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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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Regular Paper

Legume plants secrete signaling molecules called flavonoids into the rhizosphere. These molecules activate the transcription of rhizobial *nod* genes, which encode proteins involved in the synthesis of signaling compounds named Nod factors (NFs). NFs, in turn, trigger changes in plant gene expression, cortical cell dedifferentiation and mitosis, depolarization of the root hair cell membrane potential and rearrangement of the actin cytoskeleton. Actin polymerization plays an important role in apical growth in hyphae and pollen tubes. Using sublethal concentrations of fluorescently labeled cytochalasin D (Cyt-Fl), we visualized the distribution of filamentous actin (F-actin) plus ends in living *Phaseolus vulgaris* and Arabidopsis root hairs during apical growth. We demonstrated that Cyt-Fl specifically labeled the newly available plus ends of actin microfilaments, which probably represent sites of polymerization. The addition of unlabeled competing cytochalasin reduced the signal, suggesting that the labeled and unlabeled forms of the drug bind to the same site on F-actin. Exposure to *Rhizobium etli* NFs resulted in a rapid increase in the number of F-actin plus ends in *P. vulgaris* root hairs and in the re-localization of F-actin plus ends to infection thread initiation sites. These data suggest that NFs promote the formation of F-actin plus ends, which results in actin cytoskeleton rearrangements that facilitate infection thread formation.

Keywords: Fluorescently labeled cytochalasin D • Infection thread • Nodulation • Root hair • Tip growth.

Abbreviations: ADF, actin-depolymerizating factor; Cyt-Fl, fluorescently labeled cytochalasin D; F-actin, filamentous actin; IT, infection thread; Jas, jasplakinolide; LatB, latrunculin B; LOESS, locally weighted scatterplot smoothing; NF, nodulation factor; ROS, reactive oxygen species.

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^-, Ca^{2+} and H^+) exchanges, cytoplasmic alkalinization, Ca^{2+} oscillations and gene expression (Cárdenas et al. 2000, Lhuissier et al. 2001), that facilitate bacterial invasion and nodule formation. In addition, actin microfilaments and Ca^{2+} 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).

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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). Numerous 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+}] [e.g. 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 wild-type 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)

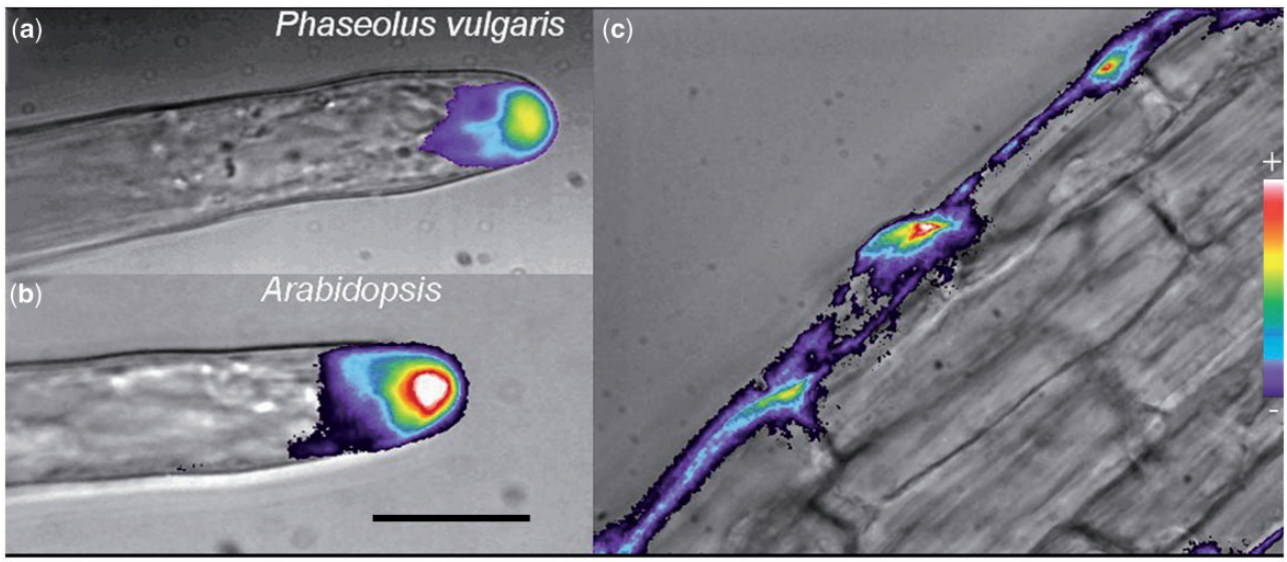

Fig. 1 *Phaseolus vulgaris* and *Arabidopsis thaliana* root hairs were treated with Cyt-Fl (50 nM). Fluorescence and DIC overlay of a (a) *P. vulgaris* and (b) Arabidopsis root hair, showing a similar distribution of microfilament plus ends. (c) The site from which a root hair is about to emerge exhibits a strong fluorescence signal. $n = 20$. Scale bar represent 20 micrometers.

(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

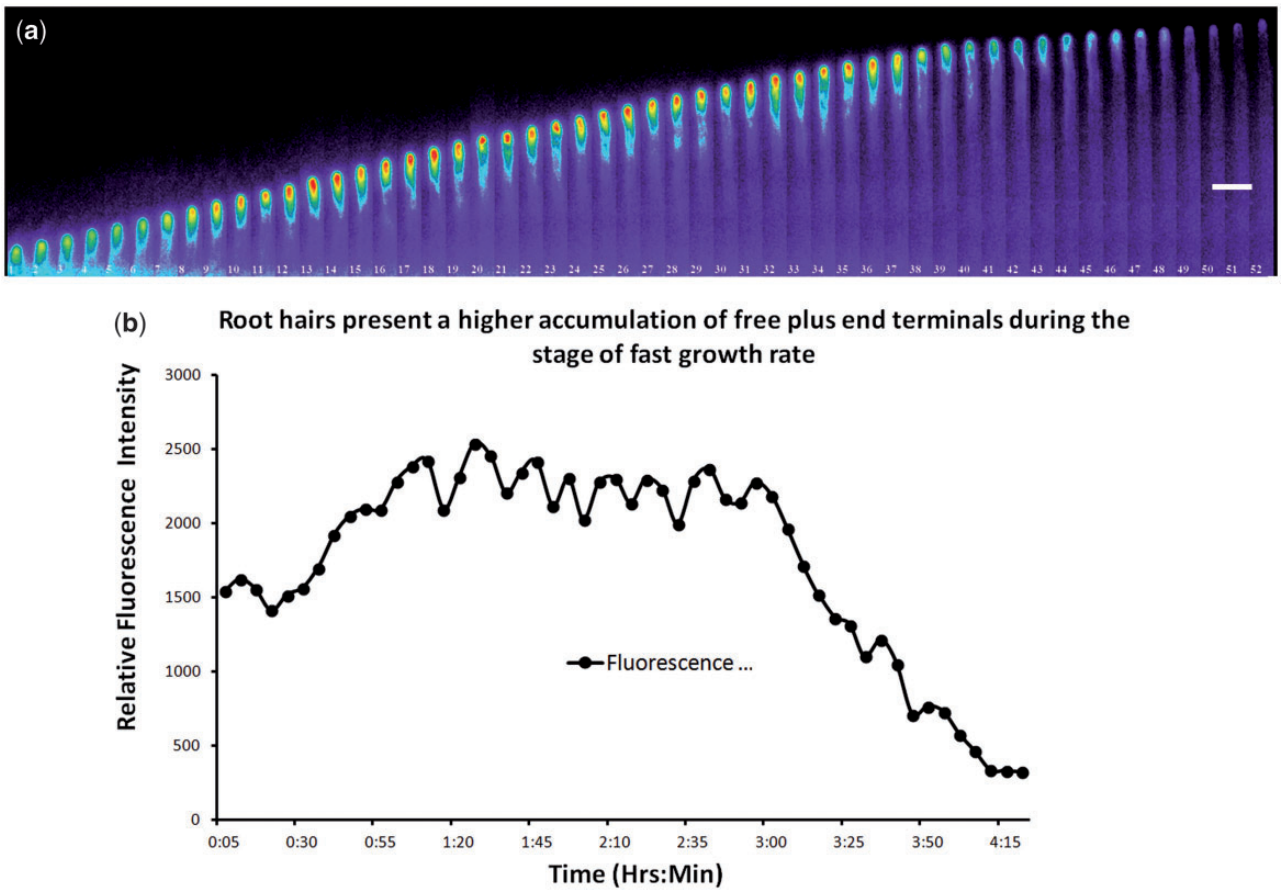

Fig. 2 Apical distribution of microfilament plus ends in Arabidopsis root hairs correlates with the different stages of root hair growth. (a) Note that the intensity of fluorescence is low during the initiation of root hair tip growth; however, the intensity increases significantly when the root hair 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.

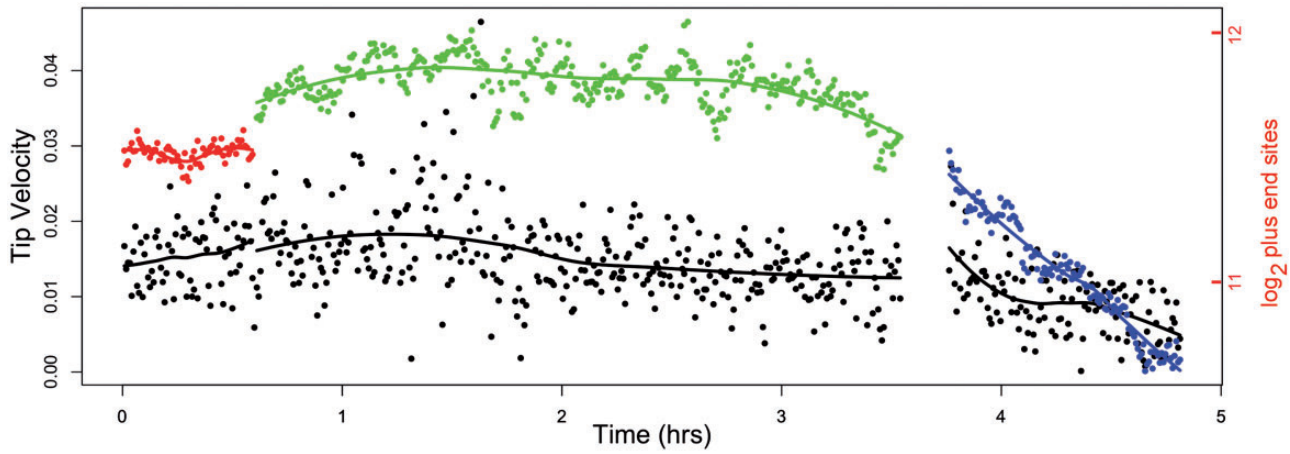

Fig. 3 Tip growth and plus end intensity over time. LOESS estimates were computed separately for early, middle and late data to remove large phasic variation, and plotted as thick lines.

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 $\log_2$ 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

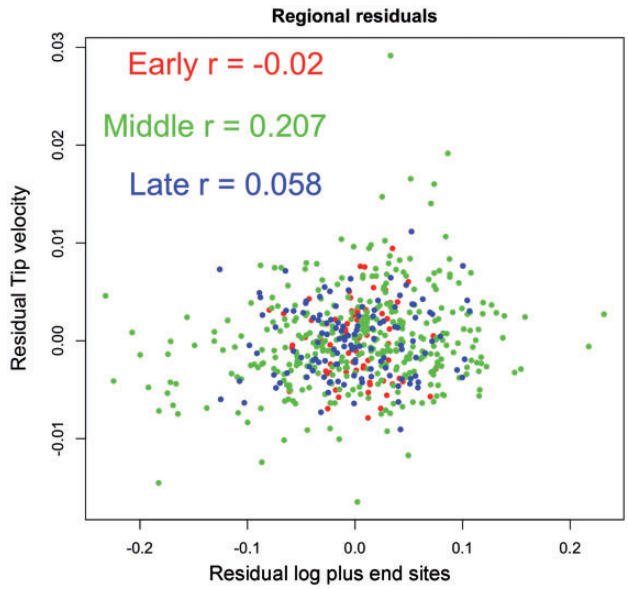

Fig. 4 Residual variability of growth is plotted against residual variability of plus end intensity for early (red), middle (green) and late (blue) root hair tip growth. The computed correlation coefficients are listed with their significance (ns = not significant at the 0.05 level; *** = very highly significant at the 0.001 level).

(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

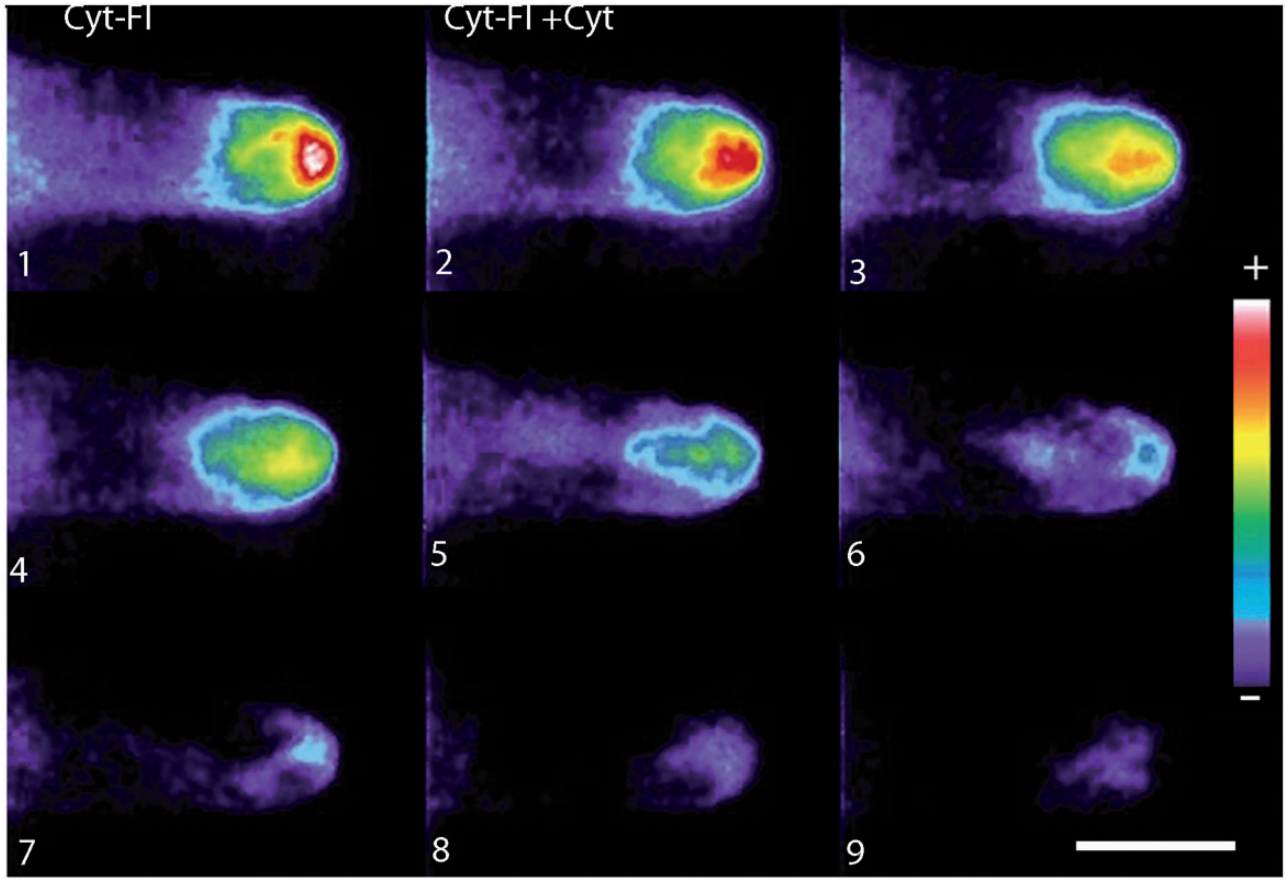

Fig. 5 Arabidopsis root hairs treated with Cyt-Fl (50 nM) and then with unlabeled cytochalasin D (50 nM). A root hair cell was treated with Cyt-Fl for 5 min (image 1) and then with unlabeled cytochalasin, and images were recorded every 15 s (image 2 onwards). Note that the fluorescence decreased after 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.

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**).

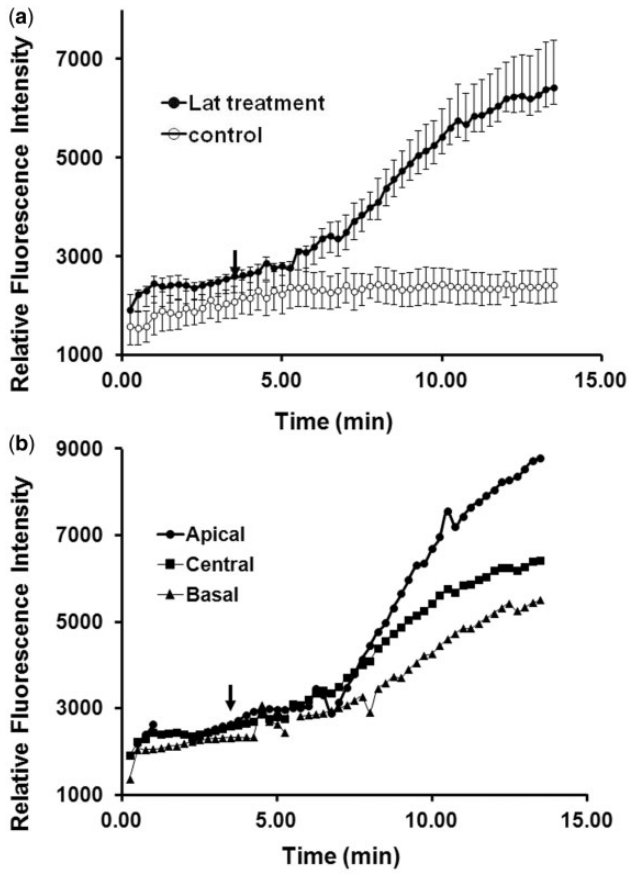

Fig. 6 Arabidopsis root hairs treated with Cyt-Fl and then with LatB exhibit increased signal at the sites of actin polymerization. (a) Filled circles depict an increased signal in the tip region in response to LatB and the open circles show control conditions with no LatB treatment. (b) Increased signal in response to LatB in various regions within the root hair cell, including the tip (circles), central part (squares) and base (triangles). Black arrows $t = 0$, $n = 20$.

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

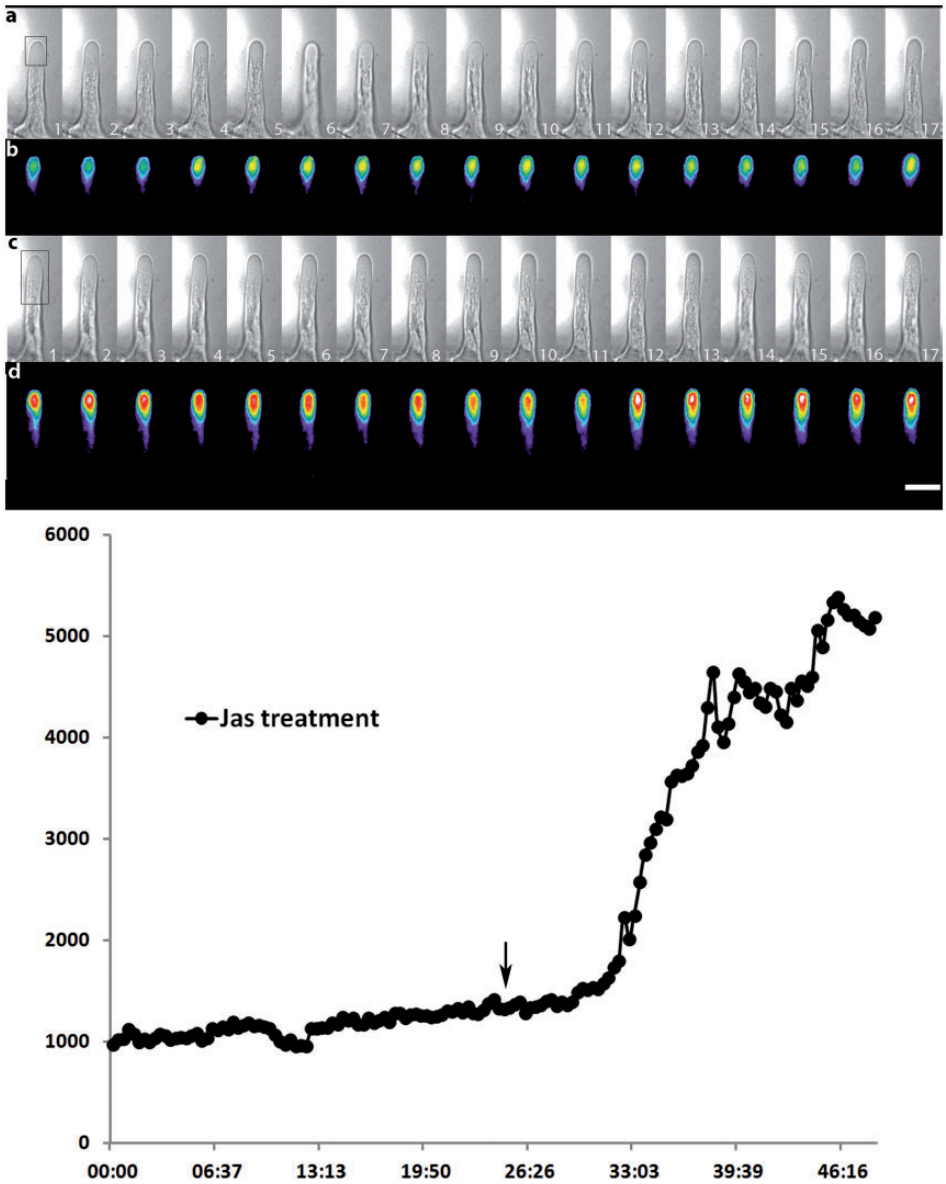

Fig. 7 The number of F-actin plus ends increases in Arabidopsis root hairs treated with Jas. Root hairs were pre-treated for 15 min with Cyt-Fl (50 nM) before treatment with Jas (2 μM). Then the accumulated fluorescence signal was measured not only in the tip but also beyond the tip dome. Upper two rows (a and b), transmitted light and fluorescence images of Cyt-Fl-loaded root hairs before treatment with Jas (control). Lower two rows (c and d), fluorescence and transmitted light images after Jas treatment. Following Jas treatment, the signal was stronger than in control conditions. Furthermore, cytoplasmic accumulation increased in the tip dome after Jas treatment, as depicted in the transmitted light and fluorescence images (see rectangle at time 1). Images were acquired at 3 s intervals. $n = 5$. Bottom, graphical representation of the fluorescence signal. Arrow indicates the addition of Jas. Scale bar represent 20 micrometers.

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).

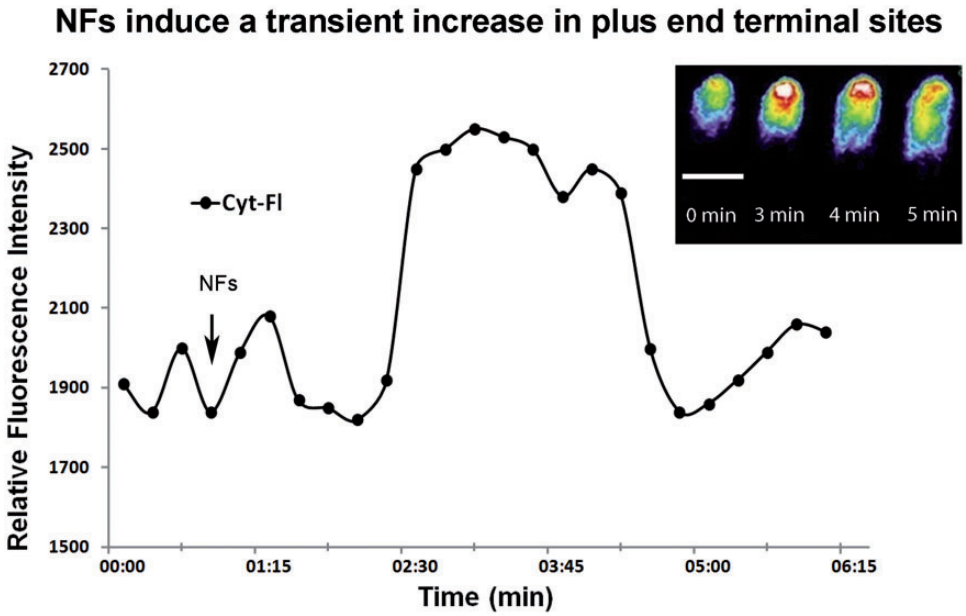

Fig. 8 Root hairs from *P. vulgaris* treated with Cyt-Fl and then challenged with NFs from *R. etli* show an increase in the number of F-actin plus ends. Inset: the fluorescence signal in the apical region expands within 5 min of treatment, and then returns to initial levels. $t = 0$ corresponds to the 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.

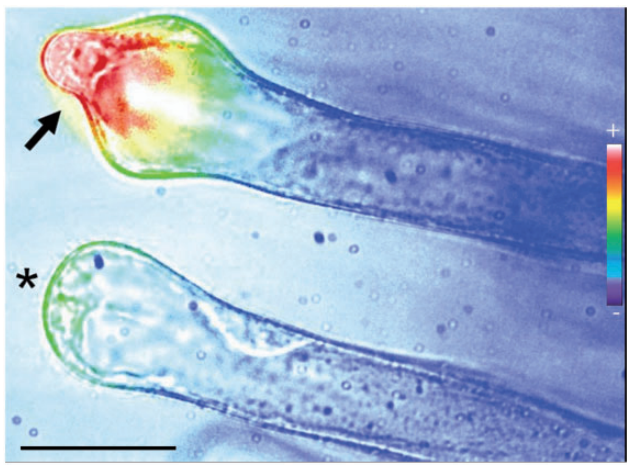

Fig. 9 F-actin plus ends are essential for polar growth. Root hairs from *P. vulgaris* were treated with Cyt-Fl and then with NFs. At 1 h after treatment with NFs, root hairs swelled up and, 1 h later, growth was re-initiated from some of the swollen cells. Swollen root hairs exhibited a low fluorescence signal (green asterisk), while root hairs that were re-initiating growth (arrow) displayed increased fluorescence (red). $n = 5$. Scale bar represents 20 micrometers.

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

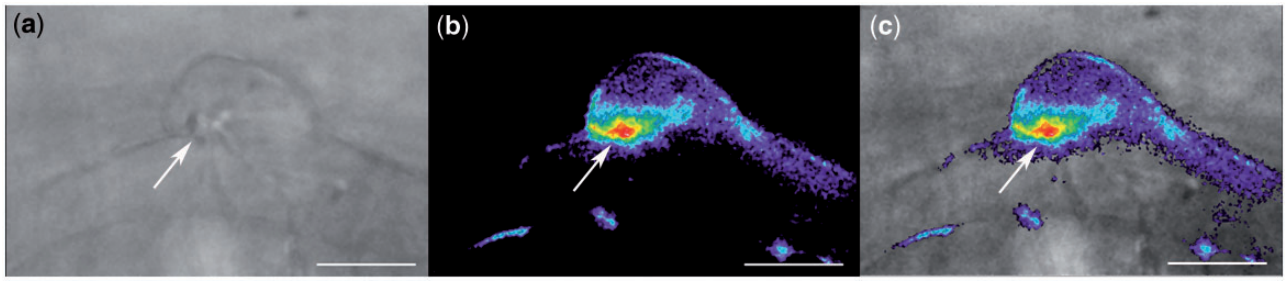

Fig. 10 The distribution of F-actin plus ends is reorganized in root hairs containing an initiated IT. *Phaseolus vulgaris* root hair cells infected with *R. etli* give rise to ITs, and actin polymerization sites are redistributed to the point of *Rhizobium* entry into the cell (arrows). This reorganization occurs within the first 72 h of infection. $n = 30$. (a) transmitted light, (b)fluorescence image and (c) merged image. Scale bar represents 20 micrometers.

microfilaments in the tip of root hairs (de Ruijter et al. 1999, Miller et al. 1999, Miller et al. 2000, Yokota et al. 2009). The role of the actin cytoskeleton in the infection process is also supported by studies in root hairs using 1 μM cytochalasin, which inhibits actin polymerization, and suppresses the morphological changes in root hair cells induced by NFs (Miller et al. 1999). At later stages of rhizobial infection, diffuse actin microfilaments are associated with the surface of the IT and with sites of IT penetration across cell junctions and cell plate-like structures (Davidson and Newcomb, 2001).

It has recently been reported using mutations in *M. truncatula* and *L. japonicus NAP1* and *PIR1*, both SCAR/WAVE complex components that impair the rhizobia–legume symbiosis, that these proteins are required for appropriate root hair, trichome and IT growth (Yokota et al. 2009, Miyahara et al. 2010). This is an interesting finding, since the ARP2/3 complex nucleates actin polymerization in all eukaryotes, and ARP2/3 is activated by the SCAR/WAVE complex, which in turn is activated by ROP-type small GTPases (Hussey et al. 2006). It has been proposed that *NAP1* directs F-actin reorganization to ensure appropriate polar growth of the IT (Miyahara et al. 2010). The role of the SCAR/WAVE complex in the control of membrane dynamics and vesicle trafficking has been well documented (Schafer 2002, Fricke et al. 2009). Therefore, it is possible that the *NAP1* and *PIR1* members of the SCAR/WAVE complex function during NF-induced reorientation of root hair growth 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 $\log_2$ 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. isototropic 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 glycosylphosphatiylinositol (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.

Materials and Methods

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 ×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

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. $\log_2$ 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

Arrighi, J.F., Godfroy, O., de Billy, F., Saurat, O., Jauneau, A. and Gough, C. (2008) The RPG gene of Medicago truncatula controls Rhizobium-directed polar growth during infection. *Proc. Natl Acad. Sci. USA* 105: 9817–9822.

Augustine, R.C., Vidali, L., Kleinman, K.P. and Bezanilla, M. (2008) Actin depolymerizing factor is essential for viability in plants, and its phosphoregulation is important for tip growth. *Plant J.* 54: 863–875.

Baluska, F., Salaj, J., Mathur, J., Braun, M., Jasper, F., Samaj, J. et al. (2000) Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol.* 227: 618–632.

Blanco, F.A., Meschini, E.P., Zanetti, M.E. and Aguilar, O.M. (2009) A small GTPase of the Rab family is required for root hair formation and preinfection stages of the common bean–Rhizobium symbiotic association. *Plant Cell* 21: 2797–2810.

Braun, M., Hauslage, J., Czogalla, A. and Limbach, C. (2004) Tip-localized actin polymerization and remodeling, reflected by the localization of ADF, profilin and villin, are fundamental for gravity-sensing and polar growth in characean rhizoids. *Planta* 219: 379–388.

Brewin, N. (2004) Plant cell wall remodelling in the Rhizobium–legume. *Crit. Rev. Plant Sci.* 23: 293–316.

Cárdenas, L., Dominguez, J., Quinto, C., Lopez-Lara, I.M., Lugtenberg, B.J., Spaink, H.P. et al. (1995) Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from Rhizobium etli. *Plant Mol. Biol.* 29: 453–464.

Cárdenas, L., Holdaway-Clarke, T.L., Sanchez, F., Quinto, C., Feijo, J.A., Kunkel, J.G. et al. (2000) Ion changes in legume root hairs responding to Nod factors. *Plant Physiol.* 123: 443–452.

Cárdenas, L., Lovy-Wheeler, A., Wilsen, K.L. and Hepler, P.K. (2005) Actin polymerization promotes the reversal of streaming in the apex of pollen tubes. *Cell Motil. Cytoskel.* 61: 112–127.

Cárdenas, L. and Quinto, C. (2008) Reactive oxygen species (ROS) as early signals in root hair cells responding to rhizobial nodulation factors. *Plant Signal. Behav.* 3: 1101–1102.

Cárdenas, L. (2009) New findings in the mechanisms regulating polar growth in root hair cells. *Plant Signal Behav.* 4: 4–8.

Cárdenas, L., Vidali, L., Domínguez, J., Pérez, H., Sánchez, F., Hepler, P.K. et al. (1998) Rearrangement of actin microfilaments in plant root hairs responding to *Rhizobium etli* nodulation signals. *Plant Physiol.* 116: 871–877.

Clifford, P., Richardson, S. and Hémon, D. (1989) Assessing the significance of the correlation between two spatial processes. *Biometrics* 45: 123–134.

Chen, C.Y., Cheung., A.Y. and Wu, H.M. (2003) Actin-depolymerizing factor mediates Rac/Rop GTPase-regulated pollen tube growth. *Plant Cell* 15: 237–249.

Chen, C.Y., Wong, E.I., Vidali, L., Estavillo, A., Hepler, P.K., Wu, H.M. et al. (2002) The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. *Plant Cell* 14: 2175–2190.

Davidson, A.L. and Newcomb, H. (2001) Changes in actin microfilament arrays in developing pea root nodule cells. *Can. J. Bot.* 79: 767–776.

de Ruijter, N.C.A., Bisseling, T. and Emons, A. (1999) Rhizobium Nod factors induce an increase in sub-apical fine bundles of actin filaments in Vicia sativa root hairs within minutes. *Mol. Plant Microbe Interact.* 12: 829–832.

Fahraeus, G. (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J. Gen. Microbiol.* 16: 374–381.

Fan, X., Hou, J., Chen, X., Chaudhry, F., Staiger, C.J. and Ren, H. (2004) Identification and characterization of a Ca^{2+}-dependent actin filament-severing protein from lily pollen. *Plant Physiol.* 136: 3979–3989.

Fischer, U., Men, S. and Grebe, M. (2004) Lipid function in plant cell polarity. *Curr. Opin. Plant Biol.* 7: 670–676.

Fisher, R.F. and Long, S.R. (1992) Rhizobium–plant signal exchange. *Nature* 357: 655–660.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A. et al. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422: 442–446.

Fournier, J., Timmers, A.C., Sieberer, B.J., Jauneau, A., Chabaud, M. and Barker, D.G. (2008) Mechanism of infection thread elongation in root hairs of Medicago truncatula and dynamic interplay with associated rhizobial colonization. *Plant Physiol.* 148: 1985–1995.

Fricke, R., Gohl, C., Dharmalingam, E., Grevelhorster, A., Zahedi, B., Harden, N. et al. (2009) *Drosophila* Cip4/Toca-1 integrates membrane trafficking and actin dynamics through WASP and SCAR/WAVE. *Curr. Biol.* 19: 1429–1437.

Gao, X.Q., Wang, X.L., Ren, F., Chen, J. and Wang, X.C. (2009) Dynamics of vacuoles and actin filaments in guard cells and their roles in stomatal movement. *Plant Cell Environ.* 32: 1108–1116.

Gibbon, B.C., Kovar, D.R. and Staiger, C.J. (1999) Latrunculin B has different effects on pollen germination and tube growth. *Plant Cell* 11: 2349–2363.

Hable, W.E., Miller, N.R. and Kropf, D.L. (2003) Polarity establishment requires dynamic actin in fucoid zygotes. *Protoplasma* 221: 193–204.

He, X., Liu, Y.M., Wang, W. and Li, Y. (2006) Distribution of G-actin is related to root hair growth of wheat. *Ann. Bot.* 98: 49–55.

Hepler, P.K., Vidali, L. and Cheung, A.Y. (2001) Polarized cell growth in higher plants. *Annu. Rev. Cell Dev. Biol.* 17: 159–187.

Hussey, P.J., Ketelaar, T. and Deeks, M.J. (2006) Control of the actin cytoskeleton in plant cell growth. *Annu. Rev. Plant Biol.* 57: 109–125.

Jones, M.A., Raymond, M.J. and Smirnoff, N. (2006) Analysis of the root-hair morphogenesis transcriptome reveals the molecular identity of six genes with roles in root-hair development in Arabidopsis. *Plant J.* 45: 83–100.

Ketelaar, T., de Ruijter, N.C. and Emons, A.M. (2003) Unstable F-actin specifies the area and microtubule direction of cell expansion in Arabidopsis root hairs. *Plant Cell* 15: 285–292.

Lhuissier, F., De Ruijter, N., Sieberer, B., Esseling, J.J. and Emons, A. (2001) Time course of cell biological events evoked in legume root hairs by Rhizobium Nod factors: state of the art. *Ann. Bot.* 87: 289–302.

Lloyd, C.W., Pearce, K.J., Rawlins, D.J., Ridge, R.W. and Shaw, P.J. (1987) Endoplasmic microtubules connect the advancing nucleus to the tip of legume root hairs, but F-actin is involved in basipetal migration. *Cell Motil. Cytoskel.* 8: 27–36.

McKenna, S.T., Kunkel, J.G., Bosch, M., Rounds, C.M., Vidali, L., Winship, L.J. et al. (2009) Exocytosis precedes and predicts the increase in growth in oscillating pollen tubes. *Plant Cell* 21: 3026–3040.

Miller, D.D., de Ruijter, N.C.A., Bisseling, T. and Emons, A.M. (1999) The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. *Plant J.* 17: 141–154.

Miller, D.D., Leferink-ten Klooster, H.B. and Emons, A.M. (2000) Lipochito-oligosaccharide nodulation factors stimulate cytoplasmic polarity with longitudinal endoplasmic reticulum and vesicles at the tip in vetch root hairs. *Mol. Plant Microbe Interact.* 13: 1385–1390.

Miyahara, A., Richens, J., Starker, C., Morieri, G., Smith, L., Long, S. et al. (2010) Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in Medicago truncatula. *Mol. Plant Microbe Interact.* 23: 1553–1562.

Monshausen, G.B., Bibikova, T.N., Messerli, M.A., Shi, C. and Gilroy, S. (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs. *Proc. Natl Acad. Sci. USA* 104: 20996–21001.

Montiel, J., Nava, N., Cardenas, L., Sanchez-Lopez, R., Arthikala, M.K., Santana, O. et al. (2012) A *Phaseolus vulgaris* NADPH oxidase gene is required for root infection by Rhizobia. *Plant Cell Physiol.* 53: 1751–1767.

Münter, S., Enninga, J., Vazquez-Martinez, R., Delbarre, E., David-Watine, B., Nehrbass, U. et al. (2006) Actin polymerisation at the cytoplasmic face of eukaryotic nuclei. *BMC Cell Biol.* 7: 1–13.

Nutman, P.S. (1959) Some observations on root-hair infection by nodule bacteria. *J. Exp. Bot.* 10: 250–263.

Pappas, C., Bhattacharya, N., Cooper, J. and Gregorio, C. (2008) Nebulin interacts with CapZ and regulates thin filament architecture within the Z-disc. *Mol. Biol. Cell* 19: 1837–1847.

Peleg-Grossman, S., Volpin, H. and Levine, A. (2007) Root hair curling and Rhizobium infection in Medicago truncatula are mediated by phosphatidylinositide-regulated endocytosis and reactive oxygen species. *J. Exp. Bot.* 58: 1637–1649.

R Development Core Team (2013) Manual of R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Sangiorgio, V., Pitto, M., Palestini, P. and Masserini, M. (2004) GPI-anchored proteins and lipid rafts. *Ital. J. Biochem.* 53: 98–111.

Schafer, D.A. (2002) Coupling actin dynamics and membrane dynamics during endocytosis. *Curr. Opin. Cell Biol.* 14: 76–81.

Schindelman, G., Morikami, A., Jung, J., Baskin, T.I., Carpita, N.C., Derbyshire, P. et al. (2001) COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. *Genes Dev.* 15: 1115–1127.

Shaw, S.L. and Long, S.R. (2003) Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. *Plant Physiol.* 131: 976–984.

Sieberer, B.J., Timmers, A.C., Lhuissier, F.G. and Emons, A.M. (2002) Endoplasmic microtubules configure the subapical cytoplasm and are required for fast growth of Medicago truncatula root hairs. *Plant Physiol.* 130: 977–988.

Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K. and Dolan, L. (2008) Local positive feedback regulation determines cell shape in root hair cells. *Science* 319: 1241–1244.

Torralba, S., Raudaskoski, M., Pedregosa, A.M. and Laborda, F. (1998) Effect of cytochalasin A on apical growth, actin cytoskeleton organization and enzyme secretion in Aspergillus nidulans. *Microbiology* 144: 45–53.

van Brussel, A.A., Bakhuizen, R., van Spronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J. et al. (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. *Science* 257: 70–72.

Vidali, L., McKenna, S.T. and Hepler, P.K. (2001) Actin polymerization is essential for pollen tube growth. *Mol. Biol. Cell* 12: 2534–2545.

Wang, Y.F., Fan, L.M., Zhang, W.Z., Zhang, W. and Wu, W.H. (2004) Ca^{2+}-permeable channels in the plasma membrane of Arabidopsis pollen are regulated by actin microfilaments. *Plant Physiol.* 136: 3892–3904.

Wasteneys, G.O. and Galway, M.E. (2003) Remodeling the cytoskeleton for growth and form: an overview with some new views. *Annu. Rev. Plant Biol.* 54: 691–722.

Wymer, C.L., Bibikova, T.N. and Gilroy, S. (1997) Cytoplasmic free calcium distributions during the development of root hairs of Arabidopsis thaliana. *Plant J.* 12: 427–439.

Yang, Z. (1998) Signaling tip growth in plants. *Curr. Opin. Plant. Biol.* 1: 525–530.

Yokota, K., Fukai, E., Madsen, L.H., Jurkiewicz, A., Rueda, P., Radutoiu, S. et al. (2009) Rearrangement of actin cytoskeleton mediates invasion of lotus japonicus roots by Mesorhizobium loti. *Plant Cell* 21: 267–284.