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# Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana*

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## Summary

**The polarized growth of cells as diverse as fungal hyphae, pollen tubes, algal rhizoids and root hairs is characterized by a highly localized regulation of cell expansion confined to the growing tip. In apically growing plant cells, a tip-focused $[Ca^{2+}]_c$ gradient and the cytoskeleton have been associated with growth. Although actin has been established to be essential for the maintenance of elongation, the role of microtubules remains unclear. To address whether the microtubule cytoskeleton is involved in root hair growth and orientation, we applied microtubule antagonists to root hairs of *Arabidopsis*. In this report, we show that depolymerizing or stabilizing the microtubule cytoskeleton of these apically growing root hairs led to a loss of directionality of growth and the formation of multiple, independent growth points in a single root hair. Each growing point contained a tip-focused gradient of $[Ca^{2+}]_c$. Experimental generation of a new $[Ca^{2+}]_c$ gradient in root hairs pre-treated with microtubule antagonists, using the caged-calcium ionophore Br-A23187, was capable of inducing the formation of a new growth point at the site of elevated calcium influx. These data indicate a role for microtubules in regulating the directionality and stability of apical growth in root hairs. In addition, these results suggest that the action of the microtubules may be mediated through interactions with the cellular machinery that maintains the $[Ca^{2+}]_c$ gradient at the tip.**

## Introduction

Root hairs are projections from epidermal cells of the root that have been proposed to play critical roles in the uptake of water and nutrients, and in anchoring the plant to the soil (Peterson and Farquhar, 1996). Root hairs elongate by tip growth where deposition of new wall and membrane material is confined to the expanding tip. Since it provides new cell wall and plasma membrane material for the expanding cell, localization of the polarized exocytosis to the apex of the root hair and directional transport of material toward this region are critical for continued elongation (Schnepf, 1986). Several vital components have been identified in the development and growth of root hairs (Ridge, 1995) and other tip-growing systems (Sievers and Schnepf, 1981). Among these factors, cytoplasmic calcium ($[Ca^{2+}]_c$) and the cytoskeleton have received increasing attention (Fowler and Quatrano, 1997; Miller *et al.*, 1997). However, the mechanisms by which calcium and the cytoskeleton act, and potentially interact, to regulate the orientation and rate of root hair growth remain unknown.

The presence of a tip-focused $[Ca^{2+}]_c$ gradient has been well documented in root hairs (Bibikova *et al.*, 1997; Felle and Hepler, 1997; Hermann and Felle, 1995; Wymer *et al.*, 1997), pollen tubes (Malho and Trewavas, 1996; Pierson *et al.*, 1996; Rathore *et al.*, 1991), fungal hyphae (Hyde and Heath, 1997), and algal rhizoids (Brownlee and Pulsford, 1988; Hodick *et al.*, 1991). Increases in $[Ca^{2+}]_c$ correlate with pulses of rapid growth at the tip of pollen tubes (Pierson *et al.*, 1996), and dissipating the calcium gradient with calcium buffers or channel blockers arrests tip growth in several types of tip-growing cells including root hairs (Hermann and Felle, 1995; Wymer *et al.*, 1997). Therefore, the tip-focused $[Ca^{2+}]_c$ gradient appears to be an important regulator of growth in most of the known tip-growing systems (Malho, 1998).

In roots of *Arabidopsis* we have previously shown that root hairs grow approximately 90 degrees from the surface of the root, maintain a single growth point, and deviate from straight growth by less than 2 degrees along their length (Bibikova *et al.*, 1997). Our previous data have also shown that redirecting growth by touch causes a shift in the position of the tip-focused $[Ca^{2+}]_c$ gradient and growth. In addition, localized calcium ionophore application, which results in the asymmetrical influx of calcium at the growing tip, elicits a transient change in the orientation of root hair growth toward the new $[Ca^{2+}]_c$ gradient (Bibikova *et al.*, 1997). A similar phenomenon has been observed in pollen tubes wherein UV photoactivation of caged calcium triggers pollen tubes to grow toward elevated calcium (Malho and Trewavas, 1996). These elevated levels of calcium have recently been shown to lead to increased levels of a calcium-dependent protein kinase (Moutinho *et al.*, 1998) which in turn could promote calcium mediated exocytosis (Blackbourn and Battey, 1993) and/or calcium-related

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changes in cytoskeletal activity (Kohno and Shimmen, 1987). Therefore, in addition to its role in maintaining growth, $[Ca^{2+}]_c$ has also been shown to be important in determining the directionality of tip growth. However, the transient nature of root hair reorientation in response to a reorientation of the $[Ca^{2+}]_c$ gradient suggests that this gradient is itself under the control of some other positional information. The cytoskeleton is an obvious candidate for this controlling factor.

The actin cytoskeleton has been found to be essential for the maintenance of elongation in tip-growing plant cells (Cai *et al.*, 1997; Kropf *et al.*, 1998) and was proposed to be an important factor in the altered growth control in root hairs responding to Nod factors (Cardenas *et al.*, 1998). The role of the microtubule cytoskeleton is more controversial. Apically growing plant cells treated with actin disrupting drugs immediately show inhibition of growth (Cai *et al.*, 1997; Kropf *et al.*, 1998). In contrast, tip-growing plant cells treated with microtubule antagonist show a variety of effects. Anti-tubulin drugs applied to several tip-growing cell types either had no effect on tip growth (Franke *et al.*, 1972; Sievers and Schnepf, 1981) or caused growth inhibition (Emons *et al.*, 1990; Mizukami and Wada, 1983). Furthermore, disrupting the microtubules in moss protonemata, fungal hyphae and pollen tubes resulted in either tip swelling (Heslop-Harrison *et al.*, 1988) or the formation of multiple growth points (Doonan *et al.*, 1988; Mizukami and Wada, 1983; Schmiedel and Schnepf, 1980; That *et al.*, 1988). However, the *ton 1* and *ton 2* mutants of *Arabidopsis*, which exhibit disrupted microtubule organization, showed no disruption of root hair growth (Traas *et al.*, 1995). This indicates that the microtubule regulatory events mediated by TON1 and TON2 are not essential for the morphogenesis of *Arabidopsis* root hairs. However, there may be other genes encoding regulators of microtubule organization that are specific for root hairs.

The organization of the cytoskeleton has been visualized extensively in root hairs of various plant species using both immunofluorescence and electron microscopy (Derksen and Emons, 1990). In particular, helical (Lloyd, 1983; Lloyd and Wells, 1985) and longitudinal arrays of microtubules have been described in root hairs (Emons *et al.*, 1990; Traas *et al.*, 1985) and proposed to interact with, and possibly order, the actin cytoskeleton (Tominaga *et al.*, 1997). However, the relationship of microtubules to growth and cell wall deposition in the growing tip of a root hair remains obscure (Emons and Mulder, 1998; Miller *et al.*, 1997). Importantly, very little is known about the role of the microtubule cytoskeleton in the directional control of tip-growth in root hairs and how this component of the cytoskeleton interacts with the $[Ca^{2+}]_c$ gradient at the tip.

In the experiments described in this paper, we show that stabilizing or depolymerizing microtubules results in a loss of directionality of root hair growth, and leads to the formation of multiple growth points in a single root hair. Confocal ratio-imaging revealed that a tip-focused $[Ca^{2+}]_c$ gradient was associated with each growing point, and artificially elevating $[Ca^{2+}]_c$ in root hairs with altered microtubules resulted in the formation of a new tip at the site of elevated calcium. This indicates that the microtubule cytoskeleton is involved in regulating the direction of root hair growth and stabilizing growth to a single point at the apex of each elongating root hair. This action of the cytoskeleton may be mediated through its interaction with the cellular machinery maintaining the $[Ca^{2+}]_c$ gradient at the tip.

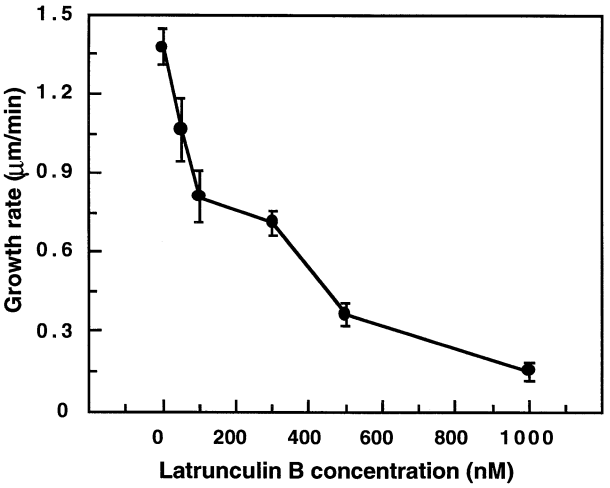


**Figure 1.** Dose–response curve of root hair growth inhibition by latrunculin B.
Seedlings were treated with the indicated concentrations of latrunculin B and root hair growth was measured after 1 h. Concentrations at or above 500 nM caused maximum growth inhibition, while concentrations as low as 50 nM caused 25% growth inhibition. Results are means ± SE, $n = 20$ root hairs from five separate roots.

## Results and discussion

To test the involvement of the cytoskeleton in root hair growth, we applied cytoskeletal disrupting drugs to root hairs of *Arabidopsis* and observed the effect on tip growth. Growth rates of root hairs were inhibited by the actin antagonist latrunculin B in a concentration dependent manner. Latrunculin B concentrations as low as 50 nM caused 25% inhibition in growth rate, while concentrations at or above 500 nM almost completely inhibited apical growth (Figure 1). Under these conditions deviations from straight growth were rarely seen. This result implies a role for actin in the mechanism of localized growth, as previously noted in many apically growing systems of plants (Cai *et al.*, 1997; Kropf *et al.*, 1998), fungi (Bachewich and Heath, 1998; Steer, 1990), and algal rhizoids (Kropf, 1997). On the other hand, the microtubule stabilizing drug, taxol, and the microtubule depolymerizing drug, oryzalin,

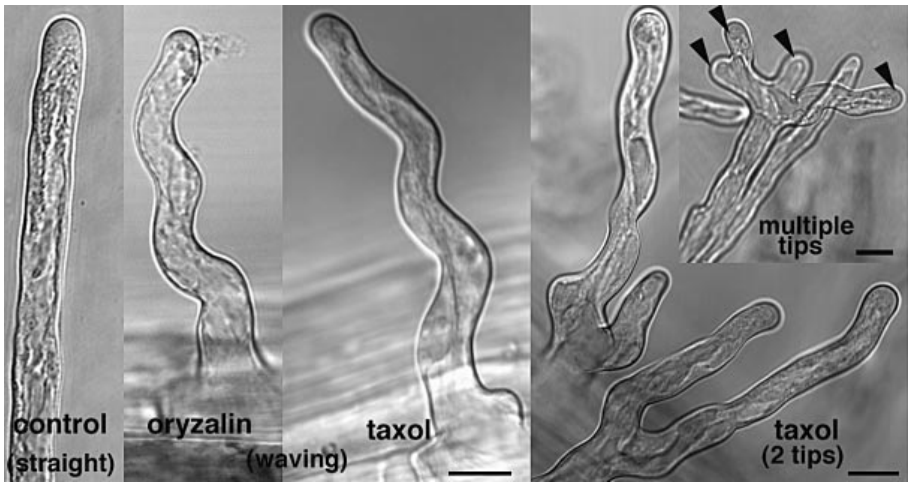


**Figure 2.** Effect of microtubule disrupting compounds on *Arabidopsis* root hair morphology and orientation.
Non-treated root hairs (controls) grow straight and maintain a near 90 degree angle from the main root axis. Both oryzalin (5 μM) and taxol (5 μM) altered the directionality of root hair growth. Root hairs treated with the above compounds displayed either a waving growth pattern or, at higher concentrations (2.5–10 μM), a branching of root hairs resulting in the formation of two or more tips (arrowheads). Representative of > 100 root hairs from > 10 separate roots. Scale bars: 10 μm.

led to a dose-dependent loss of directionality in root hair growth and morphology, characterized by a 'waving' of the root hair as it elongated (Figure 2). However, analysis of variance ($P > 0.05$) showed that the growth rate of root hairs of untreated plants (1.21 ± 0.22 μm min$^{-1}$; mean ± standard deviation, $n = 14$) was not significantly different from the growth rate of root hairs that displayed the 'waving' growth pattern after treatment with 1 μM oryzalin (1.00 ± 0.30 μm min$^{-1}$, $n = 14$) or taxol (1.19 ± 0.24 μm min$^{-1}$, $n = 14$). As drug concentration was increased to 10 μM, the root hairs exhibited an increased angle of waving (Figure 3a,b) but the growth rate was not affected (1.13 ± 0.16 μm min$^{-1}$ for taxol; 1.08 ± 0.15 μm min$^{-1}$ for oryzalin). These observations suggest that the drugs disrupted the cellular mechanism that maintains straight growth. Furthermore, root hairs treated with microtubule antagonists no longer maintained a consistent course of growth away from the root surface. Although the average angle at which untreated root hairs (88.9 degrees, $n = 80$) grew away from the root surface was similar to the angle of taxol- (92 degrees, $n = 80$) and oryzalin-treated (85.8 degrees, $n = 80$) root hairs, the standard deviation for taxol- and oryzalin-treated root hairs was ± 19 and 28 degrees, respectively, compared to only ± 6 degrees for controls (see also Bibikova *et al.*, 1997). This larger variability of growth angle suggests that the drugs affected the directionality of root hair growth and therefore indicates that the microtubule cytoskeleton may be part of the endogenous machinery that maintains a fixed angle of growth away from the root surface (Bibikova *et al.*, 1997).

In addition to the proportion of root hairs that displayed a higher angle of waving (Figure 3a), increasing concentrations of taxol or oryzalin led to a greater proportion of root hairs that formed multiple tips (Figure 3c). Prior to the initiation of new tips, root hairs initially exhibited vacuolation and swelling at the site of new tip formation. These multiple tips resulted in several elongating branches on a single root hair (see Figure 2). Because tip growth rate is maintained for both waving root hairs and the newly formed elongating multiple tips, the microtubule cytoskeleton does not appear to be part of the cellular machinery required for tip growth to proceed, but is involved in the control of its directionality. The increase in the proportion of root hairs with multiple growing tips at high inhibitor concentrations (Figures 2 and 3c) also suggests that an intact microtubule cytoskeleton is required to stabilize a single growth point.

To assess whether these drugs were in fact having an effect on microtubule structure, root hair microtubules were visualized by indirect immunofluorescence. Microtubules in control root hairs were arranged parallel to the longitudinal axis of the root hair (Figure 4a). This was similar to the pattern previously reported for root hairs of *Arabidopsis* (Traas *et al.*, 1995) and other plant species (Derksen and Emons, 1990). Oryzalin-treated root hairs exhibited diffuse staining throughout the cytoplasm (Figure 4b), which is a typical feature of other plant cells (Baluska *et al.*, 1996; Baskin *et al.*, 1994), or tip-growing algal rhizoids (Braun and Sievers, 1994) treated with microtubule depolymerizing drugs. On the other hand, taxol-treated root hairs exhibited thick filamentous strands and a brighter fluorescent signal (Figure 4c), probably reflecting microtubule stabilization and bundling. These effects are typically observed in other plant cell types treated with taxol (Baluska *et al.*, 1997; Baskin *et al.*, 1994; Blancaflor *et al.*, 1998). These observations indicate that taxol and oryzalin were having their expected effects on the microtubules of the root hairs and thus their effects on growth were most likely attributable to alterations in microtubule structure.

We also observed 'waving' of the root hairs induced by the microtubule disrupting drugs in other plant species, including cucumber, tobacco, barley and collards (data not shown), but the formation of multiple tips was most evident in root hairs of *Arabidopsis*. The formation of multiple

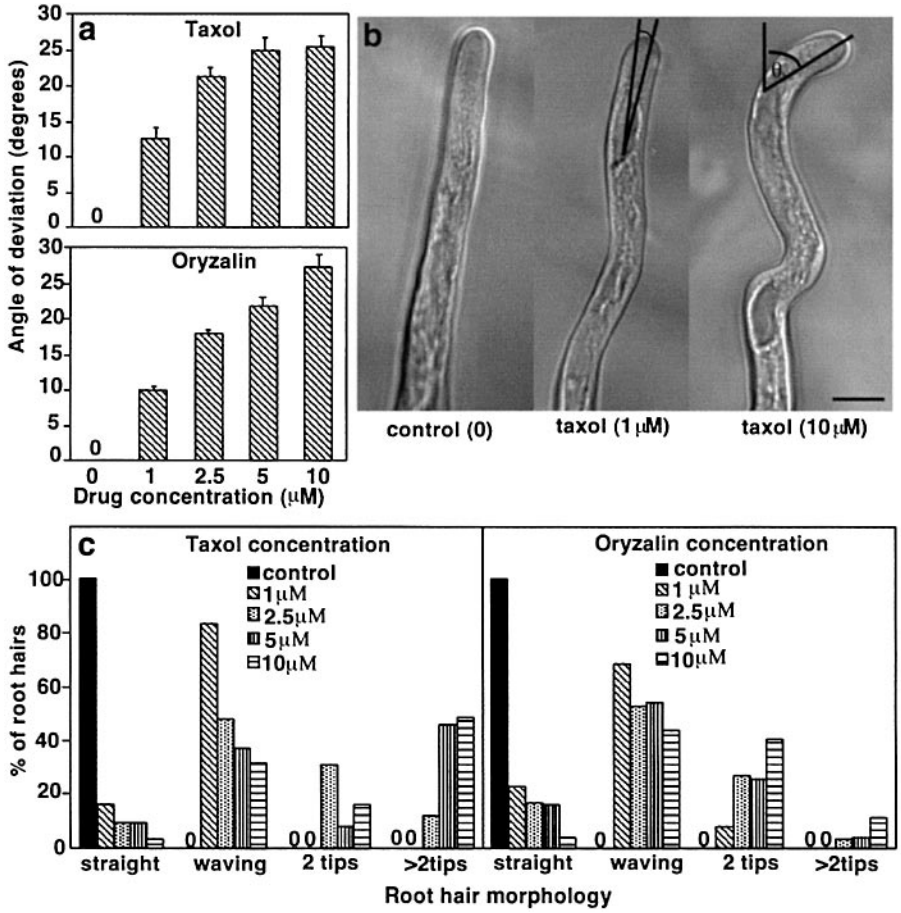


**Figure 3.** Loss of directionality of root hair tip growth.
(a) Angle of deviation (0) from straight growth. The angle of deviation from straight growth became more pronounced with higher inhibitor concentrations.
(b) Extremes of deviation from straight growth exhibited by waving root hairs at 1 μM and 10 μM taxol concentration. The angle (0) of deviation from straight growth was measured in root hairs that displayed the waving growth pattern as illustrated in this panel.
(c) Distribution of root hairs showing the various morphologies illustrated in Figure 2 after incubation in different concentrations of microtubule disrupting compounds. Oryzalin and taxol treatments led to a dose-dependent loss of directionality in root hair growth. At lower drug concentrations, a higher percentage of root hairs displayed the waving growth pattern while higher drug concentrations (2.5–10 μM) resulted in a greater percentage of root hairs exhibiting branching and multiple growing tips. At the highest inhibitor concentration used (10 μM), the percentage of root hairs with multiple tips was higher in taxol- compared to oryzalin-treated roots. Scale bar: 10 μm.

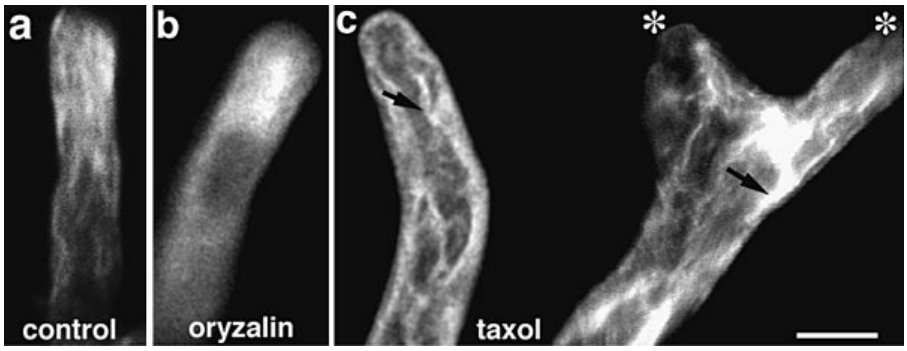


**Figure 4.** Immunofluorescence images of microtubules in *Arabidopsis* root hairs after treatment with microtubule disrupting compounds for 1 h.
(a) Microtubules in control root hairs are oriented parallel to the growth direction.
(b) Diffuse staining and the absence of filamentous microtubular structures characterize oryzalin-treated (5 μM) root hairs.
(c) Thicker filamentous structures (arrows) which are most likely bundled microtubules are prominent in taxol-treated (5 μM) root hairs that exhibit the waving growth pattern or branching (*). Representative of > 30 root hairs from > 10 separate roots. Scale bar: 10 μm.

growth points in response to microtubule antagonists has also been reported in other tip-growing systems. In caulonemal cells of *Funaria*, and protonema of *Ceratodon* and *Physcomitrella*, and *Bryopsis* cells, the microtubule depolymerizing drugs colchicine, cremart, and oryzalin caused the formation of new lateral outgrowths (Doonan *et al.*, 1988; Mizukami and Wada, 1983; Schmiedel and Schnepf, 1980; Schwuchow *et al.*, 1990). In *Neurospora*, the anti-tubulin drug benomyl induced the formation of multiple germ tubes (That *et al.*, 1988). Taxol treatment also resulted in the formation of multiple apical tips in protonema of *Physcomitrella* (Doonan *et al.*, 1988). The role of microtubules in maintaining growth to a single point could, therefore, be a common feature for many tip-growing cells.

Although the above studies clearly show that disruption of the microtubule cytoskeleton alters the polarity of tip growth, little is known about the mechanisms by which microtubules limit growth to a single apical focus. Since root hair tip growth is associated with a tip-focused $[Ca^{2+}]_c$

gradient which is required for growth (Bibikova *et al.*, 1997; Felle and Hepler, 1997; Hermann and Felle, 1995; Wymer *et al.*, 1997), the microtubules could potentially interact with the cellular machinery that maintains this calcium gradient. To determine whether an equivalent $[Ca^{2+}]_c$ gradient was present in root hairs showing altered growth patterns, we monitored $[Ca^{2+}]_c$ in taxol-treated root hairs using confocal ratio imaging of root hairs acid loaded with the calcium indicating fluorescent dye, Indo-1. In waving root hairs, or root hairs forming multiple growth points, a tip-focused $[Ca^{2+}]_c$ gradient was always observed to be directed to the center of each growing point (Figure 5a,b). Thus, a $[Ca^{2+}]_c$ gradient is associated with the growth point, even in root hairs with anomalous growth patterns.

The above results suggested that if the tip-focused $[Ca^{2+}]_c$ gradient is an important factor in directing the tip growth machinery, experimental manipulations that alter the gradient should allow us to control subsequent growth. In addition, this growth manipulation should be facilitated in taxol-treated root hairs, where control of tip growth appears relaxed. Therefore, we applied caged-calcium ionophore and a touch stimulus to taxol-treated root hairs. Both manipulations were previously characterized as eliciting a transient reorientation of both the $[Ca^{2+}]_c$ gradient and tip growth in root hairs (Bibikova *et al.*, 1997). Localized UV activation of caged calcium-ionophore generated an artificial $[Ca^{2+}]_c$ gradient focused to the site of UV irradiation (Figure 5c,d; see also Bibikova *et al.*, 1997). In taxol-treated root hairs, this artificial $[Ca^{2+}]_c$ gradient was sufficient to form a new growth point at the site of the new gradient, even when this gradient was generated in the vacuolar region of the hair more than 10 μm away from the established tip growing point (Figure 5d). This behavior

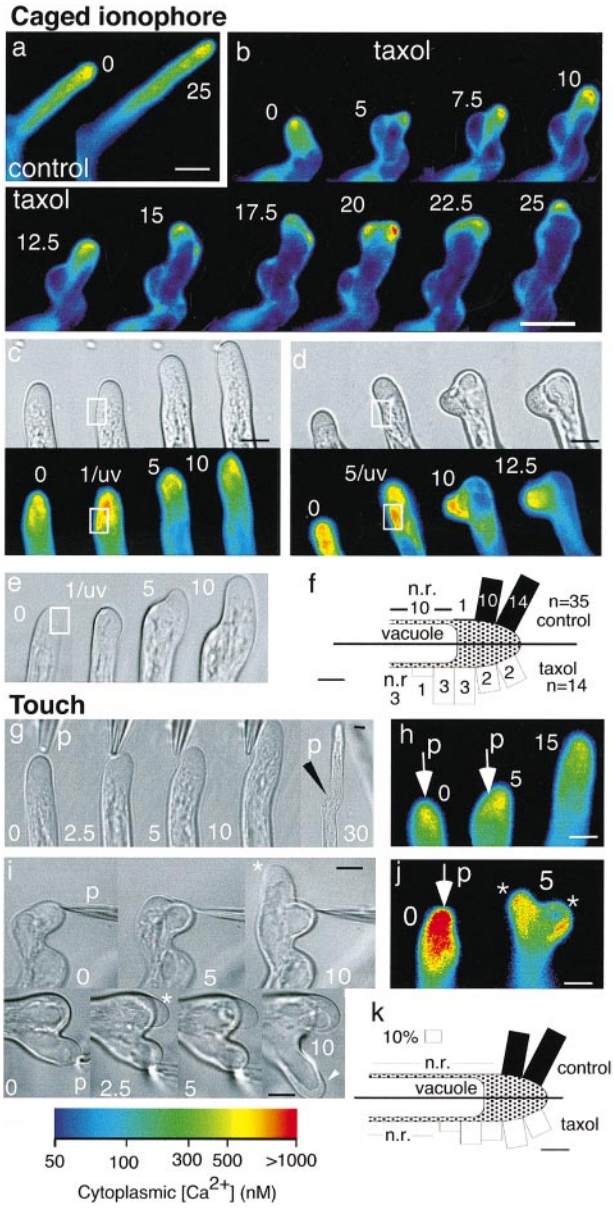


**Figure 5.** Changes in $[Ca^{2+}]_c$ as root hairs reorient in response to caged calcium ionophore and touch.

Cytoplasmic calcium in an untreated, control root hair (a) and a root hair treated with 10 μM taxol (b). Numbers represent time of observation in min. Note the tip-focused $[Ca^{2+}]_c$ gradient associated with the growing point of both root hairs, including the taxol-treated root hair that initiates two growing tips 20 min after the start of imaging. Representative of more than 21 root hairs from at least five separate roots. Cytoplasmic calcium in control (c) or 10 μM taxol-treated (d) root hairs after local photoactivation of 10 μM dimethoxynitrophenylethyl-esterified-Br-A23187 (caged calcium-ionophore). UV irradiation was applied using a 0.7 sec scan of the confocal UV confined to the region denoted by the box in (c–e). Note the lack of reorientation of growth in controls where UV activation of ionophore is away from the tip (c). In addition, reorientation of growth is transient and relatively limited in controls responding to localized ionophore activation at the growing tip (e). Substantial redirection of tip growth is evident in the taxol-treated root hair, even though ionophore is being activated away from the tip (d). Representative of more than nine root hairs from five separate roots. (f) Diagram of the number of root hairs showing redirection of tip growth to regions around the growing root hair tip induced by localized ionophore activation. The reorientation of growth in controls (black bars) was much more limited than in taxol-treated root hairs (white bars). n.r., region where no reorientation of tip growth could be induced. (g) Reorientation of root hair growth and (h) $[Ca^{2+}]_c$ at the tip elicited by touching a mechanical object. The 'hook' (arrow) seen at 30 min in (g) represents the largest reorientation seen in response to touch in untreated root hairs. (i) Redirection of growth elicited by a 10 μm taxol-treated root hair touching a mechanical object (microinjection pipette tip, p). The tip that encounters the barrier is prevented from further growth and induces the growth of an adjacent tip (*). The lower panel shows that removal of the pipette results in the resumption of tip growth (arrowhead). (j) Cytoplasmic calcium at the tip of the reorienting taxol-treated root hair. Note that the taxol-treated root hair has formed two growing tips and a tip focused $[Ca^{2+}]_c$ gradient is associated with both tips (*). Numbers show min of observation. Representative of more than 14 root hairs from six separate roots. (k) Diagram showing regions of root hairs where redirection of tip growth was observed after touch stimulation of the growing tip. Note the reorientation of growth in controls (black bars, $n = 35$) was much more limited than in taxol-treated root hairs (white bars, $n = 17$). n.r., region where no reorientation of tip growth was observed. Calcium levels were monitored by confocal ratio imaging of Indo-1 loaded root hairs as described previously (Wymer *et al.*, 1997), or calcium-green/rhodamine-injected root hairs for caged ionophore experiments (Bibikova *et al.*, 1997). Calcium levels have been pseudo-color coded according to the inset scale. Scale bars: (a, b) 10 μm; (c–l) 5 μm.

was not observed in control root hairs, which showed only transient reorientation of growth, and only if the new $[Ca^{2+}]_c$ gradient was imposed across the apical, cytoplasm-rich region of the tip where growth was already occurring (Figure 5e,f). Thus, lateral gradients of $[Ca^{2+}]_c$ generated away from the apical cytoplasm could not elicit new tip formation in control root hairs (Figure 5c,f).

Control root hairs also showed only a transient reorientation of growth and the $[Ca^{2+}]_c$ gradient in response to touch. Furthermore, these root hairs never showed formation of multiple growth points away from the site of touch stimulation (Figure 5g,h). On the other hand, root hairs treated with taxol showed a pronounced redirection of growth on encountering the touch stimulus, and even showed formation of a new growth point away from the site of touch stimulation. In addition, removal of the pipette used to apply the touch stimulus allowed growth to resume in the tip that had previously been in contact with the pipette (Figure 5i). The redirection of growth upon touch stimulation was associated with a redirection of the tip-focused $[Ca^{2+}]_c$ gradient (Figure 5j). As in the experiments with localized ionophore photoactivation, the reorientation of growth in controls in response to touch was much more limited to the apical cytoplasmic rich region of the root hair tip where growth was already occurring (Figure 5k). This is again suggestive of a destabilization of the directional control of the apical growth machinery in taxol-treated root hairs such that under touch stimulation the apical growth machinery could migrate to a new point.

These observations indicate that microtubules are intimately involved in restricting the growth machinery to the apex of an elongating root hair and may be acting through restricting the movement of the tip-focused $[Ca^{2+}]_c$ gradient. Figure 5 indicates that the $[Ca^{2+}]_c$ gradient alone is sufficient to recruit and stabilize the new growth point. Thus, the microtubules may be localizing calcium-dependent secretory elements such as annexins (Blackbourn and Battey, 1993), protein kinases (Moutinho *et al.*, 1998) and vesicle fusion events (Homann and Tester, 1997), whilst the calcium sensitivity of microtubule structure (Cyr, 1991) may also imply a feedback of calcium on microtubule activity within this apical region of the growing hair.

Rhizobial Nod factors alter root hair morphology as part of the infection process leading to root nodule formation. Nod factor treatment also leads to vacuolation and the formation of a new growth point at the root hair tip (Miller et al., 1997), reminiscent of the induction of the branching induced by microtubule antagonists seen in this study. Calcium and the actin cytoskeleton have been implicated in intracellular signaling (Ehrhardt *et al.* 1996) and the altered growth patterns seen in root hairs responding to these Nod factors (Cardenas *et al.*, 1998; de Ruijter *et al.*, 1998). It would be interesting to determine if the microtubule cytoskeleton and calcium were also interacting to alter root hair growth control in Nod factor treated plants.

Because both microtubule stabilizing and depolymerizing drugs had similar effects on the morphogenesis of root hairs in our experiments, it appears that the dynamic nature of the microtubules (Wordeman and Mitchison, 1994) may be critical for this directional control. This dynamic nature consists of the effects of the polymerization and depolymerization rate, frequency of catastrophic microtubule depolymerization, and frequency of rescue of individual microtubules. *In vivo*, microtubule dynamics are highly regulated by factors such as microtubule associated proteins, GTP hydrolysis, calcium and phosphorylation/dephosphorylation events (Wordeman and Mitchison, 1994). Thus, application of drugs that promote polymerization (taxol) or depolymerization (oryzalin) are both likely to alter this controlled dynamic nature and therefore lead to similar morphological effects. It is interesting to note that as observed in our experiments on root hairs, similar effects of microtubule stabilization and fragmentation have also been observed in other plant cell types. For example, both taxol and oryzalin have been reported to induce swelling in the cells of the elongation zone of roots (Baskin *et al.*, 1994; Blancaflor *et al.*, 1998), and cell suspension cultures treated with taxol or colchicine display similar morphologies (Weerdenburg and Seagull, 1988). Therefore, as in root hairs, a dynamic microtubule cytoskeleton appears critical in maintaining the highly polarized nature of plant cell growth in both diffuse and tip-growing cells.

Alternative explanations for the similarity between the effects of oryzalin and taxol could include disruption of microtubule-dependent events such as the ordering of actin filaments at the growing tip (Tominaga *et al.*, 1997), interference with vesicle transport to the apex (Cai *et al.*, 1997), disruption of the spatial organization of membrane proteins (e.g. ion channels) regulated by their interactions with the cytoskeleton (Thuleau *et al.*, 1998), or changes in the activity of a microtubule-based motility mechanism (e.g. kinesin; Wu *et al.*, 1998). The tip growth machinery of plants has been shown to involve the action of such elements as monomeric G-proteins, profilin, myosin and calcium (Clarke *et al.*, 1998; Lin and Yang, 1997; Lin *et al.*, 1996; Malho, 1998; Miller *et al.*, 1995; Vidali and Hepler, 1997). The challenge now is to define the molecular links between the microtubule regulatory system, the $[Ca^{2+}]_c$ gradient, and these other components of tip growth.

## Experimental procedures

### *Plant material and application of microtubule disrupting drugs*

Seedlings of *Arabidopsis thaliana* (Columbia ecotype) were grown as described previously (Wymer *et al.*, 1997). Plants were used 4–

5 days after planting at which time the roots were 1–3 cm long. Stock solutions of 10 mM taxol (Molecular Probes Inc., Eugene, OR, USA), oryzalin (Chem Services, West Chester, PA, USA) or latrunculin B (Calbiochem, La Jolla, CA, USA) were prepared in 100% DMSO. The required volumes of the stock solutions were diluted in *Arabidopsis* growth media (see Wymer *et al.*, 1997 for composition of media) to achieve the desired concentration (1–10 μM), and applied to separate sets of roots for 1 h. Growth media with the corresponding concentration of DMSO (maximum concentration 0.1% v/v) were used as controls.

### *Growth and angle measurements*

Cover slips containing the roots treated with the cytoskeletal antagonists or controls were placed on the stage of a Nikon Diaphot 300 microscope (Nikon Inc., Melville, NY, USA). An individual root hair was then observed using a 40× dry, 0.7 numerical aperture objective and differential interference contrast optics as described previously (Bibikova *et al.*, 1997; Wymer *et al.*, 1997). Images of root hairs were captured at 10 min intervals for 1 h using a Hamamatsu C2400 video camera (Hamamatsu, Tokyo, Japan) and a Scion LG-3 frame grabber (Scion Corp. Frederick, MD, USA) attached to a Quadra 800 computer (Apple Computer Inc., Cupertino, CA, USA). All angle and length measurements were obtained from digitized images of root hairs using IPLabs Spectrum image analysis software (Signal Analytics, Vienna, VA, USA). Growth rates were measured over a 1 h period, 30 min after application of cytoskeletal antagonists. Root hairs with an initial length of between 25 and 50 μm were selected for growth analysis. Under our growth conditions, root hairs of this length show a sustained and stable rate of elongation for at least 2 h (Wymer *et al.*, 1997).

### *Plunge freeze fixation and immunocytochemistry*

Fixation of root hairs for immunofluorescence was as described in Baskin *et al.* (1996) with minor modifications. *Arabidopsis* seedlings were removed from the Petri dish incubation chamber (Wymer *et al.*, 1997) and the gel was carefully lifted to expose the seedlings. Individual seedlings were picked up with a fine pair of forceps and plunged rapidly and deeply into liquid propane held at –180°C with liquid nitrogen. After holding roots in liquid propane for at least 15 sec, they were transferred into cryogenic vials held at liquid nitrogen temperature. The vials were then transferred into a – 80°C freezer. The specimens were freeze substituted in dry acetone for 48 h and allowed to warm to room temperature over an 18 h period. Acetone was replaced with pure ethanol and seedlings were then rehydrated by replacing ethanol with PHEMD buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl2, and 5% v/v DMSO, pH 7). Seedlings were then attached to cover slips by placing a thin film of agar (0.75% w/v) over the seedlings. The cover slips, with seedlings attached, were incubated in 1% w/v cellulase YC, 0.5% w/v pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) containing 0.1% w/v BSA and 0.1% v/v Triton X-100 in PHEMD buffer for 15 min. After three buffer washes, samples were incubated in a monoclonal anti-tubulin antibody (YOL 1/34; Accurate Chemical and Scientific Corp., Westbury, NY, USA) for 6–12 h followed by incubation in FITC-labeled secondary antibody (goat anti-rat IgG, Sigma) for 4 h. After rinsing the secondary antibody with PHEMD buffer, roots were mounted in 20% (w/v) Mowiol 4–88 (Calbiochem, La Jolla, CA, USA) in phosphate-buffered saline, pH 8.5, containing 0.1% (w/v) p-phenylenediamine. Microtubules in root hairs were imaged using an LSM-410 confocal scanning laser microscope (Zeiss, Thornwood, NY, USA) using excitation by a 488 nm laser, 488 nm dichroic mirror and 515–560 nm emission. Images from the confocal microscope were assembled with Photoshop 4.0 (Adobe systems Inc., Mountain View, CA, USA) and printed on an 850 stylus color printer (Epson America inc., Torrance, CA, USA).

### *Ratiometric measurement of $[Ca^{2+}]_c$*

For fluorescence ratio-imaging of $[Ca^{2+}]_c$, the roots were acid-loaded with 20 μM Indo-1 (Molecular Probes, Eugene, OR, USA) or simultaneously microinjected with the fluorescent calcium indicator calcium-green-2 linked to a 10 kDa dextran and rhodamine linked to a 10 kDa dextran as described previously (Bibikova *et al.*, 1997). Fluorescent ratio imaging of cytoplasmic calcium concentration was carried out on a Zeiss Axiovert inverted microscope attached to a LSM410 laser scanning confocal microscope and imaged using a Zeiss 40×, 0.75 N.A., dry objective as described previously (Bibikova *et al.*, 1997; Wymer *et al.*, 1997). Transmission images were also taken for each ratio image using the transmission detector of the confocal microscope and illumination by the 633 nm He/Ne laser of the confocal microscope attenuated to 10% with neutral density filters. Pseudocolor ratio images of the $[Ca^{2+}]_c$ distribution were calculated as described previously (Bibikova *et al.*, 1997; Wymer *et al.*, 1997). Image processing was carried out on a PowerMac 8100 using IP Labs Spectrum image analysis software. Ratio-images were calibrated using calcium calibration standards from Molecular Probes as described previously (Wymer *et al.*, 1997).

### *Loading and photoactivation of the caged molecules*

All procedures involving caged probes were performed in the dark or under dim safe light conditions. *Arabidopsis* root hairs were treated for at least 20 min with 20 μM 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNPE-caged) Br-A23187 (Molecular Probes), diluted from 10 mM stock in DMSO. UV irradiation was applied using a 0.7 sec scan of the UV laser of a Zeiss LSM-410 confocal scanning laser microscope as described previously (Bibikova *et al.*, 1997).

### *Application of touch stimulus*

Glass microinjection micropipettes (0.5 μm tip diameter) were filled with growth medium and positioned 10–20 μm away from the tip of growing root hairs using a Narashige M2 micromanipulator (Narashige Inc, Tokyo, Japan). The root hair was then allowed to grow into the micropipette such that the pipette touched the center of the cell tip (see Bibikova *et al.*, 1997). The electrode was maintained in a fixed position throughout each touch experiment.

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