AT* Atrichoblasts, *CW* cell wall, *T* trichoblasts. Bars = 1.5 μm (**A, B, D**), 2.5 μm (**C**)

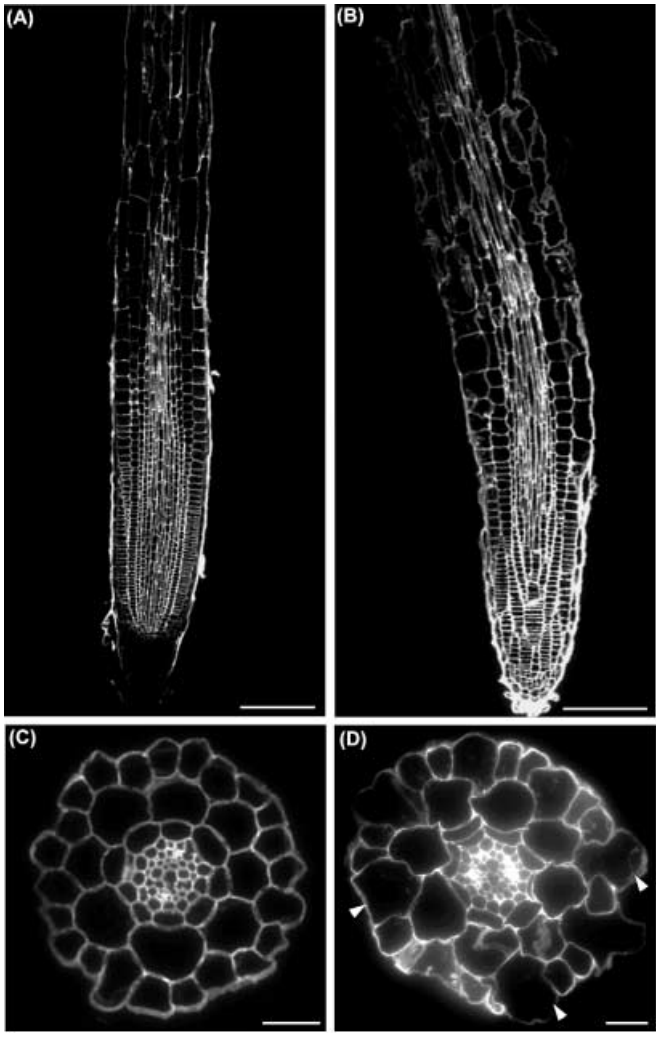

Fig. 6. Fluorescence micrographs of root longitudinal (**A, B**) and cross-sections (**C, D**) showing staining with the mAb JIM5 for wild type (**A, C**) and *reb1-1* (**B, D**). Cross-sections were made in the elongation zone where swollen trichoblasts are found (*arrowheads*). Bars = 100 μm (**A, B**), 25 μm (**C, D**)

atrichoblasts appeared similar to that of the wild type (Fig. 7A, B), the cell wall of trichoblasts differed: the two PATAg-reactive layers were less distinct than in the wild type and the outermost fibrillar layer was stained irregularly and appeared disrupted (Fig. 7D).

We examined the secretory apparatus (endoplasmic reticulum, Golgi stacks, and transport vesicles), in root tips prepared by high-pressure cryo-fixation and freeze-substitution; in *reb1-1*, these organelles were indistinguishable ultrastructurally from those of the wild type in both epidermal cell types (data not shown).

Alterations of cortical microtubules in *reb1-1*

Because the swelling of the trichoblasts in *reb1-1* suggests that a component of the machinery controlling morphogenesis has been affected, we examined microtubules. To do so, we took advantage of an *Arabidopsis* line expressing GFP fused to a microtubule-binding domain from human MAP 4 (Marc et al. 1998; Granger and Cyr 2001). We crossed this line into *reb1-1* and recovered F2 progeny that were mutant and had brightly fluorescent microtubules. In wild-type roots

Table 1. Thickness of outer cell walls of trichoblasts and atrichoblasts in wild type and *reb1-1 Arabidopsis thaliana*. Measurements were made in the elongation zone, where swollen trichoblasts are found in *reb1-1*. Data represent the mean ± SD ($n = 30$)

Epidermal cell type	Cell wall thickness (nm)	
	Wild type	*reb1-1*
Trichoblast	623 ± 27	203 ± 24
Atrichoblast	964 ± 67	818 ± 92

where root hairs emerge, cells including the non-hair part of the trichoblast had helical microtubules (Fig. 8A, C). In a similar comparable region of *reb1-1*, while atrichoblasts had helical microtubules, many of the bulging cells had no detectable microtubules (Fig. 8B). In bulbous cells that nevertheless formed a partial root hair, microtubules appeared but with aberrant organization (Fig. 8D). In roots prepared by high-pressure cryo-fixation and observed with transmission electron microscopy, microtubules were observed readily in the wild type, typically in parallel arrays (Fig. 9A, B) but were rare in the bulging trichoblasts of *reb1-1* and rarely oriented uniformly (Fig. 9C). Taken together, these results show that the phenotype of *reb1-1* includes defects in the organization and abundance of cortical microtubules.

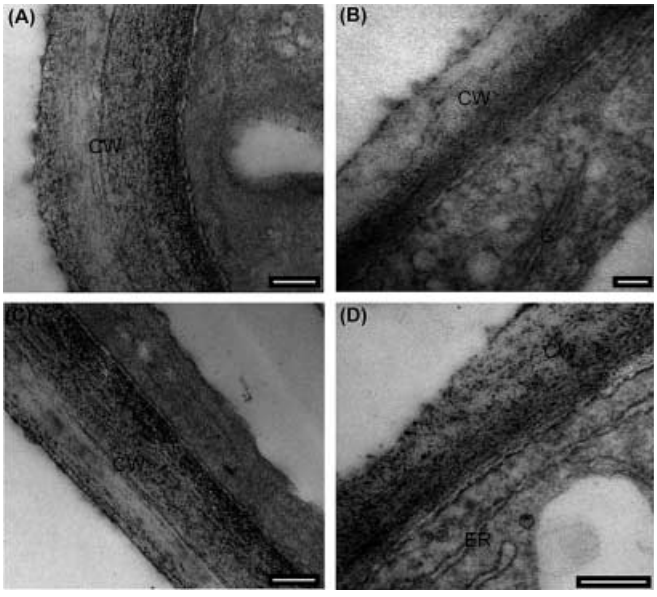

Fig. 7. Transmission electron micrographs of PATAg-stained root epidermal cell walls showing atrichoblasts (**A, B**) and trichoblasts (**C, D**) in the wild type (**A, C**) and *reb1-1* (**B, D**). *CW* Cell wall, *ER* endoplasmic reticulum, *G* Golgi stack. Bars = 0.3 μm

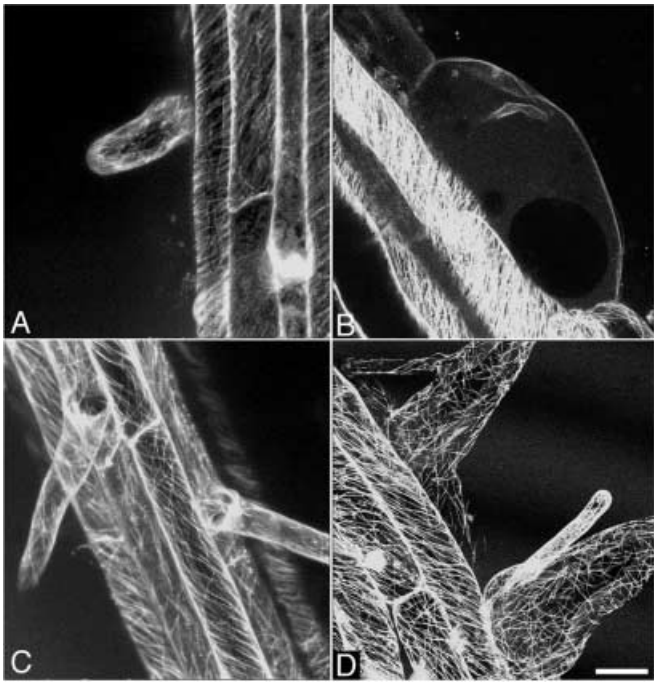

Fig. 8A–D. Confocal fluorescence micrographs of microtubules in living root epidermal cells of plants expressing GFP::MBD. **A, C** Wild type; **B, D** *reb1-1*. Panels show the zone where root hairs emerge and elongate, and are combined projections of different numbers (12–24) of optical sections. Bar in **D** = 15 μm

Discussion

Producing organs with defined and heritable shape requires precise control over the directionality of cell expansion. To understand this control, we studied a mutant, *reb1-1*, in which expansion is abnormal among selected root cells. We found that bulging in the roots of *reb1-1* begins in the zone of elongation and occurs in trichoblasts only. Trichoblast bulging appeared to result from decreased arabinogalactan-protein in the cell wall, as evident from a loss of staining by antibodies recognizing arabinogalactan-protein epitopes specifically in trichoblasts. The loss of arabinogalactan-protein

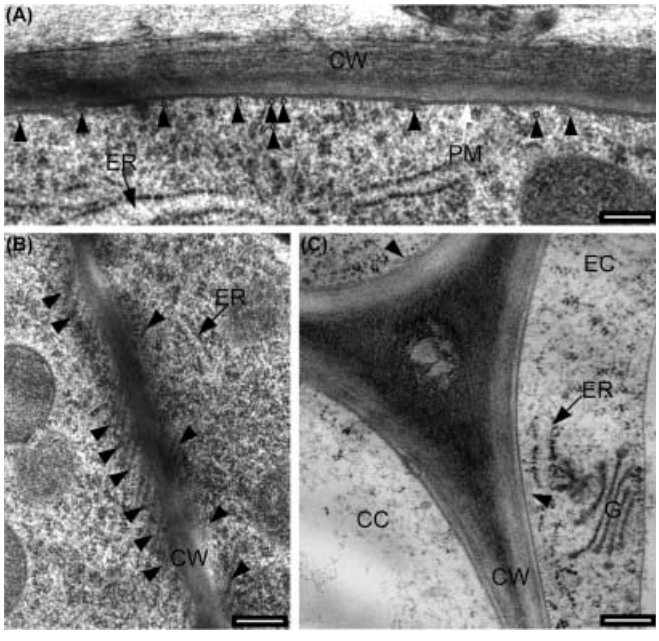

Fig. 9A–C. Electron micrographs of cortical microtubules in high-pressure-frozen/freeze-substituted roots. **A** Wild-type epidermal cell: note the abundant microtubule cross-sections (*arrowheads*). The *white arrow* indicates the plasma membrane. **B** Wild-type trichoblast and cortical cell: note the parallel microtubules viewed longitudinally (*arrowheads*). **C** *reb1-1* trichoblast: microtubules were infrequent and rarely oriented uniformly (*arrowhead*). The *black arrows* (**A–C**) indicate the endoplasmic reticulum. *CC* Cortical cell, *CW* cell wall, *EC* epidermal cell, *ER* endoplasmic reticulum, *G* Golgi stack, *PM* plasma membrane. Bars = 0.3 μm (**A**), 0.5 μm (**B**), 0.35 μm (**C**)

contrasted with the wild type where, in the meristem, trichoblasts stained more intensely with these antibodies compared to atrichoblasts. Additionally, bulging was accompanied by changes in the organization of cortical microtubules, a finding that implies an interaction between microtubules and arabinogalactan-proteins. We hypothesize that the *REB1* gene product functions to prevent a trichoblast from swelling all over while it swells in a limited area to form a root hair.

The trichoblast-specific phenotype of *reb1-1*

All of the phenotypes we have seen for *reb1-1* are consistent with its acting predominantly in trichoblast files. Inspection of scanning electron micrographs of *reb1-1* roots, as well as of cross-sections of the root, showed that swelling occurs predominantly, if not exclusively, in trichoblast files. Compared to atrichoblasts either in the wild type or in the mutant, cell walls in *reb1-1* trichoblast files are thinner, disrupted in overall structure, and contain less or abnormal arabinogalactan-proteins. Additionally, specifically in trichoblast files, cortical microtubules are disorganized and often absent. For these reasons, we conclude that REB1 acts predominantly in the trichoblast files.

Consistently, *reb1-1* has been recently discovered to be allelic to *root hair defective1* (*rhd1*; Wubben et al. 2001), a mutant isolated in a visual screen for plants with absent or defective root hairs (Schiefelbein and Somerville 1990). In the pathway of root hair differentiation, *rhd1* has been assigned a structural as opposed to a regulatory role (Ryan et al. 2001), an assignment that is consistent with the cell wall and microtubule anomalies detected here.

There is, however, one presumptive exception to this: *reb1-1* roots elongate more slowly than those of the wild type (Baskin et al. 1992). This implies that atrichoblasts as well as all of the other cells in the root must have lower elongation rates, and therefore that cell elongation throughout the root may require REB1. This interpretation is a possibility, not a certainty; instead, elongation rate may decrease as a coordinated regulation of growth in response to trichoblast bulging. Until the role of cell–cell interactions in regulating organ expansion is better understood, the diminished elongation rate of *reb1-1* roots cannot be interpreted unambiguously to mean that REB1 is active in most or all root cells.

The role of *REB1* in trichoblast differentiation

Although root hairs emerge near the base of the elongation zone, trichoblasts are distinct from atrichoblasts at much more apical positions, possibly even up to the epidermal initials. This is evident, for example, by the dense cytoplasmic staining of meristematic trichoblasts (Dolan et al. 1993; Galway et al. 1994), by the fact that atrichoblasts are longer than trichoblasts throughout most if not all of the meristem (Beemster and Baskin 1998), and by various reporter constructs which show the expression of markers going right up to the tip in one type of file but not in the other (Masucci and Schiefelbein 1996). We can now add to such observations the finding that certain arabinogalactan-protein epitopes are expressed more strongly in trichoblasts than atrichoblasts in most of the wild-type meristem, well before the initiation of the root hair.

What is the significance of the enhanced presence of arabinogalactan-protein? Given that the expression pattern is inverted in *reb1-1*, and that subsequently *reb1-1* trichoblasts bulge, we hypothesize that the arabinogalactan-protein is required to restrain radial expansion in the face of the process that causes a localized bulge forming the root hair. In this connection, it is noteworthy that the plant hormone ethylene apparently plays a role in the induction of root hair initiation and growth in trichoblasts (Masucci and Schiefelbein 1994; Tanimoto et al. 1995). However, ethylene is also produced by roots subjected to various types of stress, such as low oxygen or compact soil, and under these conditions, the entire root swells (He et al. 1996). Therefore, it is plausible that trichoblasts require a specific mechanism to prevent the ethylene needed for root hair formation from causing cell swelling. The restraint mechanism, according to our hypothesis, requires REB1. When larger quantities of ethylene are produced under stress, this restraint is either overwhelmed or repressed, and thus epidermal cells swell. The hypothesis predicts that ectopic, overproduction of REB1 would prevent ethylene-induced root swelling and that, in *reb1-1*, eliminating the response to ethylene would eliminate epidermal bulging. The latter prediction has recently been affirmed by Wubben et al. (2001) who showed that blocking ethylene biosynthesis in *rhd1-4* completely prevented the bulging of trichoblasts.

Cortical microtubules and arabinogalactan-proteins

We have found that microtubules in the swollen trichoblasts of *reb1-1* are absent or disorganized. From the results here, one may propose with equal validity that the microtubule defect caused the cell wall defect, or vice versa, or even that they are independent consequences of the mutation. However, *REB1* has recently been cloned, and found to belong to a family of UDP-glucose-4-epimerase-like genes and to have the appropriate activity for synthesizing D-galactose (G. Seifert, John Innes Institute, Norwich, UK; personal communication). This gene is active in sugar metabolism with a plausible role in synthesizing arabinogalactan-proteins; it is implausible, though not impossible, that the epimerase has evolved a separate function in microtubule organization despite retaining its catalytic activity. Therefore, knowledge of the gene function allows us to argue that the cell wall defect causes the defective microtubule organization.

Whether the microtubules are disorganized by the missing or defective arabinogalactan-proteins or by some other component of the cell wall, these results begin to define a link between cell wall structure and microtubule organization. Affecting the microtubule organization by drugs might be useful to test further such a link. Most of the attention paid to cortical microtubules concerned their role in orienting the cellulose microfibrils of the cell wall (Baskin 2001). Nevertheless, how the microtubules themselves become organized remains persistently enigmatic (Williamson 1990; Hush and Overall 1996; Marc 1997). Models have been developed in which the mechanical status of the cell wall is transduced into the cytoplasm and guides the organization among microtubules (Williamson 1990; Pickard 1994; Fisher and Cyr 1998). However, experimentation has lagged behind theory and there is little direct evidence even to say that information does flow from cell wall to cell interior, let alone any evidence to identify the molecules participating in the transmission. Our results here support the hypothesis that plasma-membrane arabinogalactan-proteins mediate interactions between the cell wall and the cortical array of microtubules, interactions that regulate the direction of expansion and that may be further elucidated in the *reb1-1* mutant.

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