

Unstable F-Actin Specifies the Area and Microtubule Direction of Cell Expansion in Arabidopsis Root Hairs

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Plant cells expand by exocytosis of wall material contained in Golgi-derived vesicles. We examined the role of local instability of the actin cytoskeleton in specifying the exocytosis site in Arabidopsis root hairs. During root hair growth, a specific actin cytoskeleton configuration is present in the cell's subapex, which consists of fine bundles of actin filaments that become more and more fine toward the apex, where they may be absent. Pulse application of low concentrations of the actin-depolymerizing drugs cytochalasin D and latrunculin A broadened growing root hair tips (i.e., they increased the area of cell expansion). Interestingly, recovery from cytochalasin D led to new growth in the original growth direction, whereas in the presence of oryzalin, a microtubule-depolymerizing drug, this direction was altered. Oryzalin alone, at the same concentration, had no influence on root hair elongation. These results represent an important step toward understanding the spatial and directional regulation of root hair growth.

INTRODUCTION

Plant cells grow when Golgi-derived vesicles fuse with the plasma membrane while inserting cellulose synthases (Kimura et al., 1999) into it (Emons and Mulder, 2000) and at the same time delivering cell wall matrix into a flexible cell wall under turgor pressure (Cosgrove, 1997). Root hairs grow at one site only, the tip. What are the roles of actin filaments and microtubules in specifying growth? The answer remains elusive. In root hairs, a cytoskeleton configuration of subapical, net-axial fine bundles of actin filaments together with a tip area free of detectable actin filaments correlate with root hair tip growth in *Vicia sativa* (Miller et al., 1999) and *Arabidopsis* (Ketelaar et al., 2002). In full-grown hairs, this typical actin configuration is not present, but in hairs that are terminating growth, it can be reinduced within 3 min by a signal molecule (bacterial Nod factor) (de Ruijter et al., 1999). This type of actin configuration is hypothesized to deliver Golgi-derived vesicles to the apical area (de Ruijter et al., 1999; Miller et al., 1999, 2000). Recently, cortical fine F-actin was reported to occur also in intercalary

growing *Arabidopsis* cells at the site of and correlating with the early stages of cell expansion (Fu et al., 2002).

In *V. sativa* root hairs, the subapical fine bundles of actin filaments, further called fine F-actin, are more sensitive to the actin-depolymerizing drug cytochalasin D (CD) than are the thicker bundles of actin filaments farther down in the root hair tube (Miller et al., 1999). Repeated CD application every 10 min led to the complete disappearance of the fine F-actin and growth stopped, whereas bundles of actin filaments remained present in the hair tube, where cytoplasmic streaming remained normal (Miller et al., 1999). Indeed, when plant cells were cultured in the actin-depolymerizing drug latrunculin A (LA), cell growth was inhibited (Baluška et al., 2001). Before tip elongation in axons, low concentrations of actin-destabilizing drugs stimulated tip growth (Bradke and Dotti, 1999), and in pancreatic acinar cells, the depolymerization of cortical actin triggered exocytosis (Muallem et al., 1995; Valentijn et al., 1999).

In the present study, we investigated whether an increase in instability (i.e., turnover) in the subapical fine F-actin in root hairs can trigger the exocytosis process by which plant cells grow. Therefore, we studied the reaction of *Arabidopsis* root hairs to the actin-depolymerizing drugs CD and LA. The results show that an increase in instability of the actin cytoskeleton can induce cell expansion over a larger area. We further show that the orientation of polarized growth depends on an intact microtubule cytoskeleton, as is known for intercalary growing cells. These results challenge the widely held views that tip growth is actin

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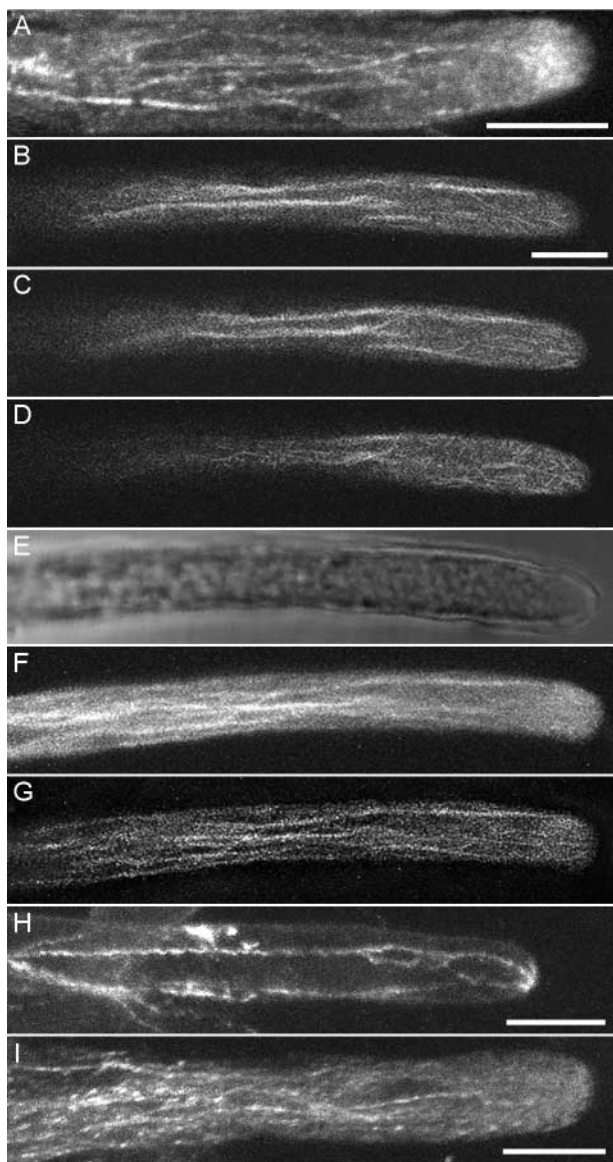


Figure 1. Actin Visualization in Arabidopsis Root Hairs.

(A) Immunofluorescence image of a whole-mount, freeze-fixed, growing Arabidopsis root hair labeled with anti-actin antibodies.
(B) to (G) Root hairs in which the actin cytoskeleton is visualized by the improved ester-aldehyde-choline method.
(B) to (D) Projections of several images from a confocal Z-stack through a whole growing root hair. **(B)** and **(C)** show median projections, and **(D)** shows a cortical projection.
(E) Reflection image of the root hair.
(F) and **(G)** Projections of the whole confocal Z-stack without deconvolution **(F)** and after deconvolution **(G)**.
(H) In full-grown root hairs, actin filaments loop through the tip.
(I) A 5-min treatment with 0.1 μM CD gives a decrease in fluorescence in the (sub)apical area, whereas the thicker bundles of F-actin toward the root hair base remain intact.
(A) and **(G)** show typical images of the actin cytoskeleton in a growing root hair: subapical fine F-actin and thicker bundles of actin filaments in the basal part of the cell. Bars = 10 μm .

based and intercalary growth depends on microtubules (Kropf et al., 1998).

RESULTS

The actin filament configuration in Arabidopsis root hairs was studied by two different methods: rapid freeze fixation followed by immunodetection of actin, and phallotoxin staining after an optimized fixation procedure using the ester-aldehyde-choline method (Miller et al., 1999; Emons and de Ruijter, 2000), as described previously for Arabidopsis (Ketelaar et al., 2002). Rapid freeze fixation and freeze substitution was followed by treatment with cell wall-degrading enzymes to facilitate antibody penetration for immunolocalization. We used antibodies because after rapid freeze fixation and freeze substitution, the reactivity of filamentous actin to fluorescently labeled phalloidins was lost for unknown reasons. Both methods gave comparable results, although the immunocytochemistry technique after freeze fixation suffered from the fact that details of the fine F-actin in the subapex were obscured, probably by antibody attached to actin monomers (Figure 1A), an effect reported previously by Miller et al. (1999). The ester-aldehyde-choline staining method had better resolving power, especially in the subapical area of growing root hairs (Figures 1B to 1G).

In both growing and full-grown Arabidopsis root hairs (Ketelaar et al., 2002), the general configuration of the actin cytoskeleton is the same as that described for *V. sativa* (Miller et al., 1999). Growing hairs have a tip area free of detectable bundles of actin filaments, subapical net-axially aligned fine F-actin, and thicker bundles of actin filaments basal to the subapical area (Figure 1). In full-grown hairs, only the latter are present and loop through the tip (Figure 1H). There is debate about the presence of filamentous actin in the apical area of growing root hairs. The expression of green fluorescent protein (GFP) fused to the actin binding site of animal talin (Kost et al., 1998), which binds to actin filaments, was studied in Arabidopsis root hairs (Baluška et al., 2000). These authors observed fluorescence in the apex of growing root hairs. However, this fluorescence was not localized in filamentous structures, which could be attributable to the insufficient time resolution of the light microscopes used for the imaging of the highly dynamic actin cytoskeleton. Whether there are very dynamic actin filaments with a high turnover rate or no actin filaments at all in the extreme apex of growing root hairs remains to be proven.

In all growing root hairs tested ($n = 5$ per treatment), a 5-min treatment with 0.05 to 0.5 μM CD or 0.01 μM LA caused a decrease in actin fluorescence (Figures 1I and 2). Figure 2 shows that the fluorescence intensity of the fine F-actin after CD or LA treatment was lower than that in control cells. CD treatment had no visible effect on cytoplasmic streaming in the basal part of root hairs of these species, as

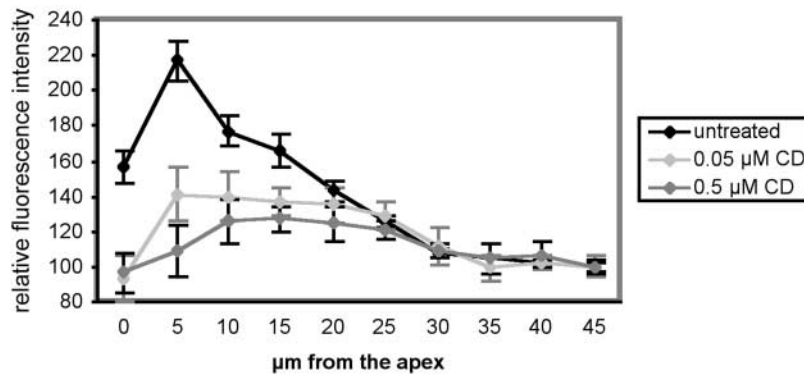


Figure 2. Relative Fluorescence Intensity Plot of Actin Immunolabeling at 5 min after CD Treatment.

The mean intensity of 5- μm -thick transverse blocks outlining the root hair shape was measured. The intensity of the block at 45 to 50 μm from the tip was set to 100, and the error bars indicate standard deviations ($n = 5$ per treatment). Application of CD leads to a significant decrease in the intensity of actin labeling in the apical area compared with the untreated control. The cytoarchitecture of the root hair does not change significantly at the concentrations of CD used; therefore, the decrease in fluorescence in the apical area cannot be attributed to increased vacuolization.

has been demonstrated for *V. sativa*, and at this low concentration did not stop cell expansion.

The higher sensitivity of the fine F-actin to a brief treatment with CD or LA shows that it is less stable than the thicker bundles of actin filaments in the basal part of a growing hair or the actin filament bundles in a full-grown hair. Thus, the actin cytoskeleton is more unstable/dynamic where growth takes place.

To examine changes in the area of cell expansion, we added 0.01 to 1 μM CD or 1 to 10 nM LA in medium to roots with living hairs and followed hair growth in time. We measured the surface expansion rate of growing root hairs in time-lapse recordings from 10 min before to 10 min after the application of 0.5 μM CD to the medium. During this interval, the growth speed after CD application was 1.03 ± 0.10 times the growth speed before application, indicating that the insertion of Golgi-derived vesicles into the plasma membrane of the apical area was not disturbed by the drug treatment. Approximately 6 min after application, tips of growing root hairs became blunt, and after 8 min, an increase in root hair width was observed at the tips where growth occurred (Figure 3). At 0.5 μM CD, the diameter of cells continued to increase until ~ 20 min after application; thereafter, cells continued to grow with this wider diameter for ~ 45 min. At low concentrations (≤ 0.1 μM CD or ≤ 10 nM LA), the diameter of the swelling was concentration dependent; cell tips reached their maximum width at 0.1 μM CD (Figure 4). After removal of the CD, root hairs continued to grow and the diameter of the growing tips decreased to normal values (Figure 5A). LA treatment (10 nM) produced a similar increase in tip width in growing root hairs, but root hairs did not recover to normal growth after the LA was washed away and contin-

ued to grow with irregular diameters (Figure 5B). When CD or LA were given repeatedly at 10-min intervals, as in *V. sativa* (Miller et al., 1999), the area with fine F-actin shrunk in length and disappeared and tip growth stopped, whereas cytoplasmic streaming continued. Together with these older results, our data show that fine F-actin is needed for vesicle delivery but not for their insertion into the plasma membrane. At still higher concentrations of CD (>50 μM) or LA (>0.5 μM), cytoplasmic streaming was arrested in all root hairs and growth was inhibited in growing hairs as a result of the complete depolymerization of all filamentous actin, as reported previously in root hairs of maize (Baluška et al., 2000) and other cells (Baluška et al., 2001).

In *Saccharomyces cerevisiae* and mammalian cells, changes in endocytosis have been observed after actin depolymerization (Engqvist-Goldstein et al., 1999). In growing root hairs, endocytosis takes place abundantly in the subapical area (Emons and Traas, 1986). Changes in the amount of endocytosis would lead to either a disturbance of the equilibrium between exocytosis and endocytosis or a change in growth speed. If the equilibrium between exocytosis and endocytosis is disturbed, one should be able to observe an excess or shortage of inserted plasma membrane, which we did not see. If the amounts of both endocytosis and exocytosis are affected similarly, one would observe changes in growth speed. We have shown that this is not the case at the drug concentrations used here (see above).

Although we have shown that the growth speed did not change during treatment with this low concentration of CD or LA, there may be changes in the area where endocytosis takes place, leading to changes in the area where exocytosis takes place, and thus a change in cell shape (i.e., an

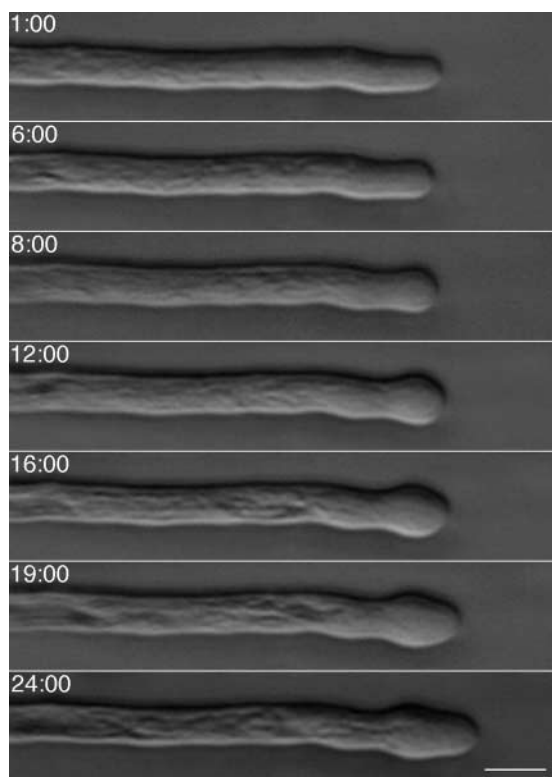


Figure 3. Response of a Growing Arabidopsis Root Hair to the Application of 0.5 μM CD.

At 6 min after application, tips of growing root hairs appear more blunt, whereas after 8 min, an increase in root hair width is observed at the growing tip. The diameter of cells continues to increase until ~ 20 min after application, after which cells continue to grow with this wider diameter. Bar = 10 μm .

increase in diameter). To test this hypothesis, we loaded Arabidopsis root hairs with 2 μM of the amphiphilic styryl dye FM4-64, which is internalized by endocytosis (Fischer-Parton et al., 2000). (A similar amphiphilic styryl dye, FM 1-43, was discussed by Emans et al. [2002].) The loading of root hairs with this dye, pretreated and grown in 0.5 μM CD, was not inhibited as expected. In addition, FM4-64 loading did not reveal significant changes in the cellular distribution of the dye (Figure 6). We conclude that endocytosis is not inhibited and takes place over a similar area of the plasma membrane during drug treatments and in control cells.

Summarizing these results, we observed that a mild treatment with actin-depolymerizing drugs induces a wider root hair tip. We conclude that a local increase in the instability of filamentous actin promotes exocytosis over an expanded cell area.

Application of actin-depolymerizing drugs did lead to an increase in root hair width, but it did not affect root hair

growth direction during recovery (Figures 4 and 5). If, as suggested by the work of Bibikova et al. (1999), microtubules determine the orientation of root hair growth, the growth direction should change during and after CD treatment in the absence of microtubules. Destruction of the fine F-actin by a CD pulse in the absence of microtubules should lead to root hair tip growth recovery to the original root hair diameter in a random direction. To test this hypothesis, we first tested the effect of oryzalin on root hairs. Oryzalin is a dinitroaniline herbicide that specifically depolymerizes the microtubule cytoskeleton of plant cells (Anthony et al., 1998). One micromolar oryzalin had a profound effect on root growth, a complete loss of anisotropic growth, as reported by Baskin et al. (1994), but it had hardly any effect on Arabidopsis root hair tip growth. We observed a slight waviness of the root hairs (Figure 7A), as has been reported by Bibikova et al. (1999). We then applied 1 μM CD to roots in the absence and presence of oryzalin for 30 min. In the absence of oryzalin, the root hairs continued to grow in the same direction ($7.2 \pm 6.4^\circ$ [absolute deviation \pm SD]) (Figure 5A). However, in the presence of oryzalin, with no microtubules present (Figures 7B and 7C), recovery to the original root hair width after a 30-min CD application occurred in a random orientation from the hemisphere ($90.0 \pm 24.5^\circ$ [absolute deviation \pm SD]) (Figures 7D to 7E). We conclude that although microtubules do not play a role in tip growth itself, they act as a scaffold that imposes the direction of growth during recovery from disturbance of the growth machinery (Figure 8). It is possible that a similar mechanism is involved, as has been suggested for neural growth cones, in which polymerizing microtubules activate site-directed actin filament assembly (Rochlin et al., 1999).

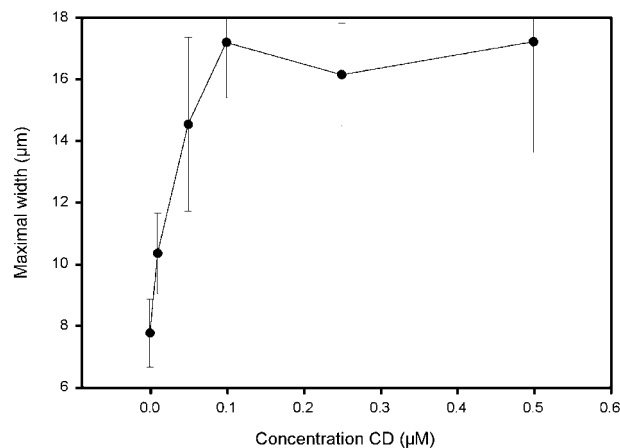


Figure 4. Maximal Arabidopsis Root Hair Diameter after the Application of Cytochalasin D.

$n > 20$ per concentration. Error bars indicate standard deviations.

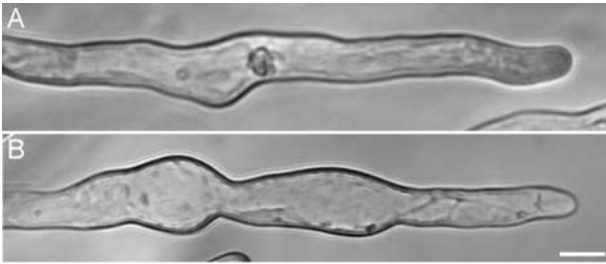


Figure 5. Recovery of Root Hairs after CD and LA Washout.

(A) After 1 μM CD washout, root hair growth continues, but the diameter decreases to the values observed in controls.

(B) After 10 nM LA washout, the diameter of growing root hairs remains variable for at least 1 h after washout.

Bar = 5 μm .

DISCUSSION

Our results represent an important step toward understanding the role of the actin cytoskeleton in the spatial regulation of growth in root hairs. They imply that actin instability specifies the size and location of the area of exocytosis. By regulating the area where F-actin is more dynamic, a plant cell could regulate where it expands. Miller et al. (1999) have shown that complete depolymerization of the subapical fine F-actin inhibits root hair growth. This finding demonstrates that the presence of fine F-actin in the zone adjacent to the growth zone is essential for cell growth. They hypothesize that fine F-actin is involved in the delivery of Golgi-derived vesicles to the site where exocytosis takes place. Similar observations have been made by Gibbon et al. (1999) in maize pollen tubes and by Vidali et al. (2001) in *Lilium longiflorum* pollen tubes. The concentrations of actin-depolymerizing drugs we used were lower than the concentration used by Miller et al. (1999). Apparently, we did not inhibit Golgi-derived vesicle delivery, because cell expansion proceeded at the same rate as in control cells but increased the cell surface area where expansion takes place. This result indicates that the actin cytoskeleton has two distinct functions in cell expansion: (1) it delivers Golgi-derived vesicles to the area where exocytosis takes place and retains them in that area (Miller et al., 1999); and (2) it must remain unstable at the plasma membrane surface area where exocytosis takes place (our results). CD and LA are both actin-depolymerizing agents, but root hairs respond somewhat differently to these two drugs. After CD is washed away from the medium, root hairs recover to their normal diameter in a matter of minutes, whereas root hairs continue to grow with irregular diameters after LA washout before total recovery for a period of up to several hours (Figure 5).

What could cause the somewhat different responses of

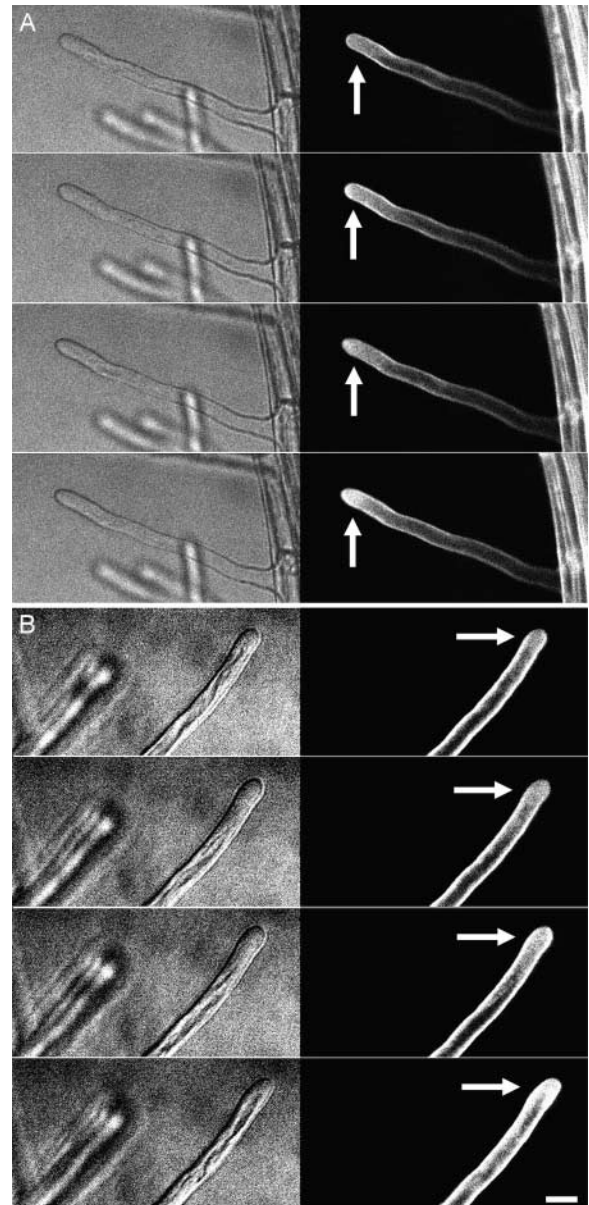


Figure 6. Loading of Arabidopsis Root Hairs with the Amphiphilic Dye FM4-64.

Images were collected on a confocal microscope with a completely opened pinhole at 0, 2.5, 5, and 7.5 min after application. The right side of each image shows the fluorescent FM4-64 loading, and the left side shows the reflection image. Image sequence **(A)** shows the loading of a control root hair with 2 μM FM4-64. Image sequence **(B)** shows a root hair pretreated for 1 min with 0.5 μM CD and loaded with 2 μM FM4-64 in the presence of CD. In both sequences, dye loading and imaging did not inhibit root hair growth. Comparison of **(A)** and **(B)** demonstrates that endocytosis is not inhibited by 0.5 μM CD treatment. The increase in diameter of the CD-treated root hair in **(B)** is not clearly visible in this time scale but can begin to be observed in the last frame. Bar = 10 μm .

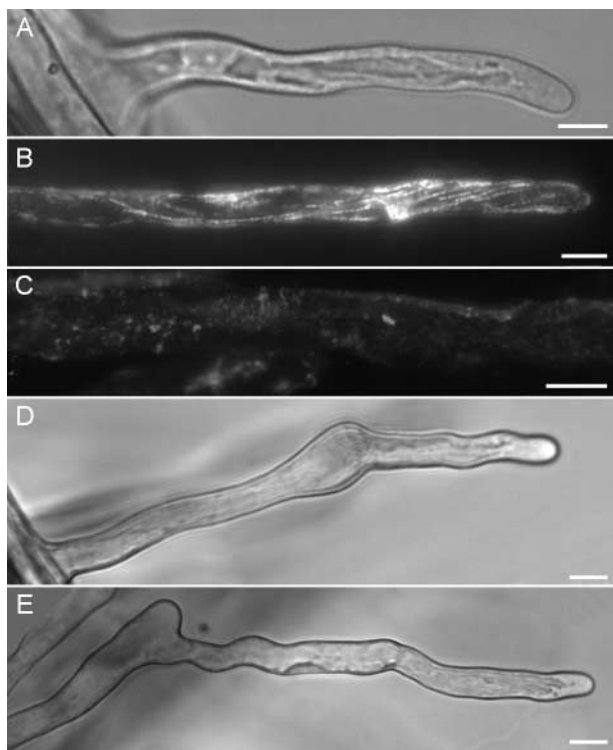


Figure 7. Combined Microtubule and Actin Drug Treatment in *Arabidopsis* Root Hairs.

(A) Permanent oryzalin treatment does not change root hair orientation and causes a slight waviness.

(B) and **(C)** Freeze fixation and immunolocalization of the microtubules in *Arabidopsis* root hairs shows that the orientation of the microtubules is net-axial **(B)** and that the microtubules are depolymerized after a 5-min treatment with 1 μ M oryzalin **(C)**.

(D) and **(E)** When CD is applied in the presence of oryzalin, a 30-min 1- μ M CD pulse leads to an increase in the diameter of growing root hairs, as expected, but also to the recovery of growing tips with normal diameter, now in a random orientation.

Bars = 10 μ m.

root hairs to LA and CD? The answer could lie in the different working mechanisms of the two drugs. LA binds 1:1 to G-actin and prevents the incorporation of G-actin into filaments, leading to a change in the equilibrium between G- and F-actin, and thus induces actin depolymerization (Gibbon et al., 1998). CD caps the barbed ends of growing actin filaments, preventing further polymerization (Cooper, 1987). At the low concentrations used, both drugs depolymerize part of the fine F-actin pool, leading to an increased concentration of G-actin available for actin polymerization and thus increased actin dynamics. A differential washout efficiency of LA and CD might explain the differences in recovery after washout of both drugs. LA binds to G-actin with a K_d of 74 nM (calculated from maize pollen actin) (Gibbon et al.,

1999), thus slowly dissociating from G-actin during washout, whereas CD-capped dynamic F-actin depolymerizes quickly, leading to release and efficient washout of CD.

In pollen tubes, which are tip-growing cells, an actin configuration has been reported that is comparable to that in root hairs: an apical area without detectable actin filaments, followed subapically by an area with a dense actin meshwork (Doris and Steer, 1996; Miller et al., 1996; Gibbon et al., 1999; Geitmann et al., 2000). Miller et al. (1996) confirmed by immunogold electron microscopic analysis after freeze substitution that the apical area is essentially free of filamentous actin. However, Fu et al. (2001) recently demonstrated that very dynamic short actin bundles, correlating with growth, are present in the apex of tobacco pollen tubes when expressing LAT52::GFP(S65C)-mTalin. Their experiments suggest that Rop is involved in regulating the polar growth of

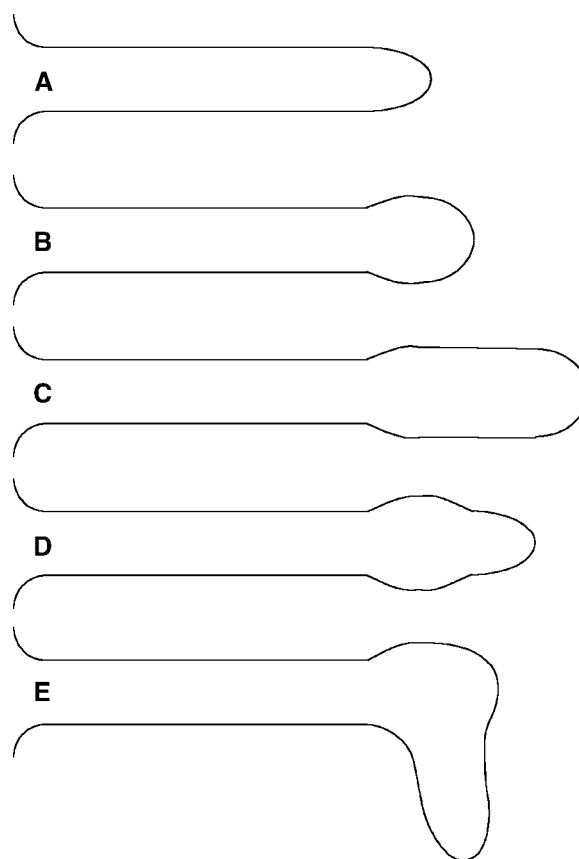


Figure 8. Cartoon Showing the Effects of CD and Oryzalin Treatments on Growing Root Hairs.

A normal growing root hair **(A)** responds to treatment with CD by an increase in exocytosis area at the tip **(B)**. After prolonged treatment, the root hair will continue to grow in this wider diameter **(C)**. After removal of the CD, root hairs respond by recovering to their normal width in the original growth orientation **(D)**. A CD pulse, in the absence of microtubules, leads to recovery in a random orientation **(E)**.

pollen tubes at least partially through the control of the organization of dynamic F-actin at the apex of pollen tubes. Both our results and the results of Fu et al. (2001) demonstrate the importance of the dynamics of the (sub)apical actin cytoskeleton in determining the area of cell expansion. In root hairs, overexpression of constitutively active Rop2 has been demonstrated to increase the diameter of the tube while forming a network of fine F-actin in the tip (Jones et al., 2002), indicating that Rop2 likely is involved in the control of the subapical actin cytoskeleton.

In intercalary growing cells, it is widely accepted that microtubules limit the direction of cell expansion (for review, see Ketelaar and Emons, 2001). Our data show that in root hairs, microtubules perform a similar function that becomes apparent only when the actin-based cell growth process has been disturbed. Bibikova et al. (1999) demonstrated that disturbance of the tip-focused gradient of cytoplasmic free calcium ions in the absence of microtubules leads to a permanent change in growth direction, whereas disturbance of the calcium ion gradient in the presence of microtubules leads to only a transient change in growth direction. These observations, in combination with our findings, suggest that the gradient of cytoplasmic free calcium directs the fine F-actin.

Future work could focus on testing the hypothesis that localized actin instability, leading to a local decrease in stable bundles of filamentous actin, combined with an underlying meshwork of fine F-actin, could provide a general mechanism for the determination of the area of cell growth while microtubules limit the growth orientation of plant cells.

METHODS

Plant Growth, Drug Treatment, and Bright-Field Microscopy

Roots of *Arabidopsis thaliana* plants (ecotype Columbia) were grown as described previously (Ketelaar et al., 2002). Drugs were applied by gradually replacing the medium with identical medium containing added drugs in a plant culture room to avoid temperature shocks. Cytochalasin D and latrunculin A were dissolved as stocks of 10 mM in DMSO. The control plant medium was replaced by medium containing the same percentages of DMSO given to the experimentally treated plants. Root hairs were analyzed by placing the plant growth chamber on the stage of a Nikon Diaphot 200 microscope (Tokyo, Japan) equipped with Hoffman modulation contrast. Time-lapse images were recorded with a Hamamatsu Argus-20 system (Herrsching, Germany) and processed with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Calculation of Root Hair Surface Increase

High-resolution images were taken at 30-s time intervals from 10 min before drug application to 10 min after drug application. Sequential images of the root hairs were aligned, and a transverse line was placed every micrometer, starting at 10 μm from the apex of the first image until the apex was reached. The increase in root hair surface

was estimated by measuring the maximal diameter of the root hair in every individual slice; thereafter, the values obtained were extrapolated to the three-dimensional circular circumference, and the individual slide surfaces were summed. The total increase in cell surface before and after local cytochalasin D application was calculated. The standard deviation was calculated from the differences in cell surface increase between the individual images, and the changes in growth speed did not exceed the standard deviation.

Immunocytochemistry, Improved Ester-Aldehyde-Choline Fixation followed by Fluorescently Labeled Phallotoxin Staining, and Fluorescence Microscopy

After drug application, plants were either fixed by the ester-aldehyde-choline method and stained with Bodipy-FL phalloidin as described previously (Ketelaar et al., 2002) or frozen rapidly in liquid propane and freeze substituted in a freeze substitution device as described (Ketelaar et al., 2002). As anti-tubulin antibody, we used the clone DM1A (Sigma), and as anti-actin antibody, we used the antibody against pea actin, as described by Andersland et al. (1994). Microscopy was performed as described previously (Ketelaar et al., 2002). Deconvolution was performed by opening the raw confocal laser scanning microscopy data in OpenLab 3.0 (Improvision, Coventry, UK) and performing 25 iterations of the Volume Deconvolution function.

FM4-64 Staining

Arabidopsis roots in a growth chamber were placed on the stage of a Bio-Rad MRC-600 confocal laser scanning microscope. The medium was replaced gradually with medium containing 2 μM FM4-64, after which the root hairs were imaged using the DM 488 BA 522 DF35 filter block. Imaging was performed with a completely opened pinhole to image the whole root hair.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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Unstable F-Actin Specifies the Area and Microtubule Direction of Cell Expansion in Arabidopsis Root Hairs

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