Visualization of Highly Dynamic F-Actin Plus Ends in Growing Phaseolus vulgaris Root Hair Cells and Their Responses to Rhizobium etli Nod Factors

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Introduction

A molecular dialog is established during the symbiotic interaction between rhizobia and legume plants. Legume roots exude flavonoids that induce the expression of bacterial nodulation genes, which encode proteins involved in the synthesis and secretion of Nod factors (NFs) (Fisher and Long 1992). These NFs signal back to the plant root, triggering various physiological responses, such as ion (K⁺, Cl⁻, Ca²⁺ and H⁺) exchanges, cytoplasmic alkalization, Ca²⁺ oscillations and gene expression (Cárdenas et al. 2000, Lhuissier et al. 2001), that facilitate bacterial invasion and nodule formation. In addition, actin microfilaments and Ca²⁺ gradients undergo dramatic changes in the tips of root hair cells responding to NFs (Miller et al. 1999, Shaw and Long. 2003, Yokota et al. 2009).

In plants, the actin cytoskeleton is a pivotal regulator of cell polarity and growth; it controls cytoplasmic streaming, and it also appears to contribute to the force that drives tip growth in fungal hyphae, characean cells and pollen tubes (Gibbon et al. 1999, Vidali et al. 2001, Braun et al. 2004, Cárdenas et al. 2005). In addition, actin plays a key role in axis establishment and polar growth in fucoid zygotes, as demonstrated by studies using several actin-disrupting drugs (Hable et al. 2003). Furthermore, actin microfilaments regulate stomatal aperture by modulating the activity of ion channels, in a similar manner to that reported in animal cells (Wang et al. 2004, Gao et al. 2009). In root hair cells, actin is organized in longitudinal bundles that extend along the cell up to the base of the tip (Cárdenas et al. 1998, Miller et al. 1999, Lhuissier et al. 2001, Ketelaar et al. 2003). During polar growth, actin microfilaments transport Golgi-derived vesicles, which contain cell wall precursors. These vesicles accumulate in the apical domain, where they eventually fuse with the plasma membrane, secrete their contents and thereby support tip growth (Hepler et al. 2001).
It has been suggested that the G-actin pool in the tip of growing pollen tubes and root hairs (Vidali et al. 2001, Cárdenas et al. 2005, He et al. 2006) is important for driving the actin polymerization that takes place in the apex (Baluska et al. 2000, Vidali et al. 2001, Cárdenas et al. 2005). Numerious factors, such as Ca$^{2+}$, pH and reactive oxygen species (ROS) levels, regulate actin cytoskeleton dynamics. For instance, Ca$^{2+}$ and pH control polymerization and actin structure in root hair cells by modulating certain key actin-binding proteins that are sensitive to changes in [Ca$^{2+}$] such as actin-depolymerizing factor (ADF), gelsolin and villin (Chen et al. 2002, Fan et al. 2004). Furthermore, Ca$^{2+}$ channels can be modulated by ROS, as described in Arabidopsis root hairs (Foreman et al. 2003, Takeda et al. 2008, Cárdenas 2009).

Previously, we reported that actin bundles in root hair cells rapidly undergo fragmentation and reorganization in response to NF exposure (Cárdenas et al. 1998). This suggests that actin microfilaments play an important role during the legume–rhizobium interaction, probably by reprogramming root hair growth to allow the establishment of symbiotic interactions. This idea has been supported by genetic studies that examined the function of the actin cytoskeleton during rhizobial infection thread (IT) formation. For instance, it has been reported that mutation of Medicago truncatula and Lotus japonicus NAP1 and PIR1, both of which are components of the SCAR/WAVE complex, which is involved in the actin rearrangements that are essential for IT formation and colonization by rhizobia, significantly impaired both the formation and subsequent progression of ITs in the root cortex (Yokota et al. 2009, Miyahara et al. 2010). These mutants have short root hairs with mostly a transverse or web-like distribution of actin filaments, whereas wildtype root hairs contain bundles of actin filaments that are predominantly longitudinal. The nap1 and pir1 alleles also conferred a characteristic distorted trichome phenotype, demonstrating a more general role for Nap1 and Pir1 in processes that establish cell polarity or polar growth in L. japonicus (Yokota et al. 2009). However, little is known about actin polymerization during the early stages of nodulation, particularly in root hairs responding to specific NFs. In this work, sublethal (non-toxic) concentrations of fluorescently labeled cytochalasin D (Cyt-Fl) were used to observe the fast-growing (plus) ends of microfilaments. Using sublethal drug concentrations, we studied the organization and distribution of filamentous actin (F-actin) plus ends in the tips of living root hair cells from Phaseolus vulgaris during regular growth and during the interaction with Rhizobium etli NFs.

Time-lapse analysis of the fluorescence signal demonstrated a dynamic reorganization of the actin polymerization sites, which are continuously generated and are sensitive to actin-disrupting drugs. When P. vulgaris root hairs were treated with Cyt-Fl and then challenged with R. etli NFs, the intensity of the signal in the apex rapidly increased. These results support the idea that actin plus ends located in the tips of root hairs promote actin polymerization, play a pivotal role in polar growth and modulate root hair cell growth during the NF response. Furthermore, actin polymerization may contribute to the reorganization of the actin cytoskeleton in such a manner that it supports IT formation, thereby establishing a successful symbiotic interaction.

### Results

#### High density of actin microfilament plus ends in the tips of living root hair cells

To visualize microfilament plus ends in growing root hairs, we used a fluorescently derivatized form of cytochalasin D (Cyt-Fl)
(Molecular Probes), which is a fungal toxin that binds to the plus ends of microfilaments. This probe (at 50 nM) was previously used to image the plus ends of actin filaments in striated muscle and to monitor actin polymerization in the vicinity of the nuclear surface of HeLa cells (Münter et al. 2006, Pappas et al. 2008). Using the same low concentration of Cyt-Fl (50 nM) and time-lapse fluorescence imaging, we visualized the plus ends of microfilaments in growing *P. vulgaris* root hair cells. Root hairs treated with Cyt-Fl displayed a strong fluorescence signal in the apical region (Fig. 1A), and appeared to grow normally. Furthermore, root hairs from a non-legume, such as Arabidopsis, had a similar apical distribution of fluorescence signal (Fig. 1B), suggesting a general role for the distribution of microfilament plus ends in polarized cells. The observation that the region from which root hairs emerge exhibited a strong fluorescence signal demonstrated that actin microfilaments are involved in the initiation of root hair formation (Fig. 1C) before the onset of polar growth. This fluorescence pattern indicates that actin polymerization sites emerge even before the bulge of the new root hair is established, which suggests that the organization of the cytoskeleton is established before growth is initiated.

**Sublethal concentrations of Cyt-Fl do not inhibit root hair tip growth or affect cell morphology**

Because root hair cells are particularly sensitive to environmental changes, we thoroughly examined the viability and morphology of root hairs treated with Cyt-Fl. We found that 50 nM was the lowest concentration of Cyt-Fl that yielded a good signal-to-noise ratio in our system. Although root hairs treated with 50 or 100 nM Cyt-Fl did not exhibit any morphological or tip growth defects during a 4 h observation period, we decided to use the lower concentration. Time-lapse imaging of root hairs undergoing bulge formation and rapid growth showed that exposure to 50 nM Cyt-Fl did not alter the growth rate compared with the control. The time-lapse sequence presented in Fig. 2a shows the different developmental stages of root hairs (n = 8), including bulge formation, the initiation of tip growth, the rapid growth stage, and maturation and termination of root hair growth.

![Fig. 2](image-url) Apical distribution of microfilament plus ends in Arabidopsis root hairs correlates with the different stages of root hair growth. (a) Note that the intensity of fluorescence is low during the initiation of root hair tip growth; however, the intensity increases significantly when the root hair undergoes rapid growth and decreases when growth ceases. Images were acquired at 4.6 min intervals. (b) Fluorescence quantification at the tip of the same root hair presented in (a). The higher intensity indicated with an underlined gray region corresponds to the rapid growth stage. Time-lapse images were acquired over a 4 h period. n = 8. Scale bar represent 40 micrometers.
polar growth. As depicted in Fig. 2, a fluorescence signal, indicating microfilament plus ends, was clearly visible during bulge formation; however, the signal became more intense when the root hair underwent rapid growth and decreased to basal levels when the root hair stopped growing. Furthermore, the fluorescence intensity fluctuated during periods of rapid growth (Fig. 2b, gray underline). These results suggest that treatment with Cyt-Fl does not affect the growth rate and morphology of the root hair cells. In addition, root hairs treated with Cyt-Fl exhibited all the developmental stages present in control root hair cells. Furthermore, the intensity of the fluorescence signal in the tip seemed to correlate with the growth rate and developmental stage (Fig. 2b). Therefore, we chose to use 50 nM Cyt-Fl in further experiments.

Cross-correlation analysis of the increase in the concentration of plus ends terminals and growth rate

The phasic variation in both the intensity and velocity data was estimated using the LOESS (locally weighted scatterplot smoothing) non-parametric regression method (see Fig. 3), and was improved using separate regressions for the early, middle and late data. The predicted mean of the smoothed log₂ intensity and the tip velocity data was calculated at the individual sampling times using the R predict function and subtracted from the data to reveal residual high frequency deviations (Fig. 4). The computed correlations, r, of the residual intensity and velocity variations are reported in Fig. 4. Whereas the early and late data correlations were not significant at the 0.05 level, the middle data correlation (r = 0.207) was very highly significant (P < 0.001, t = 3.94, df = 348).

Unlabeled cytochalasin competes with Cyt-Fl for plus end binding sites of actin microfilaments

Arabidopsis root hairs were treated for 5 min with Cyt-Fl (50 nM) and 15 min later with unlabeled cytochalasin (50 nM). Under these conditions, a decreased signal was observed within 5 min of the addition of unlabeled cytochalasin, indicating that both labeled and unlabeled cytochalasin molecules bind to actin and compete for the same binding site (Fig. 5).

Cyt-Fl signal is increased in root hair cells treated with latrunculin B or jasplakinolide

Since Arabidopsis roots are smaller and easier to manipulate than those of bean, we performed a series of experiments in
Arabidopsis. We visualized the fluorescence distribution of Cyt-Fl in real time while the root hair cells underwent tip growth arrest due to the effect of actin-disrupting drugs, such as latrunculin B (LatB) and jasplakinolide (Jas). Root hair cells were treated with Cyt-Fl and 5 min later with LatB (50 nM), and the fluorescence intensity of the actin polymerization sites was recorded and analyzed. We found that LatB treatment resulted in an increase in signal intensity, not only in the tip, but throughout the root hair cell (Fig. 6). LatB treatment enhanced fragmentation of the actin cytoskeleton (Vidali et al. 2001), thereby increasing the number of available plus end to which Cyt-Fl can bind and resulting in a stronger signal. Indeed, after LatB treatment, the area in which fluorescence was observed increased, not only in the tip, but also in patches beneath the apical dome (Supplementary Fig. S1a, see white arrow). There was initially a strong response to the LatB treatment; however, the fluorescence signal seemed to stabilize after 5 min of treatment (Supplementary Fig. S1b). It is noteworthy that root hair cells treated with Cyt-Fl and then with LatB at low frequency (20%) underwent fluctuations in fluorescence signal after 10 min of treatment (Supplementary Fig. S1a). In some cases (20% of the tested root hair cells), a clear oscillation in fluorescence signal was observed (Supplementary Fig. S2b), which indicates that new plus ends are generated. After 10 min of LatB exposure, cytoplasmic streaming was also noticeably slower and growth was arrested.

The finding that LatB treatment inhibited root hair growth, increased the Cyt-Fl signal and induced fluctuations in the fluorescence signal prompted us to repeat this experiment using a different microfilament-disrupting drug, Jas. Jas is a widely used drug that induces actin polymerization and stabilizes actin filaments (Cárdenas et al. 2005). To examine the effect of Jas on actin microfilament plus ends, we treated Arabidopsis root hair cells with Cyt-Fl and then with Jas (at 2 μM). The upper two rows in Fig. 7a and b depict a control living root hair cell, and the lower two rows (Fig. 7c, d) show the same root hair after Jas treatment. Fig. 7a and c correspond to the transmitted light image and Fig. 7b and d to the fluorescence image. Note that the cytoplasmic distribution and Cyt-Fl signal were typical of normal growing root hair cells (Fig. 7a, b). However, there was a clear increase in signal after Jas treatment (Fig. 7d). Both the region containing an intense fluorescence signal and the amount of cytoplasm in the tip increased in response to Jas treatment (Fig. 7, compare region of interest in Fig. 7a and c).

Fig. 5 Arabidopsis root hairs treated with Cyt-Fl (50 nM) and then with unlabeled cytochalasin D (50 nM). A root hair cell was treated with Cyt-Fl for 5 min (image 1) and then with unlabeled cytochalasin, and images were recorded every 15 s (image 2 onwards). Note that the fluorescence decreased after 1 min and almost disappeared after 2 min, which indicates that the two compounds compete for the same binding site. Images were taken at 15 s intervals. n = 5. Scale bar represent 20 micrometers.
Interestingly, in contrast to the effect of LatB on cytoplasmic streaming, Jas treatment caused an apparent increase in cytoplasmic activity in the tip dome. This response was reversible, since removal of the drug allowed apical growth to resume (data not shown). A more striking result, however, was the observation that, after 5 min, Jas induced a stronger accumulation of fluorescence signal in the tip (Fig. 7b) that differed from the non-treated condition (Fig. 7a). Taken together, these results indicate that sublethal concentrations of Cyt-Fl provide a useful tool for labeling the plus ends and for examining the correlation between the distribution of plus ends and the effect of actin-disrupting drugs.

**Rhizobium etli** Nod factors promote the formation of plus ends in living root hair cells

Growing *P. vulgaris* root hair cells were treated with Cyt-Fl and then with *R. etli* NFs at $10^{-8}$ M, and time-lapse imaging was used to monitor the signal in the apical region (Fig. 8, inset). We found that this fluorescence signal transiently increased in response to NFs, since pentamers (i.e. chitin oligomers) did not induce a similar response at the same concentration. The increased fluorescence occurred within the first 2 min of treatment with NFs, peaked at 3–4 min and decreased by 5 min, and is thought to reflect an elevation in the number of plus ends at the tip region of the root hair cell. The graph in Fig. 8 shows that the fluorescence signal increased in the tip region a few minutes after NF treatment. When root hairs from Arabidopsis were treated with a similar concentration of NFs at $10^{-8}$ M, we did not observe a change in the fluorescence signal (data not shown).

The cytoplasm is unevenly distributed in root hair cells, as a consequence of a huge vacuole occupying almost 90% of the volume and squeezing the cytoplasm towards the tip. This can result in the differential distribution of any dye that is introduced into the cell. To determine the precise subcellular distribution of actin polymerization sites, we performed a pseudoratiometric analysis. Briefly, this approach consists of introducing a reference dye (cell tracker red, Invitrogen), which does not change in response to environmental conditions and monitors the accessible volume of the cells, and then a second dye, in this case Cyt-Fl, to determine the subcellular distribution of actin microfilament plus ends. Using this approach, we found that the plus ends localized to the tip dome, in close proximity to the apical plasma membrane (Supplementary Fig. S3). We then treated these cells with NFs and recorded the signal distribution at 3 s intervals. As depicted in Supplementary Fig. S4, the signal intensity increased immediately after exposure to NFs and returned to initial values after 1–2 min. These results are similar to those described in Fig. 8 and therefore confirm that the number of plus end increases after NF treatment.

**Plus ends are essential for root hair tip growth**

Typically, legume root hair cells treated with NFs display a transient loss of polarity within 1 h of treatment, and during this time the cell undergoes isotropic growth, which is characterized by swelling at the tip and the absence of polar growth. When these swollen root hair cells were labeled with Cyt-Fl, the actin microfilament plus ends normally found in the tip disappeared (see Fig. 9, asterisk). However, swollen root hair cells eventually reinitiated polar growth in the presence of the NFs (after 1–2 h) and, when this happened, an elevated fluorescence signal was observed in the tip dome (see Fig. 9, black arrow). These data strongly suggest that the re-establishment of actin microfilament plus ends in the apical region is essential for root hair outgrowth.

**Actin microfilament plus ends define the region for infection thread initiation**

The apical region of root hairs grows around the site of bacterial attachment and induces an invagination of both the plasma membrane and the cell wall to initiate the formation of ITs. ITs are unique structures formed de novo in legume root hairs...
that allow bacteria to colonize a plant host. These structures are polar-growing invaginations of the plant cell, with the cell wall matrix on the inside of the ITs and thus extracellular to the plant. We labeled root hair cells containing an initiated IT with Cyt-Fl and found that plus ends localized to the site of early IT formation (see Fig. 10), which usually occurred close to the subapical domain. Therefore, our results substantiate the idea that the actin cytoskeleton plays a pivotal role during the Rhizobium–legume symbiosis.

Discussion

Root hairs are polarized extensions of epidermal cells. These tubular structures exhibit an apical distribution of secretory vesicles, and a well-characterized membrane polarization. Pollen tubes elongate in a similar manner to root hair cells, and several factors that modulate tip growth have been described in both models, such as Ca$^{2+}$, ROS, pH, the cytoskeleton and the cell wall (Yang 1998, Monshausen et al. 2007).
ITs are unique structures in rhizobia-infected legume roots that are initially formed by polar-growing invaginations of the plasma membrane and cell wall at the site of bacterial attachment. Interestingly, the cell wall matrix remains on the inside of the IT and thus extracellular to the plant. Once initiated, the IT follows, by an unknown mechanism, the movement of the nucleus, which usually migrates from the tip of the root hair cell to the base, in a microtubule- and actin-dependent manner (Nutman 1959, Lloyd et al. 1987). The path traveled by the IT from the root hair cell to the cortical cells is determined by many factors, including glycoproteins, ROS, small GTPases, phosphoinositides and the cytoskeleton (Peleg-Grossman et al. 2007, Arrighi et al. 2008, Fournier et al. 2008, Blanco et al. 2009, Montiel et al. 2012). Pre-infection threads, which resemble a cell division plate that does not divide, allow the formation of a tube-like structure that becomes an IT (van Brussel et al. 1992, Brewin 2004).

The actin cytoskeleton has an important role in directing cell division and cell growth, including polar growth (Wasteneys and Galway 2003, Hussey et al. 2006). It is therefore plausible that the development of polarized ITs may require a dynamic cytoskeleton to maintain growth from the root hair tip to the cortical cells. A major function of F-actin is to guide vesicles to sites of cell growth, and root hair cells and pollen tubes have been important models to substantiate this role (Sieberer et al. 2002, McKenna et al. 2009). During the early interaction between the root hair and the rhizobia, the apical region of the root hair grows around the site of bacterial attachment and induces invagination of the plasma membrane and the cell wall. This process requires F-actin, which must be dynamically regulated during IT formation; however, we do not know anything about the role of the actin cytoskeleton in modulating IT formation. We have reported that the actin cytoskeleton is rapidly reorganized in response to purified NFs, that it undergoes rapid and persistent fragmentation in the tip region of root hair cells within 5 min of NF treatment and that this is followed by actin bundling at the base of the root hair (Cárdenas et al. 1998). This finding has been supported by other studies that showed the accumulation of fine actin

![Fig. 8](image1.png)

**Fig. 8** Root hairs from *P. vulgaris* treated with Cyt-Fl and then challenged with NFs from *R. etli* show an increase in the number of F-actin plus ends. Inset: the fluorescence signal in the apical region expands within 5 min of treatment, and then returns to initial levels. t = 0 corresponds to the same root hair pre-treated with pentamers (control conditions). Graphical representation of the fluorescence signal during NF treatment. Note that the increase in fluorescence was transient and was not observed under control conditions. n = 20. Scale bar represents 20 micrometers.

![Fig. 9](image2.png)

**Fig. 9** F-actin plus ends are essential for polar growth. Root hairs from *P. vulgaris* were treated with Cyt-Fl and then with NFs. At 1 h after treatment with NFs, root hairs swelled up and, 1 h later, growth was re-initiated from some of the swollen cells. Swollen root hairs exhibited a low fluorescence signal (green asterisk), while root hairs that were re-initiating growth (arrow) displayed increased fluorescence (red). n = 5. Scale bar represents 20 micrometers.
the role of the SCAR/WAVE complex in the control of membrane dynamics and vesicle trafficking has been well documented by modulating actin organization and the mobilization of secretory vesicles.

However, actin dynamics are the result of highly regulated polymerization and depolymerization processes, and these processes have not been analyzed in tip-growing cells, such as root hairs, or during IT development. The work described here supports the essential role of dynamic F-actin regulation. Using sublethal concentrations of Cyt-Fl, we labeled the plus ends of actin microfilaments and thus visualized the polymerization zone in growing root hairs from *P. vulgaris*. Cyt-Fl has previously been used to visualize the plus ends (Münter et al. 2006, Pappas et al. 2008). Using this approach, we studied the role of F-actin ends in root hair apical growth and during the interaction with specific NFs. We found that there was a correlation between an increased number of F-actin plus ends in the apical zone of root hair cells and polarized growth. To correlate the presence of actin plus ends with growth rate, we acquired long time-lapse sequences that spanned all developmental stages in the same root hair cell. Using this approach, we found that fluorescence intensity, a marker of plus end accumulation, increased during periods of rapid root hair growth. Furthermore, we performed a correlation analysis in which the log₂ of actin plus ends was calculated before correlating the residuals about its LOESS smooth. This was done to remove the obvious heteroskedasticity of the data, which is reduced by the log transform. It is notable, however, that the log transform did not totally reduce the heteroskedasticity, which indicates that high growth rates involve an additional factor that increases variability beyond a size-related factor. This additional variation could involve the linkage between our measure of actin polymerization and growth. This is supported by the finding that non-growing swollen root hair cells do not have a distinct region with F-actin plus ends like that present in growing root hair cells and in cells in which growth is reinitiated after NF treatment. Furthermore, NFs induced a specific transient increase in the Cyt-Fl signal and therefore in the number of plus ends. This response was not observed upon treatment with inactive chitin oligomers. We found that F-actin plus ends accumulated in the tip of growing root hair cells, and time-lapse imaging demonstrated that these polymerization sites, which are continuously generated, are highly dynamic.

The finding that F-actin plus ends are present in tip-growing plant cells supports the idea that actin plays an important role in tip growth. The dynamic fluorescence signal of growing *P. vulgaris* root hairs treated with Cyt-Fl increased within 2 min of exposure to *R. etli* NFs. This result suggests that new plus ends can be generated in the presence of NFs, due to fragmentation of microfilaments, as previously reported (Cárdenas et al. 1998). Later, when the root hairs started and stopped growing in response to NF treatment, the number of plus ends decreased significantly, suggesting that there is a correlation between a decrease in these sites and the absence of polar growth (i.e. isotropic growth). Remarkably, as soon as the root hair cells re-established the plus ends, as depicted by an elevated fluorescence signal, polar growth was re-initiated. These results indicate that actin polymerization in the apical dome is a key element during the NF response and, more importantly, during polar plant cell growth.
Root hairs are highly polarized cellular structures that result from localized growth of specific root epidermal cells. The morphogenesis of root hair cells is especially interesting and involves the interaction of multiple processes, including vesicle exocytosis, Ca\(^{2+}\) homeostasis, cytoskeleton rearrangements, activation of reactive oxygen species (ROS), and dynamic changes of various glycosylphosphatidylinositol (GPI)-anchored proteins within lipid rafts (Schindelman et al. 2001, Fischer et al. 2004, Sangiorgio et al. 2004, Jones et al. 2006). It is well established that there is a large apical G-actin pool in root hairs and pollen tubes. This observation strongly suggests that newly formed actin polymerization sites fueled by the abundant G-actin pool are an important factor in supporting tip growth (Cárdenas et al. 2005, He et al. 2006). Furthermore, the fact that there is a well-described tip-focused Ca\(^{2+}\) gradient in tip-growing cells (Wymer et al. 1997, Cárdenas et al. 2000, Lhuissier et al. 2001) supports the notion that transient increases in the concentration of Ca\(^{2+}\) and ROS, which are apically distributed in root hairs and are able to respond transiently to specific NFs (Foreman et al. 2003, Cárdenas and Quinto 2008), could regulate the above-mentioned actin polymerization dynamics. Furthermore, numerous actin-binding proteins, such as gelsolin and ADF, have been localized to the tip region of root hairs and pollen tubes (Vidali et al. 2001, Chen et al. 2003, Augustine et al. 2008). Both gelsolin and ADF are regulated by Ca\(^{2+}\) or pH and are thus likely to play a role in Ca\(^{2+}\)-modulated actin remodeling processes. It is well known that Golgi-derived vesicles, which carry the precursors for cell wall synthesis, accumulate to high levels in the tip region during polar growth and that these vesicles appear to be delivered to the tip dome in a regulated manner. Actin polymerization is thought to provide a driving force for tip growth, as demonstrated by numerous studies that show that actin-disrupting drugs alter actin dynamics and inhibit polar growth, but also vesicle motility and distribution (Torralba et al. 1998, Gibbon et al. 1999, Miller et al. 1999, Lhuissier et al. 2001, Vidali et al. 2001, Cárdenas et al. 2005). Furthermore, by means of actin instability or regulating F-actin dynamics, a plant cell can regulate where it expands and maintains a proper area of exocytosis, suggesting that actin has a key role in supporting the spatial regulation of polar growth (Ketelaar et al. 2003). Interestingly, the normal outward pattern of root hair cell growth is inverted during IT formation, and this would require reprogramming of the growth direction. This may be facilitated by the previously described reorganization of actin during the infection process (Cárdenas et al. 1998, Miller et al. 1999, Yokota et al. 2009, Miyahara et al. 2010). The generation of new F-actin plus ends may adjust the architecture of the cytoskeleton in such a manner that rhizobial infection may occur.

**Seed germination**

*Phaseolus vulgaris* seeds were surface sterilized with sodium hypochlorite (25%) for 5 min, rinsed five times with sterile water, treated with pure ethanol for 1 min and rinsed another five times with water, as described in Cárdenas et al. (1995). Sterile bean seeds were transferred to sterile steel plates lined with wet paper towels, covered with foil and transferred to an incubator set at 28°C for 2 d, at which point the seeds were fully germinated.

**Mounting living root hairs**

Two-day-old seedlings were placed in Fahraeus medium at pH 6.0, as previously described (Cárdenas et al. 1995). After 8 h, root hairs were usually well adapted to the medium. Intact seedlings containing the growing root hairs were mounted in chambers constructed from large Petri dishes that had been perforated in the center and then covered with a large glass coverslip. The seedling was visualized under the microscope (Diaphot 300, Nikon) with a x 40/1 NA water immersion lens (Nikon).

**Treatment of root hair cells with the fluorescently labeled cytochalasin**

Briefly, the Cyt-Fl probe (Molecular Probes) was dissolved in dimethylsulfoxide (DMSO; Sigma) at 2.5 μM and centrifuged for 2 min at 7,000 r.p.m. to remove non-dissolved particles. Then, 4 μl of the dye was mixed with 1 ml of Fahraeus medium (Fahraeus 1957) and added to the plate, replacing the original Cyt-Fl-free medium. After 15 min, the medium was replaced with new Fahraeus medium without Cyt-Fl. This procedure was performed carefully, to avoid any mechanical stress to the cell. When needed for pseudoratiometric analysis, the cell tracker red AM dye was used at a concentration of 20 μM. First, the cell tracker red was ester loaded for 15 min, and then the Cyt-Fl was introduced under the same conditions. After 25 min, the root hair cells were ready for analysis as described below.

**Incubation of root hairs with Nod factors**

*Rhizobium etli* NFs were purified by HPLC and applied to plant roots as previously described (Cárdenas et al. 1995). Before treatment, NFs were mixed with 0.5 ml of the same medium in which the seedlings had been growing and were then gently added to the growing root hairs to replace the NF-free medium. The final concentration of NFs in the medium was 10\(^{-8}\) M. As a negative control, 10\(^{-8}\) M penta-N-acetylchitopentaose (Seikagaku America) dissolved in CHAPS (non-denaturing, zwitterionic detergent [3-(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate) was used.

**Pseudoratiometric analysis of F-actin plus ends in root hairs**

Briefly, cell tracker red (Invitrogen), a reference dye that is freely distributed in the cytoplasm, was introduced into the cell. This fluorescent probe does not change in response to environmental conditions and is used to measure the accessible volume of the cells. A second dye, Cyt-Fl, was used to determine the subcellular distribution of actin microfilament plus ends. Using MetaMorph (Molecular Dynamics) and a fast filter wheel (lambda 10-2, Sutter Instruments), cell tracker red

**Materials and Methods**

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was excited at 555 nm and Cyt-Fl at 484 nm, and the emissions were collected at 613 and 520 nm, respectively, and independently. After background subtraction, the plus end-dependent signal was divided by the signal imparted by the reference dye.

**Image acquisition and processing**

All images were acquired with a CCD camera (Sensys, Roper Scientific) attached to a Nikon TE300 inverted microscope with a ×40/1 NA water immersion objective lens and operated with MetaMorph/MetaFluor software (Universal Imaging, Molecular Devices). Loaded cells were excited using a xenon illumination source (DG-4, Sutter Instruments), which contains a 175 W ozone-free xenon lamp (330–700 nm) and a galvanometer-driven wavelength switcher. Cells containing Cyt-Fl were excited at 484 nm and emission was collected at 530 nm (20 nm band pass). All filters used were from Chroma Technology, and image acquisition and analysis were carried out using MetaMorph/MetaFluor software (Universal Imaging, Molecular Devices). Finally, images were prepared for publication using Adobe Photoshop.

**Correlation analysis**

Transforms, e.g. log2 and LOESS smoothing of the data, were performed in the R computation environment (R Development Core Team 2013). The significance of correlation coefficients was tested using the approximation to the t-test (Clifford et al. 1989).

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


