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E Nguema-Ona

A Bannigan

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TI Baskin

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Disruption of arabinogalactan proteins disorganizes cortical microtubules in the root of *Arabidopsis thaliana*

Eric Nguema-Ona¹, Alex Bannigan², Laurence Chevalier¹, Tobias I. Baskin² and Azeddine Driouich^{1,*}

¹UMR CNRS 6037, IFRMP 23, Plate Forme de Recherche en Imagerie Cellulaire, Université de Rouen, 76 821 Mont Saint Aignan, Cedex, France, and

²Biology Department, University of Massachusetts Amherst, 611 N. Pleasant Street, MA 01003, USA

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*For correspondence (fax +33 235 14 6615; e-mail azeddine.driouich@univ-rouen.fr).

Summary

The cortical array of microtubules inside the cell and arabinogalactan proteins on the external surface of the cell are each implicated in plant morphogenesis. To determine whether the cortical array is influenced by arabinogalactan proteins, we first treated *Arabidopsis* roots with a Yariv reagent that binds arabinogalactan proteins. Cortical microtubules were markedly disorganized by 1 μM β -D-glucosyl (active) Yariv but not by up to 10 μM β -D-mannosyl (inactive) Yariv. This was observed for 24-h treatments in wild-type roots, fixed and stained with anti-tubulin antibodies, as well as in living roots expressing a green fluorescent protein (GFP) reporter for microtubules. Using the reporter line, microtubule disorganization was evident within 10 min of treatment with 5 μM active Yariv and extensive by 30 min. Active Yariv (5 μM) disorganized cortical microtubules after gadolinium pre-treatment, suggesting that this effect is independent of calcium influx across the plasma membrane. Similar effects on cortical microtubules, over a similar time scale, were induced by two anti-arabinogalactan-protein antibodies (JIM13 and JIM14) but not by antibodies recognizing pectin or xyloglucan epitopes. Active Yariv, JIM13, and JIM14 caused arabinogalactan proteins to aggregate rapidly, as assessed either in fixed wild-type roots or in the living cells of a line expressing a plasma membrane-anchored arabinogalactan protein from tomato fused to GFP. Finally, electron microscopy of roots prepared by high-pressure freezing showed that treatment with 5 μM active Yariv for 2 h significantly increased the distance between cortical microtubules and the plasma membrane. These findings demonstrate that cell surface arabinogalactan proteins influence the organization of cortical microtubules.

Keywords: arabinogalactan protein, cell wall, cryofixation, microtubules, morphogenesis, root growth.

Introduction

The extracellular matrix is a major determinant of cell growth and morphogenesis. As a material, the matrix can be considered as a fiber-reinforced composite. In animal cells the extracellular matrix comprises roughly equal amounts of protein and polysaccharide, whereas in plants the matrix, known as the cell wall, comprises mainly polysaccharide. Cell wall polysaccharides are assembled into complex macromolecules, including cellulose, hemicellulose, and pectin. Cellulose forms microfibrils, which constitute an ordered, fibrous phase, whereas pectin and hemicellulose form an amorphous matrix phase, surrounding the microfibrils. Hemicellulose is believed to regulate the linkage and mechanical interactions between microfibrils, whereas the pectin is believed to form a more or less independent gel,

influencing the mechanical behavior of the wall as well as its permeability (Cosgrove, 2005).

While the polysaccharides constitute the overall fabric of the cell wall, proteins and proteoglycans are also present. In general, these macromolecules are in low abundance and of unclear function, although in some cases roles in architecture or defense against pathogens have been established. One class that has attracted particular attention are the arabinogalactan proteins. This class consists of a polypeptide backbone decorated with branched arabinogalactans; the glycosylation is extensive, typically amounting to more than 90% of the mass of the macromolecule (Nothnagel, 1997). Arabinogalactan proteins have been implicated in a variety of cellular functions, ranging from embryogenesis to

expansion (Gaspar *et al.*, 2001; Majewska-Sawka and Nothnagel, 2000). A noteworthy feature of this cell wall component is that a majority of them (the so-called 'classical' arabinogalactan proteins; Schultz *et al.*, 2000), contain a carboxyl-terminal, glycosyl phosphatidylinositol (GPI) anchor that allows their tight association with the external leaflet of the plasma membrane (Oxley and Bacic, 1999; Svetek *et al.*, 1999; Youl *et al.*, 1998).

However, while a role for this class of proteoglycan in morphogenesis is accepted, demonstrating specific functions has proved to be difficult. In the Arabidopsis genome, there are 40 or so putative arabinogalactan-protein backbone genes (Schultz *et al.*, 2002), and for the most part, mutants in these genes have been uninformative. The function of arabinogalactan proteins has been studied with the so-called Yariv reagent. Yariv *et al.* (1967) discovered that a phenyl glycoside containing β -D-glucosyl units ('active Yariv') binds and precipitates arabinogalactan proteins, whereas related phenyl glycosides, for example containing α -D-mannosyl units ('inactive Yariv'), do not. Active Yariv reagent inhibits expansion in suspension-cultured cells (Serpe and Nothnagel, 1994) and disrupts morphogenesis in roots, shoots, and even pollen tubes (Mollet *et al.*, 2002; Vitré *et al.*, 2005; Willats and Knox, 1996). However, at least in pollen tubes, active Yariv massively disrupts cell wall assembly (Roy *et al.*, 1998), which implies that the effect of this reagent on morphogenesis could reflect a dominant, gain-of-function action of the inhibitor rather than the loss of arabinogalactan-protein function.

Their location at the cell surface has encouraged speculation that arabinogalactan proteins function in information flow between the cytoplasm and the cell wall. Among the messages that must flow between the cell and the cell wall are instructions for the orientation of cellulose microfibrils. In an elongating cell, microfibrils are aligned at right angles to the direction of maximum expansion rate; this alignment gives the wall a mechanical anisotropy that dictates the expansion anisotropy (Baskin, 2005; Green, 1980). In secondary cell walls, the angle of microfibril alignment governs wall strength and stiffness (Reiterer *et al.*, 1999). A microfibril is synthesized at the plasma membrane by a multi-subunit complex, called a rosette (Lerouxel *et al.*, 2006). The rosette incorporates glucose residues at its cytosolic side and at its extracellular side extrudes polymerized glucose chains, which crystallize into a microfibril. As they synthesize microfibrils, rosettes translate within the plasma membrane; microfibrils are thought to be aligned in a specific orientation by the cell controlling the translation of the rosettes.

Controlling the movement of the rosettes has long been ascribed to the cortical array of microtubules. These microtubules abut the plasma membrane and are highly ordered. Since their discovery in the early 1960s, cortical microtubules have been repeatedly observed to parallel microfibrils;

furthermore, inhibiting microtubule polymerization usually disturbs microfibril alignment (Baskin, 2001). Recently, the idea that cortical microtubules help align cellulose was supported by Paredes *et al.* (2006) who labeled one of the subunits of the rosette and showed, in living cells of the Arabidopsis hypocotyl, that the movement of the label paralleled cortical microtubules, and became aberrant soon after the disruption of cortical microtubules.

However, while the involvement of microtubules in directing cellulose alignment is established, the molecular details of this process remain obscure, and other roles for microtubules have been suggested (Wasteneys, 2004). Investigators have failed to detect interactions between microtubules and any of the known subunits of the rosette. And while indirect interactions have been imagined, none have been validated experimentally. Furthermore, it has been suggested repeatedly that cortical microtubules may influence morphogenesis by means additional to controlling the alignment of microfibrils. (Emons *et al.*, 1992; Sugimoto *et al.*, 2003), and various alternative targets have been proposed, including arabinogalactan proteins (Wasteneys and Galway, 2003).

Because arabinogalactan proteins are present at the plasma membrane, they are good candidates for a link in the chain between cortical microtubules and cell wall assembly. Evidence for a potential interaction between arabinogalactan proteins and cortical microtubules has come from a study of the *root epidermal bulger1 (reb1)* mutant of Arabidopsis (Andème-Onzighi *et al.*, 2002). The *reb1-1* mutant, allelic to *root hair defective1 (rhd1)*, is characterized by a reduced elongation rate of the primary root and by bulging trichoblasts (Baskin *et al.*, 1992). The *REB1/RHD1* gene belongs to a family of UDP-D-glucose 4-epimerases involved in the synthesis of D-galactose (Seifert *et al.*, 2002). Interestingly, the swollen trichoblasts of the mutant have disorganized cortical microtubules and lowered levels and aberrant synthesis of certain arabinogalactan proteins (Andème-Onzighi *et al.*, 2002; Ding and Zhu, 1997). Further evidence comes from a recent observation that tobacco tissue culture cells treated for many hours with high concentrations of active Yariv reagent have disorganized cortical microtubules (Sardar *et al.*, 2006).

Here we used Yariv reagent to test the hypothesis that cell surface arabinogalactan proteins influence the organization of cortical microtubules, but we also took advantage of monoclonal antibodies that recognize epitopes of arabinogalactan proteins, and we focused on short-term treatments, from minutes to a few hours. We assessed microtubule organization and arabinogalactan-protein status in both fixed cells with anti-tubulin antibodies and immunocytochemistry, and in living cells with GFP reporters. Finally, we used high-pressure freezing and transmission electron microscopy (TEM) to examine cell ultrastructure and cortical microtubule distribution. We find that binding arabinogalactan proteins

leads to microtubule disorganization within 30 min, supporting the idea of a linkage between these elements in the process of morphogenesis.

Results

Effect of Yariv reagents on microtubule organization

Previously, investigators have shown that active Yariv reagent causes root epidermal cells to swell (Ding and Zhu, 1997; Willats and Knox, 1996), and we confirmed this here (Figure 1). Inactive Yariv did not visibly alter elongation rate or morphology whereas active Yariv gave rise to the characteristic cell bulging. The swelling was weak in the meristem and strong in the elongation zone, which is also true for trichoblasts in *reb1/rhd1* mutant (Andème-Onzighi *et al.*, 2002). These effects were pronounced at 1 μM and apparently saturated at 5 μM . Inactive Yariv (up to 10 μM) did not cause any detectable change in morphology.

To investigate the status of cortical microtubules, seedlings were treated with Yariv reagents for 24 h and then fixed and labeled with anti-tubulin antibodies. In roots treated with 1 μM inactive Yariv, cells in the elongation zone had well-organized transverse cortical microtubules (Figure 2a–c). In contrast, cells in the elongation zone in roots treated with active Yariv (1 μM) had disorganized cortical microtubules (Figure 2d–f). Similar disorganization was observed in a transgenic line expressing GFP fused to the

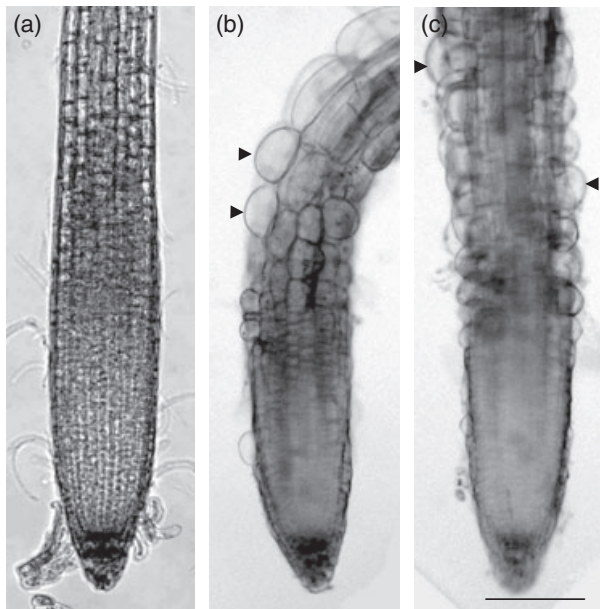


Figure 1. Effects of Yariv reagents on the morphology of 5-day-old *Arabidopsis* roots.

Treatments were: (a) 1 μM inactive Yariv, (b) 1 μM active Yariv, (c) 5 μM active Yariv. All treatments were for 24 h. Note the swelling of epidermal cells (arrowheads). Bar = 50 μm .

microtubule-binding domain from human MAP4 (data not shown). Treatment with inactive Yariv (up to 10 μM) caused no obvious microtubule disorganization (Figure S1a,b).

To determine how rapidly microtubule organization was affected, we perfused roots expressing the GFP–microtubule reporter while imaging living epidermal cells through confocal fluorescence microscopy. Whereas perfusion with buffer, or with inactive Yariv (5 μM), caused little if any net change in the status of the microtubules (Figure S1c,d), perfusion with active Yariv (5 μM) caused microtubules to become clearly disorganized by 30 min (Figure 3a–c). The first signs of disorganization appeared after 15 min in a few cells, and then progressed by 30 min to almost all the cells of the epidermis in the elongation zone. To assess the reproducibility of the time course, we quantified microtubule orientation relative to the long axis of the cell (Figure 3d–f). Transverse microtubules (i.e. those between 80° and 100°) represented 65% of the population at time zero and fell to 50% by 20 min of treatment, and to 27% by 30 min. Thus, active Yariv reagent rapidly disorganizes the cortical array.

Effects of anti-arabinogalactan-protein antibodies

Because the specificity of active Yariv reagent is open to question (Mashiguchi *et al.*, 2004; Triplett and Timpa, 1997), we took advantage of two monoclonal antibodies that had been raised against arabinogalactan-protein epitopes, JIM13 and JIM14 (Knox *et al.*, 1991). We exposed living roots expressing the GFP–microtubule reporter to antiserum and then, after 30–60 min, imaged microtubules. Compared with treatment without antibody (Figure 4a,b), treatment with JIM14 (Figure 4c,d) or JIM13 (Figure 4e,f) extensively disorganized the microtubules. In contrast, treatment with the anti-pectin monoclonal antibody, JIM5, had no obvious effect on microtubule orientation (Figure 4g,h). Likewise, microtubule orientation was unaltered following treatment with a monoclonal antibody specific for xyloglucan (CCRM-1; data not shown). These results confirm the findings with Yariv reagent that binding arabinogalactan proteins can influence the organization of cortical microtubules.

To gain insight into the localization of arabinogalactan proteins after Yariv or antibody treatments, we took advantage of a transgenic line that expresses a classical arabinogalactan-protein gene from tomato fused to GFP (Estévez *et al.*, 2006), thus allowing this particular arabinogalactan protein to be imaged in living seedlings. Preliminary experiments showed that this line grows indistinguishably from the wild type. Based on its sequence, the reporter protein is expected to be GPI-anchored and hence at the plasma membrane, which is consistent with the even staining of the epidermal cell surface in untreated roots (Figure 5a,b). However, hour-long treatments with JIM13, JIM14, or active

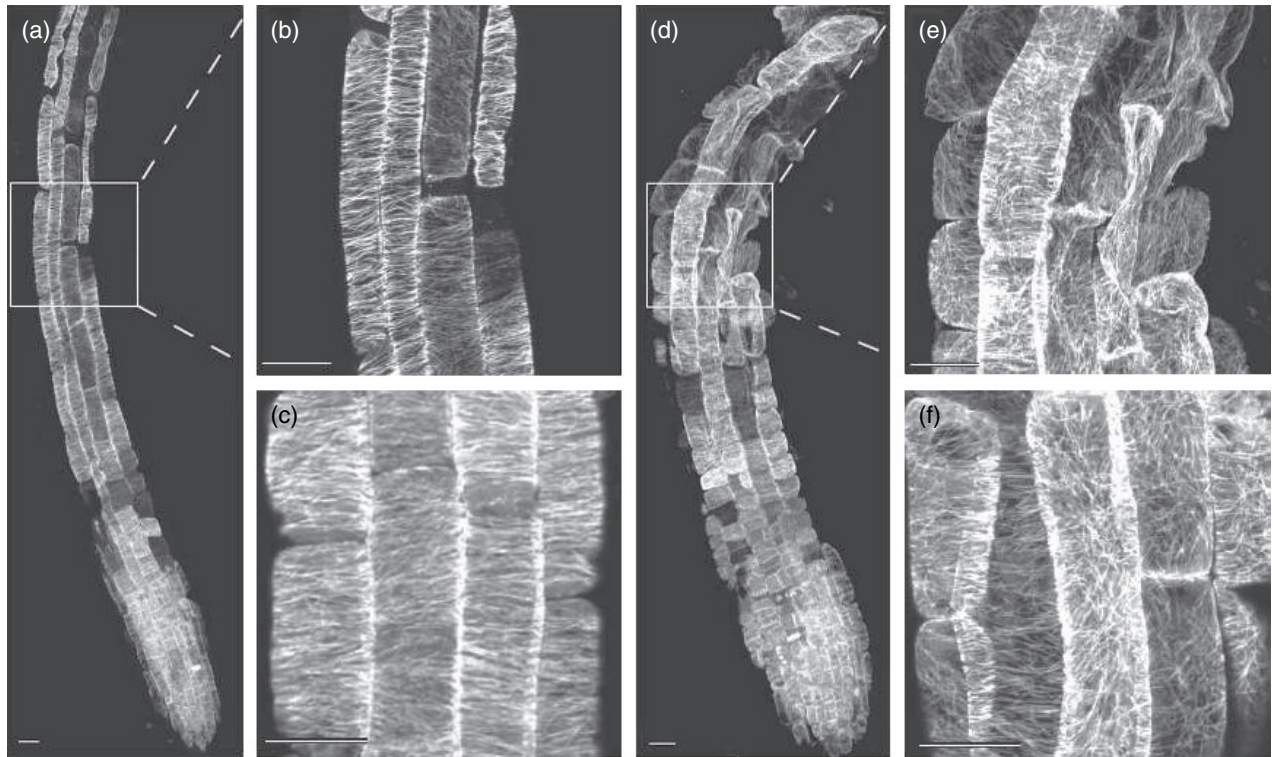


Figure 2. Effects of Yariv reagents on cortical microtubule organization.

Microtubules localized in the epidermis of fixed roots with immunocytochemistry using anti-tubulin antibodies: (a–c) 1 μM inactive Yariv, (d–f) 1 μM active Yariv. Treatments for 24 h. Confocal projections (12 to 29 optical sections). Note the transverse alignment of cortical microtubules in the control (a–c) and the disorganized arrays in the active Yariv treatment (d–e). Bars = 20 μm .

Yariv, caused a punctate staining pattern (Figure 5c–h). The punctate fluorescence increased in intensity over time and was somewhat less pronounced with JIM13 than the other two probes. No apparent alteration in staining was caused by the anti-pectin monoclonal antibody JIM5 (data not shown) or by 5 μM inactive Yariv (Figure S1e,f).

Because of the inevitable uncertainty over a heterologous reporter (i.e. expressing a tomato arabinogalactan protein in *Arabidopsis*), we examined the effect of the probes on arabinogalactan-protein distribution in wild-type root cells. Living roots were treated with antisera for 1 h, then fixed, and immediately stained with the fluorescent secondary antibody (Figure S2). When roots were incubated in buffer for 1 h and then stained for JIM14, smooth, featureless staining resulted (data not shown), similar to that seen in living cells. When living roots were incubated in JIM14, a punctate staining pattern was found, as was the case for JIM13 (data not shown); however, punctate staining was not observed following JIM5 treatment. Note that following treatment with active Yariv, immunostaining with either JIM13 or JIM14 was unsuccessful, presumably because the epitopes had been masked. Taken together, these results show that the binding probes we used rapidly alter the status of the cell surface arabinogalactan proteins.

Yariv-induced disorganization of microtubules and calcium influx

Treating lily (*Lilium longiflorum*) pollen tubes or tobacco (*Nicotiana tabacum*) suspension-cultured cells with active Yariv has been shown to elevate cytosolic calcium levels markedly (Pickard and Fujiki, 2005; Roy *et al.*, 1999). To determine whether the microtubule disorganization seen here involves calcium influx across the plasma membrane, we used gadolinium, a trivalent cation known to block a variety of calcium channels (Demidchik *et al.*, 2002, 2007), and shown previously to block the Yariv-induced influx of calcium in tobacco cells (Pickard and Fujiki, 2005). Gadolinium pre-treatment (1 h with 100 μM GdCl_3) caused little if any disturbance to the microtubules (Figure 6a). Treatment of roots with 10 mM calcium chloride depolymerized microtubules extensively and this was largely prevented by gadolinium pre-treatment (Figure 6b–d), indicating that some calcium influx channels were successfully blocked. Following gadolinium pre-treatment, the microtubule disorganization induced by active Yariv appeared undiminished (Figure 6e). These results suggest that the opening of a gadolinium-sensitive, plasma membrane calcium channel is not required for active Yariv to disorganize cortical microtubules.

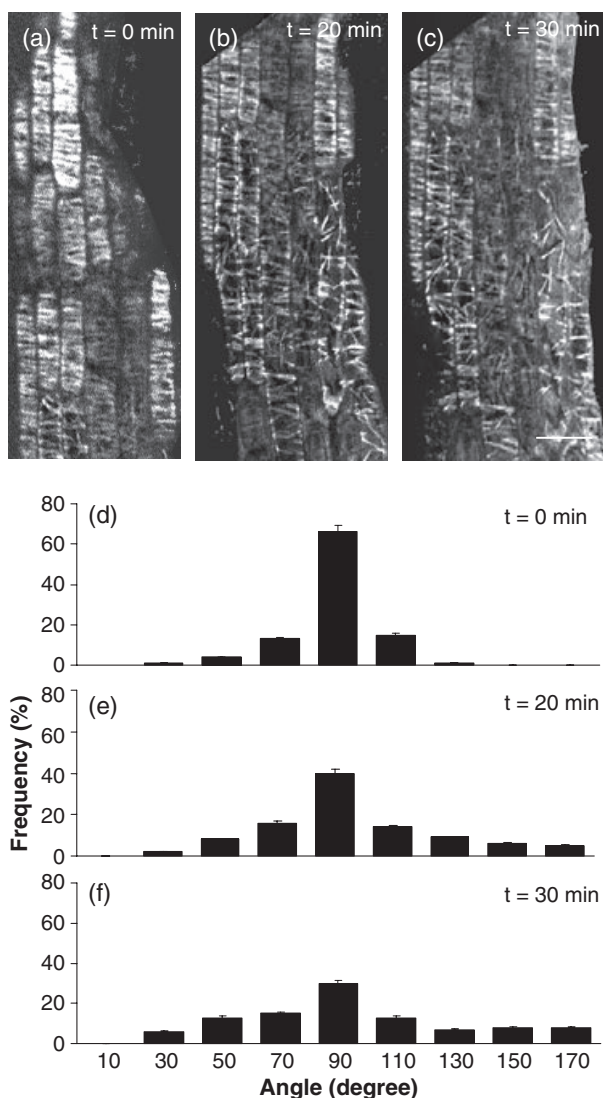


Figure 3. Time-course study of microtubule organization following treatment with Yariv reagent.

(a–c) A line expressing a GFP–microtubule reporter was perfused with 5 μM active Yariv on the stage of the confocal fluorescence microscope, as described in Experimental procedures. Images taken immediately after perfusion ($t = 0$) and at the times indicated in the upper right. Bar = 20 μm . (d–f) Distribution of microtubule angle following treatment with Yariv reagent. Roots were treated and imaged as described for Figure 3 (a–c), and microtubule angles quantified as described in Experimental procedures. A transverse orientation is indicated by an angle of 90°. Bars plot the mean percentage of total for each 10° angle class, \pm SD.

Ultrastructural effects of Yariv reagents observed with high-pressure cryofixation

To obtain further information about the consequences of binding arabinogalactan proteins, we examined ultrastructure in roots that were prepared by high-pressure cryofixation. The plasma membrane in roots treated for 2 h with inactive Yariv (1 μM) was straight and closely appressed to the cell wall (Figure S3). In contrast, in roots treated with

active Yariv (5 μM), the membrane undulated; there were apparent gaps between wall and membrane, occasional invaginations of cell wall material into the cell, and small osmiophilic deposits between wall and membrane or within the wall itself (Figure S3c,d,e). Cortical microtubules usually lay within a microtubule diameter of the plasma membrane in roots treated with inactive Yariv (Figure 7a,b), which is characteristic of the cortical array, including untreated root epidermal cells in material prepared by high-pressure freezing (Andème-Onzighi *et al.*, 2002; Kiss *et al.*, 1990). In contrast, cortical microtubules in roots treated with active Yariv were often displaced from the plasma membrane (Figure 7c,d). This observation was confirmed by measuring the distance between the membrane and the center of the microtubule in epidermal cells, which revealed the distance was increased significantly (Figure 7e). These results suggest that the disorganization of cortical microtubules seen with fluorescence microscopy following disruption of arabinogalactan proteins is associated with a loss of anchorage between microtubules and the membrane.

Discussion

The goal of this study was to test the hypothesis that cell surface arabinogalactan proteins influence cortical microtubules, as suggested earlier (Andème-Onzighi *et al.*, 2002; Sardar *et al.*, 2006). To this end, cortical microtubules in *Arabidopsis* root epidermal cells were examined following disruption of cell surface arabinogalactan proteins not only by means of active Yariv reagent but also by using the monoclonal antibodies JIM13 and JIM14. These probes alter the localization of surface arabinogalactan proteins and concomitantly disorganize cortical microtubules, probably because the microtubules become detached from the plasma membrane. These effects were specific to arabinogalactan-protein binding, insofar as microtubule alignment was unaffected by treatment with an inactive form of Yariv reagent, anti-pectin, or anti-xyloglucan antibodies. These findings demonstrate that extracellular arabinogalactan proteins can influence the organization of cytosolic cortical microtubules.

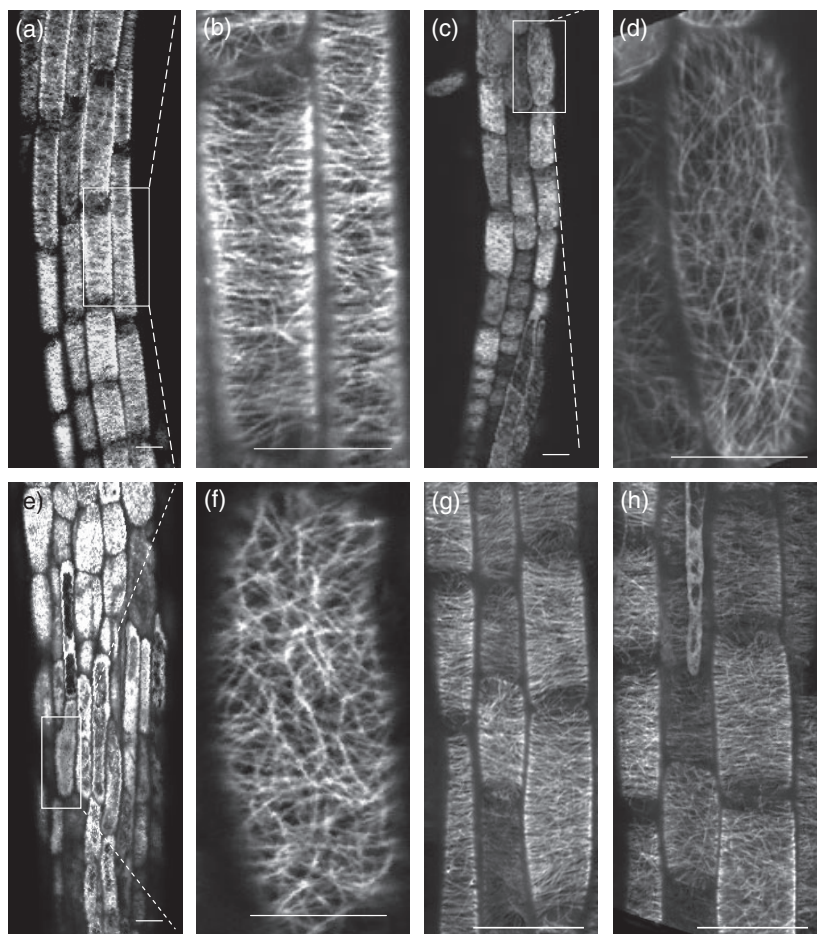
Status of surface arabinogalactan proteins

The active Yariv reagent and the antibodies alter the physical state of arabinogalactan proteins at the extracellular face of the plasma membrane, as judged by the rapid transformation of uniform membrane staining to a heterogeneous pattern. Although binding might stimulate detachment from GPI anchors or endocytosis of a population of arabinogalactan proteins, the simplest explanation is that some of these proteoglycans are mobile within the plane of the plasma membrane, and are therefore aggregated by multivalent probes such as active Yariv or antibodies. If

Figure 4. Effects of antibodies on cortical array organization.

Microtubules imaged in living root epidermis with the GFP-microtubule reporter line: (a, b) buffer; (c, d) JIM14 (monoclonal, recognizes arabinogalactan proteins) for 30 min; (e, f) JIM13 (monoclonal, recognizes arabinogalactan proteins) for 30 min; (g, h) JIM5 (monoclonal, recognizes pectic homogalacturonan) at $t = 0$ (g) and at $t = 30$ min (h).

Note that antibodies against arabinogalactan proteins disorganize the cortical array whereas the anti-pectin antibody does not. Bars = 20 μm .



arabinogalactan proteins are mobile in a time frame of minutes, then this implies that the polymers are neither cross linked to nor entangled with the polysaccharide fabric of the cell wall. This could be tested by treating cells with univalent probes such as Fab fragments of the JIM13 or JIM14 antibodies.

Regardless of their mobility, arabinogalactan proteins, along with other heavily glycosylated extracellular proteins, may constitute a periplasmic zone, similar to the glycocalyx of animal cells (Tarbell and Pahakis, 2006). This zone may be important for cell wall assembly insofar as secreted polysaccharides traverse it on their way to incorporation into the cell wall (Pickard, 2007; Vaughn *et al.*, 2007). A function in cell wall assembly for this zone, enriched in arabinogalactan proteins, could explain the manifest alteration of cell wall ultrastructure seen when growing cells are treated with Yariv reagent (Figure S3; Roy *et al.*, 1998).

Arabinogalactan proteins and microtubules: communication from cell wall to cell

The connection between arabinogalactan proteins and cortical microtubules must be indirect: microtubules are

cytosolic, whereas arabinogalactan proteins are extracellular, with their GPI anchor present only within the outer leaflet of the plasma membrane (Oxley and Bacic, 1999). Previously, a connection was inferred to exist based on observations made on *reb1* (Andème-Onzighi *et al.*, 2002) and treatments with active Yariv lasting 6 h or more (Sardar *et al.*, 2006); however, here we find that binding arabinogalactan proteins causes disorganization of the cortical array in less than 30 min. This short time frame implies that the connection between arabinogalactan proteins and cortical microtubules is reasonably close. If information on the mechanical status of the cell wall were transmitted from the cell wall to the microtubules (Fischer and Cyr, 1998; Kohorn, 2001; Pickard, 2007; Williamson, 1990), then the observations here could be interpreted by saying that the altered arabinogalactan-protein status impacts upon the mechanical properties of the cell wall. This altered status could be transmitted to the microtubules, by some unknown carrier, and result in altered microtubule organization. Arabinogalactan proteins could themselves be part of the load-bearing fabric of the cell wall, or alternatively could interact with load-bearing elements, and in either case information on the status of the cell wall could be communicated to the microtubules.

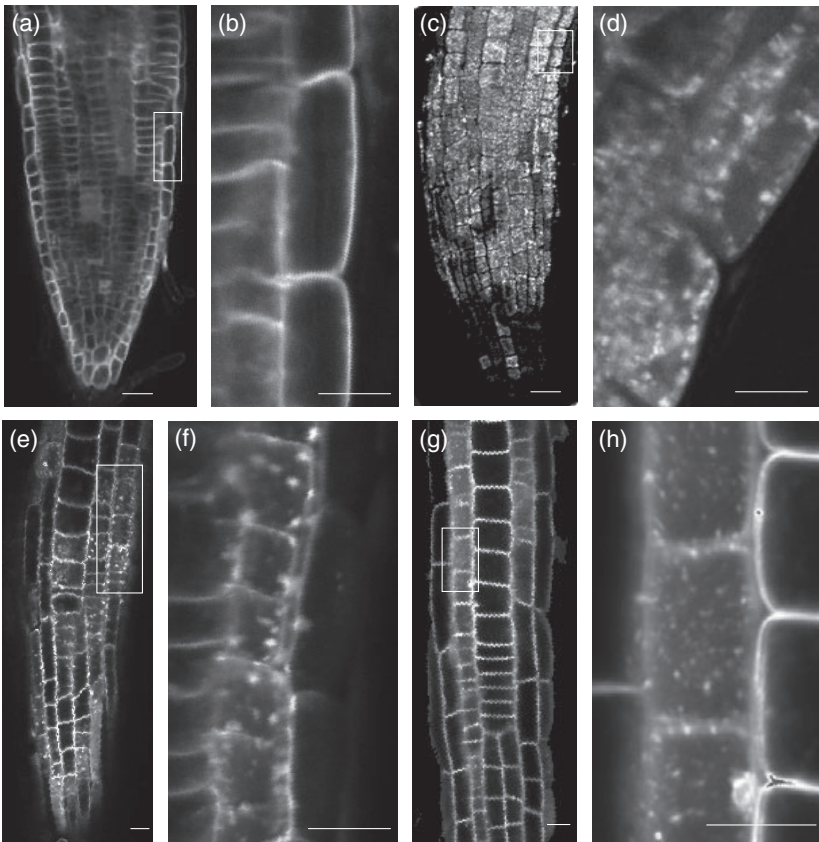


Figure 5. Effects of antibodies and active Yariv on the distribution of arabinogalactan proteins, imaged in living root epidermal cells expressing an arabinogalactan protein tagged with GFP. Treatment times are 30 min: (a, b) buffer; (c, d) 5 μM active Yariv; (e, f) JIM14; (g, h) JIM13. Images (b), (d), (f) and (h) are enlarged views of the boxed regions in (a), (c), (e) and (g), respectively. Bars = 20 μm .

Consistent with the idea that arabinogalactan proteins help the flow of communication from cell wall to cytosol, active Yariv reagent dramatically elevates cytosolic calcium within a few minutes in lily pollen tubes and tobacco suspension-cultured cells (Pickard and Fujiki, 2005; Roy *et al.*, 1999). Although neither study confirmed the specificity of this effect with an anti-arabinogalactan-protein antibody, given the well-known influence of calcium on microtubules (Hepler, 1994), elevated calcium levels could be involved in the effects of arabinogalactan-protein binding seen here. Nevertheless, we think it is unlikely that elevated calcium levels are necessary and sufficient to disorganize microtubules, for two reasons. First, the recognized action of calcium on microtubules is depolymerization rather than disorganization (Hepler, 1994; Sivaguru *et al.*, 2003). Second, gadolinium did not prevent active Yariv from disorganizing cortical microtubules. While it is impossible to rule out the involvement of calcium release from intracellular stores (which gadolinium would be unlikely to reach) or the existence of a plasma-membrane calcium channel that is insensitive to gadolinium, this compound has been successfully employed to limit calcium influx into cells in many instances (Demidchik *et al.*, 2002, 2007; Sivaguru *et al.*, 2003). Most notably, Pickard and Fujiki (2005) elegantly demonstrated in tobacco cells expressing a

chameleon reporter that gadolinium totally prevented the Yariv-induced rise in cytosolic calcium. Effects on microtubule organization and on calcium channels could be two independent consequences of the disruption of arabinogalactan proteins at the plasma membrane (Pickard, 2007).

Arabinogalactan proteins and microtubules: from cell to cell wall

An indirect connection between cortical microtubules and arabinogalactan proteins can also be sought within the outward flow of information, from cytoplasm to cell wall. Cortical microtubules provide information to govern cell wall assembly, establishing an anisotropic framework required for morphogenesis (Wasteneys, 2004). Whenever microtubules are disrupted, expansion tends toward isotropy, as indicated by swollen cells or organs. Our finding that active Yariv disorganizes cortical microtubules provides a proximal cause for the cell swelling elicited by this compound (Ding and Zhu, 1997; Willats and Knox, 1996).

One action of cortical microtubules, although not necessarily the only one, is to align cellulose microfibrils (Baskin, 2001; Paredes *et al.*, 2006). According to one model, the cellulose synthase walks along the microtubule, in the manner of a motor protein; another model posits that

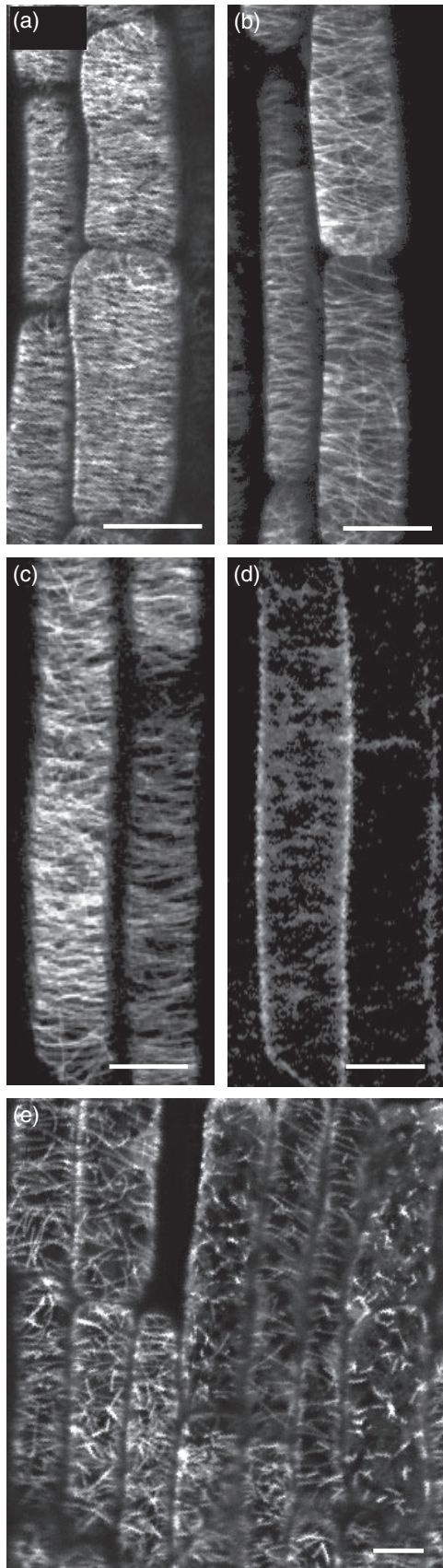


Figure 6. Effects of gadolinium on cortical microtubule organization. Microtubules imaged in living root epidermis with the GFP-microtubule reporter line: (a) 100 μM gadolinium for 1 h; (b) 100 μM GdCl_3 for 1 h followed by 10 mM CaCl_2 for 15 min; (c, d) epidermal cells before (c) and after (d) treatment with 10 mM CaCl_2 for 15 min; (e) 100 μM GdCl_3 for 1 h followed by 5 μM active Yariv for 30 min. Bar = 20 μm .

the synthase is constrained between parallel microtubules, which act as 'guard rails' (Giddings and Staehelin, 1991). Both models see the cortical microtubules interacting with the rosette, an interaction that might disrupt cellulose synthesis insofar as rosette subunits require uninterrupted cooperation to build a highly crystalline microfibril (Waste-neys, 2004). An alternative model was recently proposed in which microtubules interact with the nascent microfibril by means of a scaffold, or raft, of membrane proteins (Baskin, 2001; Baskin *et al.*, 2004). The scaffold is envisioned to bind microtubules cytosolically and nascent microfibrils extracellularly.

Arabinogalactan proteins could be a component of the scaffold and might function therein to facilitate the crystallization of glucans or to prevent unwanted interactions between microfibrils and the plasma membrane. Binding arabinogalactan proteins within such a scaffold might alter

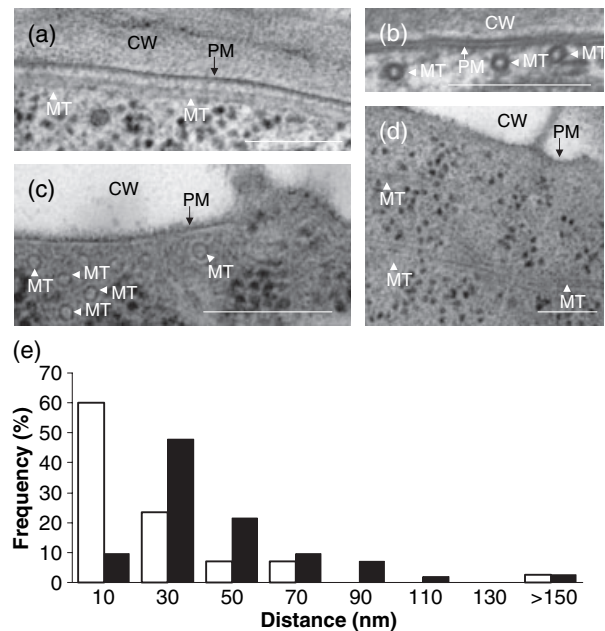


Figure 7. Electron micrographs showing the status of cortical microtubules in epidermis of roots prepared by high-pressure freezing. Treatments were for 2 h: (a, b) 1 μM inactive Yariv; (c, d) 5 μM active Yariv. Note that unlike in control cells, cortical microtubules in treated cells are distant from the plasma membrane. CW, cell wall; MT, cortical microtubule; PM, plasma membrane. Bars = 200 nm. (e) Frequency distribution of the distance between microtubule center and plasma membrane. White bars are control, black bars are active Yariv treated. $n = 91$ for control and $n = 88$ for active Yariv treated. Equivalence of the distributions is rejected at $P < 0.001$ by the chi-squared test.

associated transmembrane proteins, perhaps by aggregating them. This could disorder microtubule-binding sites or reduce their affinity, causing disorganization of cortical microtubules or dissociation from the membrane. This view is premised on a physical linkage between microtubules, transmembrane proteins, and extracellular proteins including arabinogalactan proteins. The linkage may usually function to transmit information from the microtubules to the cell wall, but when extracellular components are manipulated experimentally, as here, the linkage would also transmit information from the cell wall to the cytosol.

That microtubules are attached to the plasma membrane rather than simply adjacent has been demonstrated by several lines of evidence. In electron micrographs, cross bridges between microtubules and the plasma membrane are apparent (Shibaoka, 1994; Vesik *et al.*, 1996). When protoplast ghosts are prepared, cortical microtubules adhere to the membrane and substrate, unless the protoplasts are pre-treated with a protease (Akashi and Shibaoka, 1991). When living maize (*Zea mays*) roots are sectioned, turgor pressure ejects the cytoplasm except for a skein of cortical microtubules and underlying membrane (Tian *et al.*, 2004). Finally, membrane proteins have been purified that bind microtubules (Marc *et al.*, 1996). Taken together, these observations offer compelling support for the idea that microtubules associate with plasma membrane proteins.

In conclusion, our results substantiate the existence of a close linkage between cortical microtubules and arabinogalactan proteins, as suggested earlier (Andème-Onzighi *et al.*, 2002; Sardar *et al.*, 2006). Such a link is likely to function in the flow of morphogenetic information between the cell and the cell wall. The challenge now is to discover the components forming the link: a difficult task but one that should illuminate mechanisms of morphogenesis in vascular plants.

Experimental procedures

Plant material and growth conditions

Plant material used was *Arabidopsis thaliana* L. (Heynh), ecotype Columbia, and two transgenic lines in the same background: the first harbors a construct fusing GFP to the microtubule-binding domain (MBD) of human microtubule-associated protein 4 (Granger and Cyr, 2001), and the second harbors a construct fusing GFP to a classical arabinogalactan protein from tomato (*Lycopersicon lycopersicum*) (Estévez *et al.*, 2006). Seeds were treated as described in Nguema-Ona *et al.* (2006) and 4–6-day-old seedlings were used.

Yariv synthesis, antibody preparation, and treatment

Active Yariv (β -D-glucosyl) was synthesized according to the experimental procedure described by Yariv *et al.* (1962). Inactive Yariv (α -D-mannosyl-Yariv) was purchased from Biosupplies Australia Pty (<http://www.biosupplies.com.au/>). For synthesis, phloroglucinol and *p*-aminophenyl- β -D-glucopyranoside were

purchased from Sigma (<http://www.sigmaaldrich.com/>). For some experiments, Yariv reagents were the generous gift of Gene Nothnagel (University of California, Riverside, CA, USA). Gadolinium chloride and calcium chloride were from Sigma. The monoclonal antibodies used in this study were desalted using a PD-10 column Sephadex G-25 M (Amersham Biosciences; <http://www5.amershambiosciences.com/>) to remove sodium azide, reconcentrated and diluted at 1:4 in fresh culture medium before use. Seedlings expressing GFP-MBD were gently transferred onto glass slides and imaged under a confocal fluorescence microscope before incubation with specific probes, and then perfused with liquid growth medium supplemented as indicated.

Immunofluorescence localization of microtubules

Seedlings were transferred onto plates with growth medium supplement as needed, fixed in a buffer containing paraformaldehyde and glutaraldehyde, and immunolabeled, all as described by Bannigan *et al.* (2006). Seedlings were incubated with 1/1000 monoclonal anti- α -tubulin (Sigma) at 37°C overnight. After rinsing three times for 5 min in PBS (3 mM KH₂PO₄, 7 mM K₂HPO₄, 150 mM NaCl), the roots were incubated with 1/200 CY3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch; <http://www.jacksonimmuno.com/>) at 37°C for 3 h, rinsed again in PBS twice for 5 min each, mounted in a commercial, anti-fade medium (Vectashield, Vector Laboratories; <http://www.vectorlabs.com/>), covered and sealed. Immunofluorescence microscopy of whole roots was carried out using a Zeiss 510C Meta confocal microscope (<http://www.zeiss.com/>).

Imaging microtubules in living cells

Fluorescence from GFP-MBD was examined in living roots mounted in growth medium through a confocal laser scanning microscope (TCS SP2 AOBS, Leica; <http://www.leica.com/>). Images were acquired after 30–60 min of treatment, or for the time course study every 5 min, using the Leica software LCS Lite. Fluorescence from GFP was excited with the 488 nm line of an argon laser and signal was acquired between 500 and 530 nm. Images were captured through an oil immersion objective (40 \times , numerical aperture 1.2, U-plan apochromat). Observations were repeated with three to six individual roots per treatment. Microtubule angles were measured from single confocal sections as described in Sugimoto *et al.* (2003). An angle of 0° was defined by the root's long axis. For histograms, angles were pooled in 10° bins, centered on 90°. Between 50 and 100 cells from three treated roots were analyzed for microtubule organization.

Immunolocalization of pectic and arabinogalactan protein epitopes in roots pre-treated with antibodies

Wild-type seedlings were transferred in liquid medium supplemented with desalted antibodies diluted at 1:4 and placed at 20°C for 2 h in a growth chamber under constant light (100 μ mol m⁻² sec⁻¹). Treated roots were fixed for 1 h at room temperature in 4% (v/v) formaldehyde with 0.2% (v/v) glutaraldehyde in 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7), with 1 mM CaCl₂. Fixed roots were rinsed in 50 mM PIPES buffer with 1 mM CaCl₂ and then incubated with 1/50 fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Sigma) at 37°C for 2 h, rinsed again in PBS twice for 5 min each, mounted in Citifluor AF2 antifade (Oxford Instruments; <http://www.oxinst.com/>), covered and sealed.

High pressure freezing, freeze-substitution and electron microscopy

Samples for transmission electron microscopy were prepared using high-pressure freezing. Root apices (2–3 mm long) were first incubated in a freezing medium composed of 2-(*N*-morpholine)-ethanesulfonic acid (MES) buffer (20 mM MES pH 5.5, 2 mM CaCl₂, 2 mM KCl) containing 200 mM sucrose, for 5 min. Three to four roots were placed in gold platelet carriers pre-filled with the same freezing medium and frozen with the EMPACT freezer (Leica Microsystems) as previously described (Studer *et al.*, 2001). Freeze-substitution in acetone and osmium and embedding in Spurr's resin were performed following the procedure described by Studer *et al.* (2001). Sections were post-stained with uranyl acetate and lead citrate and finally examined in a Tecnai 12 Biotwin Microscope (FEI Company; <http://www.fei.com/>) at 80 kV. Measurements of the distance between plasma membrane and microtubule centers were performed on ultra-thin sections of epidermal cells using EM Image Analysis software (FEI Company). Only microtubules that were sectioned transversely were measured. For each treatment (control and active Yariv), about 90 microtubules were measured on sections from four different samples. Statistical analysis was performed using the chi-squared test.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Effects of inactive Yariv on cortical microtubule organization and arabinogalactan protein distribution. (a, b) Microtubules in root epidermis localized in fixed roots with immunocytochemistry. (a) Control. (b) Treatment for 24 h with 10 μM inactive Yariv. (c, d) Microtubules in living root epidermis imaged with the GFP-microtubule reporter line. Root was perfused with 5 μM inactive Yariv and imaged (c) immediately and (d) after 30 min. (e, f) Distribution of arabinogalactan proteins, imaged in living root cells expressing an arabinogalactan protein tagged with GFP. Treatments for 30 min with 5 μM inactive Yariv. Image in (f) is an enlarged view of the boxed region. Note that neither microtubule organization nor arabinogalactan protein distribution are affected by the inactive Yariv. Bars = 20 μm.

Figure S2. Effects of antibodies on the distribution of arabinogalactan proteins, imaged in fixed material. Treatments for 2 h. (a, b) JIM14. (c, d) JIM5. Bar = 20 μm.

Figure S3. Electron micrographs of epidermis in roots prepared by high-pressure freezing. Treatments for 2 h. (a, b) 1 μM inactive Yariv. Note (in a) that the plasma membrane appears as a smooth,

straight line, and is close to a cortical microtubule; at higher magnification (b), the bilayer structure of the membrane is evident. (c–e) 5 μM active Yariv. Note the presence of dense deposits located at the interface between the plasma membrane and the cell wall (arrows; c, d) and within the wall itself (arrows, e). Undulations in the plasma membrane (arrowheads; c, e) and osmiophilic particles (stars, d) are also indicated. CW, cell wall; G, Golgi stack; MT, cortical microtubule; M, mitochondria; PM, plasma membrane. Bars = 200 nm.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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