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The Dynamic Pollen Tube Cytoskeleton: Live Cell Studies Using Actin-Binding and Microtubule-Binding Reporter Proteins

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ABSTRACT Pollen tubes elongate within the pistil to transport sperm cells to the embryo sac for fertilization. Growth occurs exclusively at the tube apex, rendering pollen tube elongation a most dramatic polar cell growth process. A hallmark pollen tube feature is its cytoskeleton, which comprises elaborately organized and dynamic actin microfilaments and microtubules. Pollen tube growth is dependent on the actin cytoskeleton; its organization and regulation have been examined extensively by various approaches, including fluorescent protein labeled actin-binding proteins in live cell studies. Using the previously described GFP-NtADF1 and GFP-LlADF1, and a new actin reporter protein NtPLIM2b-GFP, we re-affirm that the predominant actin structures in elongating tobacco and lily pollen tubes are long, streaming actin cables along the pollen tube shank, and a subapical structure comprising shorter actin cables. The subapical collection of actin microfilaments undergoes dynamic changes, giving rise to the appearance of structures that range from basket- or funnel-shaped, mesh-like to a subtle ring. NtPLIM2b-GFP is used in combination with a guanine nucleotide exchange factor for the Rho GTPases, AtROP-GEF1, to illustrate the use of these actin reporter proteins to explore the linkage between the polar cell growth process and its actin cytoskeleton. Contrary to the actin cytoskeleton, microtubules appear not to play a direct role in supporting the polar cell growth process in angiosperm pollen tubes. Using a microtubule reporter protein based on the microtubule end-binding protein from *Arabidopsis* AtEB1, GFP-AtEB1, we show that the extensive microtubule network in elongating pollen tubes displays varying degrees of dynamics. These reporter proteins provide versatile tools to explore the functional connection between major structural and signaling components of the polar pollen tube growth process.

INTRODUCTION

Plants rely on a dramatic polar cell growth process—pollen tube elongation within pistil tissues—to transport sperm cells to the ovules for fertilization (Hepler et al., 2001; Lord and Russell, 2002; Cheung and Wu, 2008). Pollen grains on the stigma extrude a polar outgrowth from a germination pore to form a pollen tube. The pollen tube penetrates the extracellular matrix of stigmatic and stylar tissues and elongates basally by a tip growth process whereby cell expansion occurs only at the tube apex. As the tube apex extends away from the pollen grain, callose is deposited periodically behind the migrating tube front, thus compartmentalizing the pollen cytoplasm, the tube nucleus and two sperm cells to the most proximal segment of the pollen tube. In many species, pollen tubes extend for centimeters within the pistil, sometimes even much longer (e.g. in the maize silk), before reaching the ovules for fertilization, resulting in a tubular structure whose length is thousands of times that of the diameter of the grain or of the width of the tube.

The cellular basis of this polar cell growth process has been extensively studied in in-vitro pollen tube growth cultures, in particular those of tobacco and lily because of their robust growth properties. Elongating pollen tubes show a highly

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polarized cytoplasmic organization (Steer and Steer, 1989; Derksen et al., 1995; Hepler et al., 2001; Cheung and Wu, 2007, 2008). Readily noticeable is the almost exclusive segregation of a large density of transport vesicles to the tube apex, giving rise to the appearance of an apical domain with a relatively smooth cytoplasm. This organelle-free cytoplasm spans the apical dome and converges at about 5–15 μm distal from the tube apex, such as in tobacco and lily pollen tubes, respectively, to form an inverted cone-shaped region, referred to as the 'clear zone'. Subtending the inverted cone is a cytoplasm enriched in the more granular-appearing, metabolic, and secretory organelles. The cytoplasm is continuously engaged in a rapid streaming pattern, referred to as reverse fountain cytoplasmic streaming. Cellular contents are transported apically along the cortical cytoplasm until the subapical region subtending the clear zone, where their transport is reversed and they are trafficked distally in the core of the tube. Numerous studies in chemically fixed and living pollen tubes reveal an elaborate cytoskeleton comprising extensive networks of long actin cables and microtubules throughout the shank of the tube. They are largely aligned with the long axis of the tube, reaching the subapical region but not readily observable within the apical dome (see below). A prominent actin structure comprising shorter actin cables is consistently observed in the subapical region, but is variably referred to as a ring or a collar (Kost et al., 1998; Gibbon et al., 1999; Fu et al., 2001), a mesh (Geitmann et al., 2000; Chen et al., 2002), a funnel- or basket-like structure (Vidali et al., 2001; Hormanseder et al., 2005), and a fringe (Lovy-Wheeler et al., 2005). These seemingly variant structures would seem to suggest a structure that is constantly in flux and highly sensitive to the constantly fluctuating cytoplasmic conditions; or they may reflect a highly fragile structure easily perturbed by fixation or binding by actin reporter proteins, rendering an accurate representation difficult. Inhibitor studies show that the polar cell growth process in angiosperm pollen tubes is dependent on the actin cytoskeleton whereas actin and microtubule interactions are important for gymnosperm pollen tube growth (Anderhag et al., 2000).

The rapidity, dramatic polar characteristic, and absolute dependence of the angiosperm pollen tube growth process on the actin cytoskeleton are the basis for an attractive cell system for studying actin organization and dynamics and examining how they may be correlated to cell growth. Many actin-binding proteins regulate different aspects of actin dynamics and architecture (Staiger, 2005; Ren and Xiang, 2007). Among these, the G-actin-binding protein profilin and actin depolymerizing factors (ADF) inhibits actin polymerization and stimulates depolymerization, respectively, are probably the best characterized. Microinjection of profilin and slight overexpression of ADFs from a transgene both induce severe disruption of the pollen tube actin cytoskeleton and inhibit growth (Vidali et al., 2001; Chen et al., 2002), suggesting properly regulated actin dynamics critically underlies the normal tip growth process. The level of nascent actin filament production is apparently also critically regulated, as slight increases in the actin nucleating protein formin disrupts the normal actin organization, induces supernumerary actin cables, pollen tube growth depolarization and arrest (Cheung and Wu, 2004). Increasing or reducing the level of gelsolin-like actin severing proteins, ABP29 and ABP41 from lily, results in growth inhibition and obliteration of the actin cytoskeleton (Fan et al., 2004; Xiang et al., 2007). Moreover, the activity of many of these actin-binding proteins is regulated by ionic conditions and lipid metabolites (Ren and Xiang, 2007). Thus, the pollen tube actin cytoskeleton and growth are subject to regulation by signaling pathways that directly or indirectly impact actin dynamics. Rho GTPases, referred to as RAC/ROPs, with their multiple signaling pathways that regulate Ca^{2+}, phosphoinosides and ADF activity are known to play critical roles in regulating pollen tube growth and polarity (Nibau et al., 2006; Yang and Fu, 2007; Kost, 2008).

An expedient pollen tube growth system that permits observation of the actin cytoskeleton under live cell conditions will immensely facilitate efforts to dissect in real time the intricate network of interacting elements that regulate the pollen tube actin cytoskeleton. Towards this end, several green fluorescent protein (GFP)-labeled actin-binding proteins, in particular a GFP-labeled actin-binding domain of mouse talin (GFP-mTalin) (Kost et al., 1998; Fu et al., 2001) and ADF from tobacco and lily (GFP-NtADF1 and GFP-LlADF1) (Chen et al., 2002), have been developed in the past decade. As the GFP-ADFs have been determined to be most tolerated by growing pollen tubes (Wilsen et al., 2006), we present here an overall description of the tobacco and lily pollen tube actin cytoskeleton as revealed by these reporter proteins based on reported and additional imaging data. Recent studies have identified a novel actin-binding protein, NtWLIM1, from tobacco (Thomas et al., 2006). Using a GFP labeled, pollen-specific LIM protein from tobacco, NtPLIM2b-GFP, we describe the use of this actin reporter protein in a study that examines the functional connection between a guanine nucleotide exchange factor Rho GTPases from *Arabidopsis* (Berken et al., 2005), AtROP-GEF1, polar tube growth, and the pollen tube actin cytoskeleton.

RESULTS

GFP-NtADF1 Reveals a Dynamic Actin Cytoskeleton in Elongating Tobacco Pollen Tubes

We have shown that the pollen tube actin cytoskeleton and polar cell growth process are highly sensitive to alterations in ADF level or activity (Chen et al., 2002). Growth is retarded in tobacco pollen tubes that have been transformed by microprojectiles coated with as little as 0.1 μg of Lat52-NtADF1 DNA. Transformation by 2.5 and 5 μg of this DNA reduces the average growth rates over a 5–8-h growth period to less than 20% that of control pollen tubes. On the other hand, pollen tubes transformed by 5 μg of Lat52-GFP-NtADF1 maintained an average growth rate of about 70–80% that of control tubes. GFP-NtADF1 decorates an elaborate actin cytoskeleton structure with long cables aligned with the tube axis in the shank and a prominent mesh- and basket-like structure at the

subapical region of these transformed pollen tubes (Chen et al., 2002, 2003; Feijo and Moreno, 2004; Moreno et al., 2006; Cheung and Wu, 2008). In fact, as little as 0.5–1 μg of Lat52-GFP-NtADF1 DNA used for transformation is adequate to reveal various actin structures in transformed tobacco pollen tubes (Figure 1; Cheung and Wu, 2004, 2008; Wilsen et al., 2006). Since the Ser6 residue in NtADF1 is important for its actin depolymerizing activity (Chen et al., 2002), fusion of a GFP at its N-terminus apparently has compromised this activity while preserving actin binding. The mitigated actin disassembling activity due to the N-terminal fusion thus permits the use of GFP-NtADF1 as an actin marker over a broad range of expression levels. A relatively large number of transformed pollen tubes (Figure 1A) with elongation properties approximating those in non-transformed or control GFP-expressing transformed pollen tubes, namely with growth rates of between 20 and 40 nm s^{-1} in GM, and ~40-70 nm/s in polyethylene glycol supplemented media, are routinely observed in each transformation sample over a growth period spanning 3–8 h from the start of growth cultures.

Transformed pollen tubes elongating at rates higher than 7–8 nm s^{-1} showed a range of GFP-NtADF1 labeling patterns (Figure 1B–1E). The least fluorescent tubes tend to have an entirely cytoplasmic GFP-NtADF1 labeling pattern (not shown). At higher expression levels, GFP-NtADF1 decorates almost exclusively a subapical mesh-like structure in some tubes (Figure 1C–1E), or a mesh and a basket- or funnel-shaped subapical structure together with long actin cables along the shank of some pollen tubes (Figure 1B). The subapical structure, whether more mesh-like or more basket-shaped, shows continuous changes in its location relative to the migrating tip, morphology, expanse, and density of actin cables. These changes are readily observable in images captured in time series for elongating pollen tubes (Figure 1E; Supplemental Movie 1E; see also Chen et al., 2002; Cheung and Wu, 2004; Feijo and Moreno, 2004; Wilsen et al., 2006; Moreno et al., 2006; Cheung and Wu, 2008). Medial sections of pollen tubes with a denser subapical structure show that it spans the width of the tube cytoplasm and is evidently maintained in time series (Figure 1E). When whole tube projections are rotated to provide a more frontal view of the tube, actin cables can also be seen across much of the cross-section of the tube (Figure 1B and 1D). Rotated views of less prominent subapical structures reveal a subtle ring structure, with denser actin cables around the cortex than in the center of the tube (Figure 1C, upper panel). Longitudinal sections nonetheless reveal presence of actin cables across the width of these tubes (e.g. in the 4-μm section of Figure 1C, lower panel). In pollen tubes in which a higher density of shank actin cables is revealed than in the tube shown here, the subapical short actin cables become less discernable but appear more as a continuum with the long cables (see, e.g. Figure 2H in Cheung and Wu, 2007). Notably, in these live cell observations, short actin cables, whilst they evidently exist, usually do not persist along the apical periphery in elongating pollen tubes.

GFP-LlADF1 Reveals Prominent Subapical Actin Mesh and Basket in Elongating Lily Pollen Tubes

Lily pollen tubes are almost twice the width of tobacco pollen tubes and elongate at rates of 200–300 nm/s (see Hepler et al., 2001), making them particularly attractive for studies in which microinjections are involved (e.g. see Holdaway-Clarke and Hepler, 2003). Previous studies using 5 μg of Zmc13-GFP-LlADF1 DNA for transformation showed transformed lily pollen tubes with a prominent subapical mesh, sometimes subtended by actin cables, yielding also a basket-shaped structure (Chen et al., 2002). A subsequent study reiterated the earlier observations of a GFP-LlADF1-labeled subapical structure across the width of medial sections in transformed tubes (Wilsen et al., 2006).

The substantially larger cytoplasmic volume in lily pollen tubes requires higher levels of actin marker proteins to efficiently reveal more of the actin cytoskeleton beyond the subapical collection of microfilaments. To determine if GFP-LlADF1 may be expressed to a high enough level to provide a more complete view of the lily pollen tube actin cytoskeleton, we bombarded lily pollen grains by microprojectiles coated with either 7.5 or 10 μg of Zmc13-GFP-LlADF1 DNA. Both quantities of input DNA yielded acceptable results in that about 50% of the transformed pollen tubes elongating at rates within the growth rate range reported for wild-type lily pollen tubes show various extents of discernable GFP-labeled actin structures. A distribution profile of growth rates among these transformed pollen tubes observed from two independent bombardment samples is shown in Figure 2A. The growth rapidity of these pollen tubes is readily noticeable in the selected images within time series taken of these tubes (Figure 2B and 2C) and in Supplemental Movies 2B and 2C. Pollen tubes monitored to obtain the data shown in Figure 2A show a range of actin cytoskeleton labeling patterns. These include pollen tubes in which GFP-LlADF1 reveals only the subapical actin mesh (Figure 2B), similar to those reported previously (Chen et al., 2002; Wilsen et al., 2006). In others, the subapical structure and long actin cables in the shank can both be observed (Figure 2C and 2D). Rotated views of its subapical structure (Figure 2D, lower panel) and animation of its serial sections (Supplemental Movie 2D) show a subapical basket structure with a denser collection of actin cables closer to the cortex than in the core of the tube. Transformed pollen tubes elongating at rates close to and below 75 nm s^{-1} have more extensive GFP-LlADF1-labeled actin cables, some showing presence of actin cables in the apical dome cytoplasm. Thus, physiological studies in lily pollen tubes with a relatively normal GFP-actin-binding protein-decorated actin cytoskeleton and growth rates comparable to those observed in non-transformed controls are imminently possible.

NtPLIM2b-GFP, a New Reporter Protein for the Pollen Tube Actin Cytoskeleton

LIM proteins are a conserved family of eukaryotic proteins characterized by the common presence of what is referred

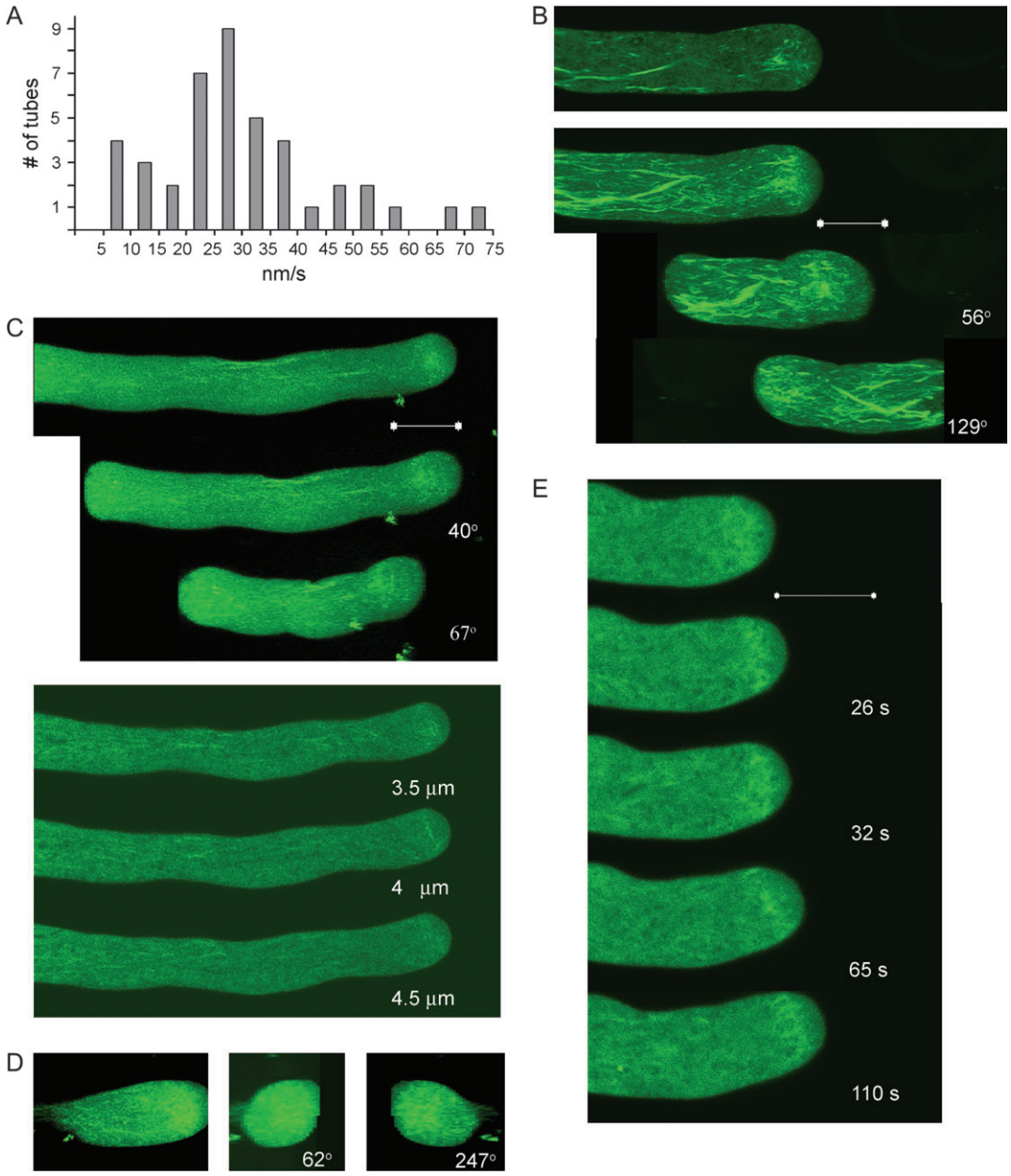

Figure 1. GFP-NtADF1 Reveals an Elaborate and Dynamic Actin Cytoskeleton in Elongating Tobacco Pollen Tubes.

(A) Growth rate distribution among pollen tubes expressing GFP-NtADF1 examined in one transient transformation experiment. Growth rates were obtained from measuring distances elongated by each pollen tube imaged at their medial planes over periods spanning ~250 to ~800 s, at either 5 or 10-s intervals. Forty-four pollen tubes were observed over a span of 3–9 h after bombardment. The distribution profile is typical of numerous experiments carried out using GFP-NtADF1 as an actin marker for different experimental purposes.

(B) A medial section (upper panel) and a whole tube projection (lower panel) of a transformed pollen tube with an extensive GFP-NtADF1-labeled actin cytoskeleton. The middle and lower images, in the whole tube projection panel, are rotated (degrees rotated clockwise are as indicated) from the axial view (upper image) to reveal a more frontal view of the tube, showing short actin cables spanning the region across the proximal pollen tube cytoplasm. Similarly, serial sections of the Z-stack (Supplemental Movie 1B) reveal short actin cables spanning the width of cytoplasm at the subapical region. The pollen tube was observed at ~5.5 h after bombardment. Arrow indicates a basket-like subapical actin structure; arrowhead indicates a mesh-like structure.

(C) (Top panel) An axial (upper) and two rotated (middle and lower) views of a whole tube projection of a pollen tube showing a moderately labeled subapical mesh-like structure. (Bottom panel) Three consecutive optical sections from the medial region of the tube reveal subtle but evident presence of short GFP-NtADF1-labeled cables spanning the subapical cytoplasm. The pollen tube was observed at ~4.5 h after bombardment.

(D) An axial (left panel) and two rotated (middle and right panels) views of a transformed pollen tube with a prominently labeled subapical actin mesh. Short actin cables spanning the width of tube are evident in the rotated views. The pollen tube was observed at ~6 h after bombardment.

to as the LIM domain—a cysteine-histidine-rich, zinc-finger-containing domain (Eliasson et al., 2000; Arnaud et al., 2007). They are found in the cytoplasm as well as the nucleus (Arnaud et al., 2007). While a clear biological role for LIM proteins remains to be revealed, a GFP-labeled tobacco vegetative cell-expressed LIM protein, GFP-WLIM1, has been shown to associate prominently with the actin cytoskeleton in BY2 cells and in *N. benthamiana* leaf cells (Thomas et al., 2006). We isolated a cDNA (NtPLIM2b) from a *N. tabacum* pollen cDNA library corresponding to a LIM protein that shares high levels of homology with another tobacco pollen-expressed LIM, NtPLIM2 (Eliasson et al., 2000) (see Supplemental Figure 1). A sunflower pollen-specific promoter, SF3, was used to drive the expression of NtPLIM2b-GFP in transformed tobacco plants. More than 20 transgenic plants were obtained, with at least 10 among these expressing readily detectable levels of NtPLIM2b-GFP by protein blots (not shown) or microscopic imaging (Figure 3). None of the transformed plants showed noticeable fertility defects, suggesting in-vivo pollen tube growth is quite tolerant of NtPLIM2b-GFP.

In-vitro-grown transformed pollen tubes reveal extensive NtPLIM2b-GFP-labeled long filamentous structures reminiscent of the actin cytoskeleton (Figure 3A). Co-localization with Texas-red conjugated phalloidin in chemically fixed transformed pollen tubes (Figure 3B) confirms that GFP-NtPLIM2b indeed associates with F-actin in these cells. Furthermore, the NtPLIM2b-GFP labeling pattern is obliterated rapidly upon treatment of elongating pollen tubes with the G-actin sequester latrunculin b (Figure 3C). These stably transformed NtPLIM2b-GFP-expressing pollen tubes elongate rapidly in vitro (Figure 3D; Supplemental Movie 3D), showing actively streaming actin cables along the shank and a discernable subapical actin structure that is more frequently basket-shaped than mesh-shaped. Whole tube projection of serial sections of these transformed tubes also shows prominent NtPLIM2b-GFP labeling of the shank actin—a subtle but still evident subapical structure (Figure 3A). Stable transformed pollen tubes expressing N-terminal fused GFP-NtPLIM2b show similar labeling patterns, although fluorescence signal tends to be weaker than in tubes expressing the C-terminal fused NtPLIM2b (unpublished observation).

Using NtPLIM2b-GFP as an Actin Reporter Protein in Sample Transient Pollen Transformation Experiments

A major goal of developing imaging markers for cellular components is providing the opportunity to assess how different experimental conditions may impact the marked cellular system under live cell conditions. When combined with transient expression systems, such as microprojectile bombardment-mediated pollen transformation, efforts to examine the functional integration of various components into specific cellular processes could be accomplished expediently. Here, we use NtPLIM2b-GFP in a series of experiments to illustrate the use of actin reporter proteins to examine how the pollen tube actin cytoskeleton responds to cellular perturbations in transient expression assays.

Average growth rates among pollen tube populations transformed by microprojectiles coated with different doses of SF3-NtPLIM2b-GFP DNA were determined initially to determine the best combination of DNA dosage, marker protein expression level and the effect of these actin-binding proteins on tip growth. This combination should permit a broad window of time for observation in normally elongating pollen tubes before optimum pollen tube growth properties begin to decline.

Using three different levels of SF3-NtPLIM2b-GFP DNA, we determined that transforming pollen with microprojectiles coated with 1.25 or 2.5 μg of DNA does not significantly affect pollen tube growth rates over a period of 3–6 h, while transformation by 5 μg of input transgenes results in obvious growth reduction (Figure 4A and 4B). Because of the inherent variability in the time an individual pollen grain takes to germinate and in pollen tube growth rates between pollen tubes in general, distribution profiles of the length of individual pollen tubes observed in this kind of experiment (Figure 4B) provide an assessment that more directly reflects the overall growth characteristic of these transformed cells. As seen in Figure 4B, the majority of control pollen tubes are between 100 and 700 μm long after 6 h of growth, while the bulk of pollen tubes transformed by 1.25 and 2.5 μg of SF3-NtPLIM2b-GFP DNA spans the range of between 100 and 600 μm. It is also evident that the majority of pollen tubes in the culture transformed by 5 μg of the transgene have not elongated much beyond 200 μm after 6 h growth. The small number of tubes in the culture transformed by 5 μg DNA that have attained longer lengths are substantially weaker in their fluorescence signals, thus permitting more rapid growth.

Based on these analyses, we routinely use between 1.5 and 2.5 μg of SF3-NtPLIM2b-GFP to coat microprojectiles for one transformation sample. Pollen tubes transformed by a higher amount of DNA produce observable fluorescent signal around 3 h after bombardment, while those transformed by lower doses of transgenes are optimum for observation at later hours after bombardment. In general and whenever comparison can be made with tubes developed from stable transformed pollen grains, transiently transformed pollen tubes begin to show higher levels of fluorescence by 2–3 h after bombardment.

(E) Selected images in a time series of a GFP-NtADF1-expressing pollen tube elongating at ~51 nm s^{-1}. The time series was taken at 5-s intervals over a 116.5-s period. A movie for this time series (Supplemental Movie 1E) shows morphological changes in the subapical structure during growth.

Pollen tubes were transformed by microprojectile bombardment and cultured in GM. 0.5-μm serial sections were obtained for the Z-stacks. Scale bars = 10 μm.

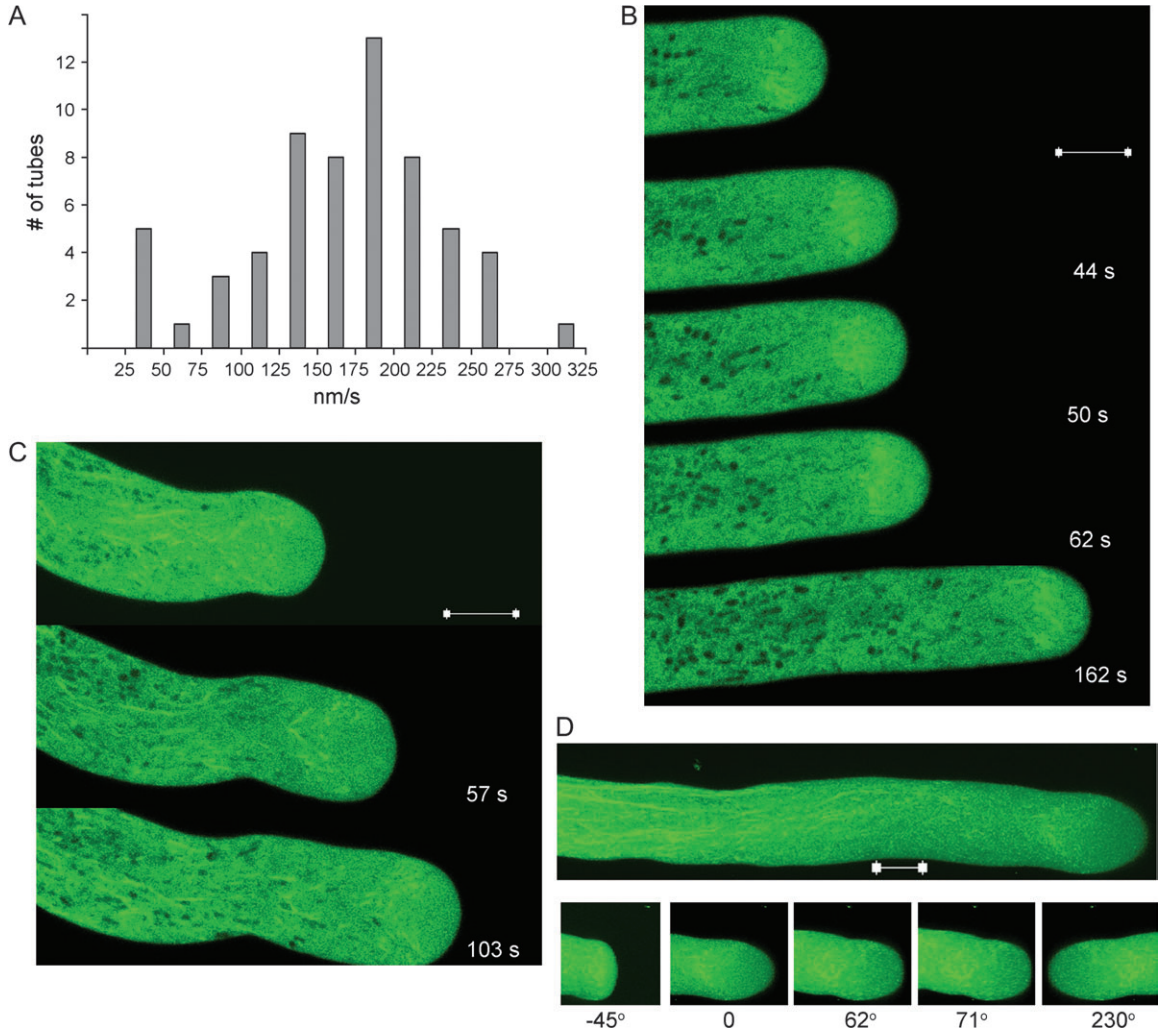

Figure 2. GFP-LlADF1 Reveals an Elaborate and Dynamic Actin Cytoskeleton in Elongating Lily Pollen Tubes.

(A) Growth rate distribution among pollen tubes expressing GFP-LlADF1 examined in two transient transformation samples. Growth rates were obtained from measuring distances elongated by each pollen tube imaged at their medial planes over periods of ~150 to ~510 s, at either 5 or 10-s intervals. Sixty-six pollen tubes were observed over the period of 3.5 to 8.5 h after bombardment.

(B) Selected sections from a time series of a GFP-LlADF1-expressing lily pollen tube showing a prominently labeled subapical mesh-like structure (arrow). The pollen tube was observed at 5 h after bombardment. The time series was taken at 5-s intervals over a 162-s period; the pollen tube was elongating at an average rate of 236 nm s^{-1}. A movie for this time series (Supplemental Movie 2B) shows obvious as well as subtle morphological changes in the subapical structure during growth.

(C) Selected sections from a time series of a GFP-LlADF1-expressing lily pollen tube with an evidently labeled subapical basket-like structure (arrowhead) as well as a network of long cables in the tube shank. The pollen tube was observed at 5.5 h after bombardment. The time series was taken at 10-s intervals over a 150-s period; the pollen tube was elongating at an average rate of 198 nm s^{-1}. A movie for this time series (Supplemental Movie 2C) shows constant morphological changes in the subapical structure during growth.

(D) A whole tube projection for the tube shown in (C) taken after the time series. The Z-stack was obtained from serial 0.6-μm steps. The lower panel shows rotated views of the tip region of the same tube. The axial view is designated as 0. Degrees rotated from 45° counter-clockwise to 230° clockwise from the axial view are as indicated. Serial sections of the Z-stack are shown in animation in Supplemental Movie 2D.

Pollen tubes were transformed by microprojectile bombardment and were cultured in LGM. Arrows indicate basket-like subapical actin structure; arrowhead indicates a mesh-like structure. Scale bars = 10 μm.

By 5–8 h, pollen tubes with a range of reporter protein expression levels would be found in these pollen cultures. Thus, observations of tobacco pollen tubes maintaining the normal pollen tube growth property and imageable levels of NtPLIM2b-GFP are possible during a span of at least 3–8 h after bombardment. Beyond 8 h, fewer tubes continue to grow with optimum properties; those that remain robustly growing can still be used for analysis.

Images shown in Figures 4C, 4D, and 5A are representative of at least half of the transformed pollen tube population

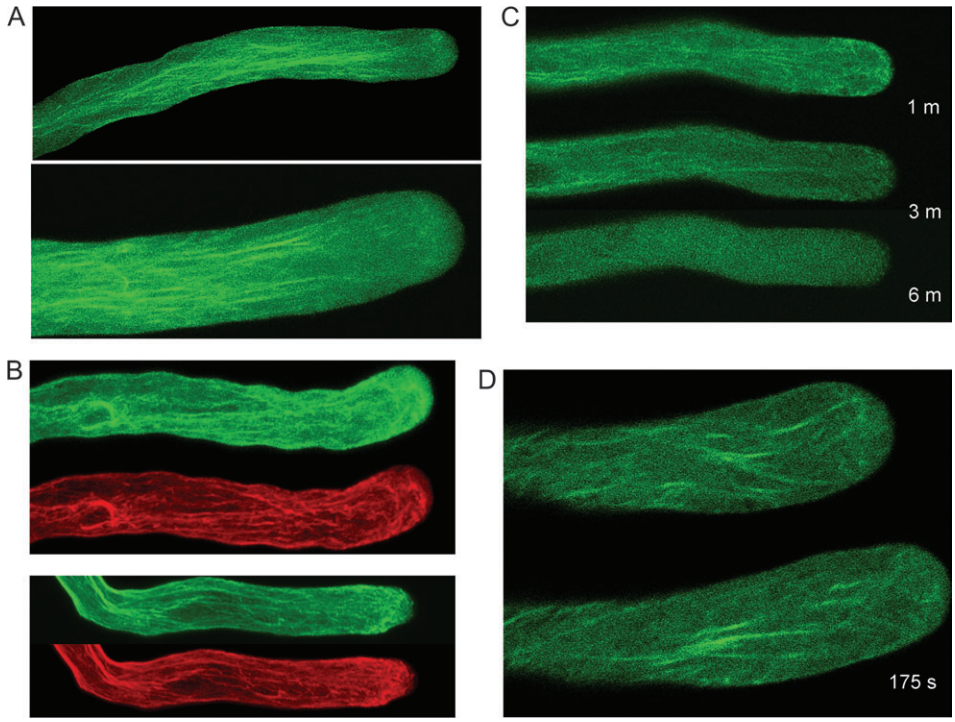

Figure 3. Actin Cytoskeleton Revealed by NtLIM2b-GFP in Stably Transformed Tobacco Pollen Tubes.

(A) Whole tube projections of two typical NtLIM2b-GFP-expressing pollen tubes. The tube shown in the lower panel was imaged after the time series shown in (D) was collected. Extensive long actin cables along the shank are prominently labeled. A subtle but evidently labeled subapical structure is also labeled.

(B) Whole tube projections of two NtLIM2b-GFP-expressing transformed tubes showing co-labeling by Texas red-phalloidin (upper) and NtLIM2b-GFP (lower).

(C) A medial section of a latrunculin b (250 nM)-treated NtLIM2b-GFP-expressing tube. The 1-m image was taken almost immediately after latrucunlin was added. The relatively high latrunculin b concentration was used to achieve rapid disassembly of actin cables. In studies in which growth and intracellular trafficking aspects are assessed, concentrations of between 12.5 and 50 nM of latrunculin b are preferred for tobacco pollen tubes (see, e.g. de Graaf et al., 2005).

(D) The first and last image of a time series of NtLIM2b-GFP-expressing pollen tubes elongating at 69 nm s^{-1}. The entire time series, taken at 10-s intervals between frames, is shown in Supplemental Movie 3D.

Z-stacks were taken at 1-μm steps. Pollen tubes were cultured in GM(P2%S). Arrows indicate basket-like subapical actin structure; arrowhead indicates a mesh-like structure. Scale bar = 10 μm.

routinely observed in one transformation sample that expresses an imageable level of NtPLIM2b-GFP. The NtPLIM2b-GFP-labeled patterns of actin cables in these transiently transformed pollen tubes are indistinguishable from those seen in stably transformed tubes (Figure 3), namely high density of actively streaming long actin cables along the shank. A subtle but discernable subapical structure is also present in some of the tubes (Figure 4C). These pollen tubes usually have elongated considerably at the time of observation, suggesting appreciable growth rates. In the specific examples shown here (e.g. Figure 4C and 4D; Figure 5A), the average growth rates for the pollen tubes shown are within the range normally observed for control tobacco pollen tubes.

Among hundreds of transiently transformed GFP-NtPLIM2b-expressing pollen tubes observed to date for different experimental purposes, a few (<10) show strong, sometimes intertwining, donut-shaped structures clustered in the cytoplasm (Figure 4E). These tubes are usually short and no longer elongating. The low frequency at which these defective pollen tubes occur and their obvious abnormality permit them to be readily recognizable as a defect and discounted from analysis.

Growth rate observations and the actin cytoskeleton revealed in NtPLIM2b-GFP-expressing tobacco pollen tubes reported here suggest it is an efficient actin reporter protein that is rather well tolerated by tip-growing pollen tubes. In a study that examines the functional role of a pollen-expressed LIM protein in lily, LlLIM1, a majority of microprojectile-mediated transformed pollen tubes with GFP-tagged LlLIM1 decorated actin cytoskeleton also maintains normal tube growth properties (Wang et al., 2008), suggesting it may also be a useful actin reporter protein in lily pollen tube live cell studies.

NtPLIM2b-GFP as a Reporter for the Actin Cytoskeleton in Functional Studies

To illustrate the versatility of live cell actin markers in functional studies, we show here a sample experiment using NtPLIM2b-GFP as the actin reporter and the Rac/Rop regulator

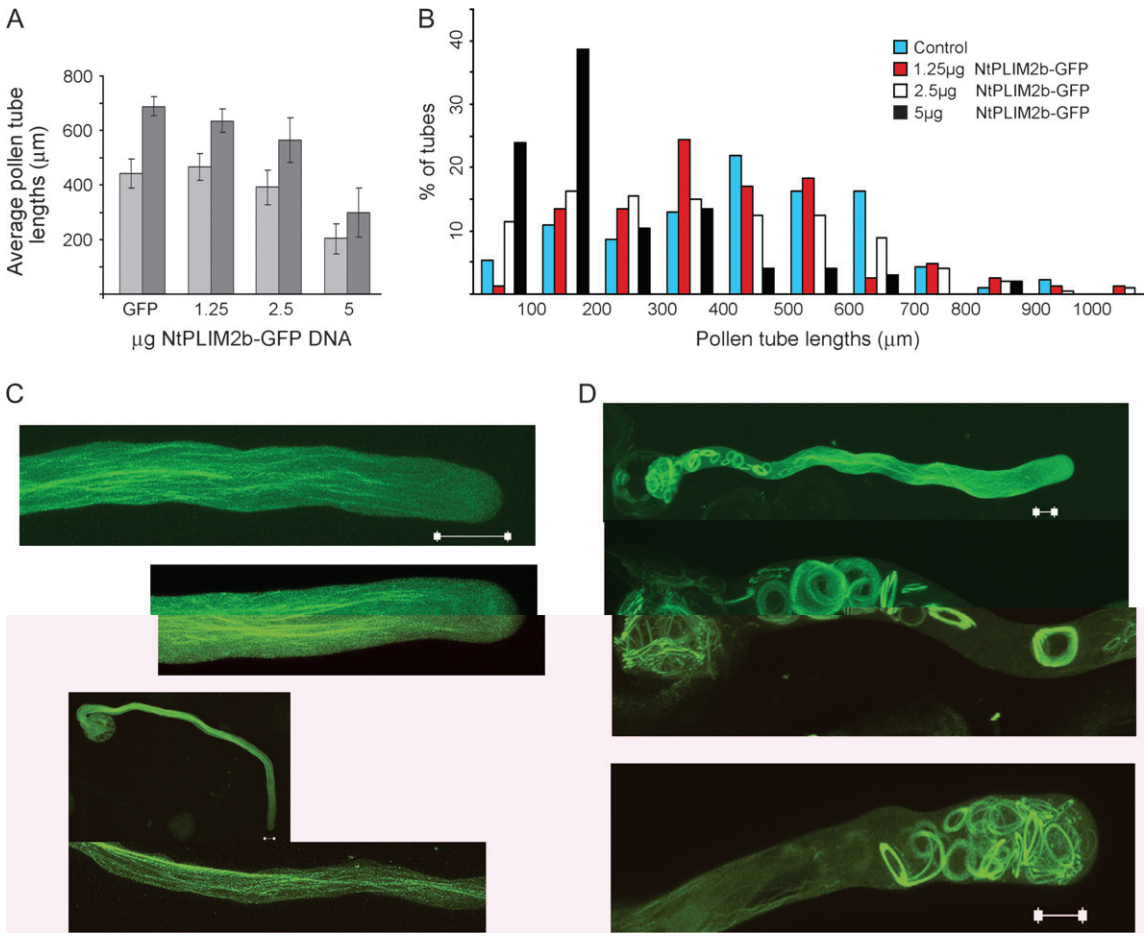

Figure 4. NtPLIM2b-GFP as an Actin Reporter Protein in Transiently Transformed Tobacco Pollen Tubes.

(A) Tube growth measurements for control (GFP-expressing) and NtPLIM2b-GFP-expressing pollen tubes transformed by microprojectiles coated with different amounts of SF3-NtPLIM2b-GFP DNA as indicated. Triplicate bombardments were made for each condition. Pollen tubes were cultured in GM, and aliquots were imaged by epifluorescence at the indicated hours after bombardment; their lengths were subsequently measured. Because of inherent differences among pollen grains in the time they take to germinate irrespectively of experimental manipulations, pollen tubes shorter than three grain lengths in the 3-h sample (light gray data bar) and those shorter than five grain lengths in the 6-h sample (dark gray data bar) were not included in the measurements. Average pollen tube lengths were calculated independently for each triplicate. Data bars shown are averages of the average pollen tube lengths calculated from the three triplicates (*n* = at least 25 tubes in each of the triplicates).

(B) Pollen tube length distribution profiles for control (GFP-expressing) and NtPLIM2b-GFP-expressing pollen tubes cultured for 6 h in GM(P5%S) after bombardment. Data were collected from one bombardment experiment. Image collection and tube length measurements were as described above. All pollen tubes observed were included in the distribution profiles. Numbers of tubes analyzed were 92, 82, 192, and 96, respectively for the control, 1.25, 1.5, and 5 μg SF3-NtPLIM2b-GFP transformed pollen tube samples.

(C, D) Whole tube projections of three NtPLIM2b-expressing pollen tubes showing prominent long actin cables in the shank and a subtle subapical mesh-like structure. Pollen tubes were transformed with microprojectiles coated with 2 μg (C) or 2.5 μg (D) of SF3-NtPLIM2b-GFP DNA. The NtPLIM2b-GFP-labeling patterns are typical of normally elongating pollen tubes among the transformed population, which routinely accounts for 40–60% of the observed samples during an imaging period of between 3 and 8 h after bombardment under similar culture conditions. Long actin cables can be seen extending throughout the proximal (C) and the distal (D, lower panel) regions of the tubes. Top and bottom images in (D) are of different magnifications to display the overall length of the tube and the NtPLIM2b-GFP-labeling pattern in the distal region of the tube. The pollen tube, imaged at 3 h after bombardment, was approximately 310 μm long, reflecting an average growth rate of ~1.73 μm min^{-1} (~28.7 nm s^{-1}) over the entire growth period.

(E) Two pollen tubes showing mild pollen tube depolarization and with NtPLIM2b-GFP-labeling pattern dominated by donut-shaped species anomalous to known actin structures in pollen tubes. The pollen tube in the upper panel of (E) is shown in two magnifications. We only observed two pollen tubes showing these elaborate ring structures (at the most, 2% of the transiently SF3-NtPLIM2b-GFP-transformed pollen tubes observed by CLSM thus far under different experimental manipulations). They have not been observed in stably transformed pollen tubes expressing this marker protein or in tubes transiently expressing other actin reporter genes we have used so far.

Scale bars = 10 μm.

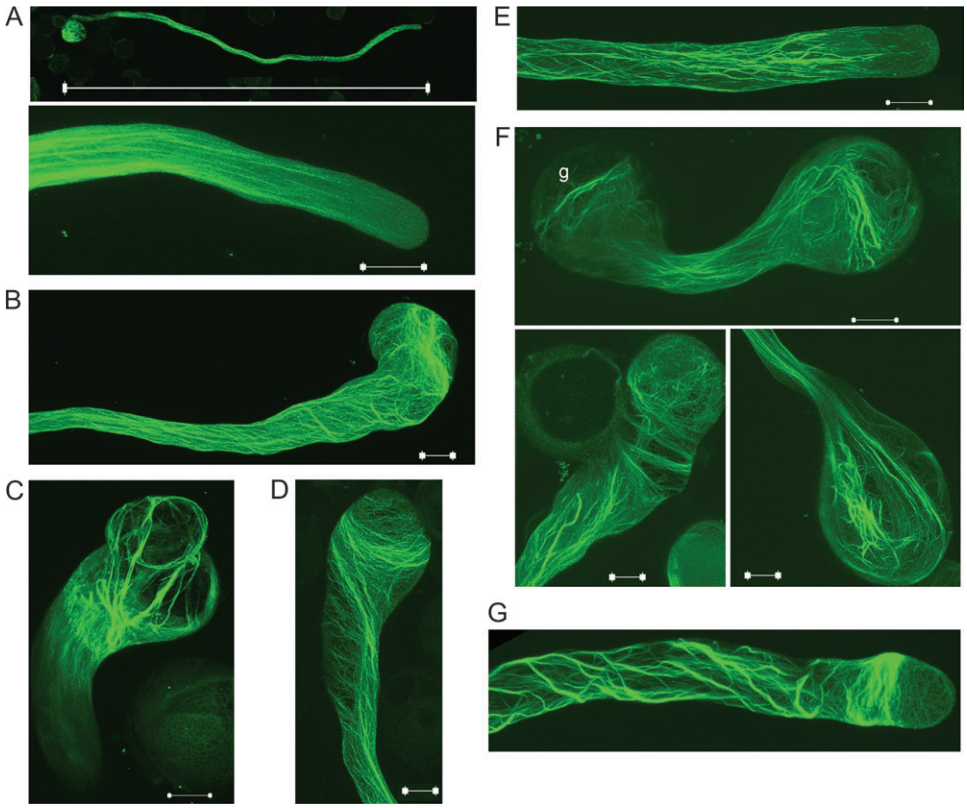

Figure 5. NtPLIM2b-GFP Reveals AtROP-GEF1-Induced Deformation of Actin Cytoskeleton in Pollen Tubes.

(A) A control transformed pollen tube expressing NtPLIM2b-GFP shown in two magnifications. This pollen tube was imaged at 4.5 h after bombardment; its length (~515 μm) reflects an average growth rate of ~1.92 μm min^{-1} (~32 nm s^{-1}).

(B–D) Pollen tubes co-transformed by SF3-NtPLIM2b-GFP and Lat52-AtROP-GEF1 expressing the respective actin marker proteins and AtROP-GEF1. The disrupted actin cytoskeleton revealed by NtPLIM2b-GFP is characteristic of all the transformants that displayed the effect of augmented activities in the Rho GTPase signaling pathway.

(E) A control transformed pollen tube expressing GFP-mTalin.

(F) Representative pollen tubes co-transformed by Lat52-GFP-mTalin and Lat52-AtROP-GEF1.

(G) A pollen tube representative of a prevalent class of GFP-mTalin-expressing pollen tubes.

Pollen was transformed by 2.5 μg of SF3-NtPLIM2b-GFP (A) and, together with 0.625 μg of Lat52-AtROP-GEF1 (B-D), or 1 μg of Lat52-GFP-mTalin (E, G), and together with 0.625 μg of Lat52-AtROP-GEF1 (F), and cultured in GM(P5%S). Pollen tubes were observed between 4 and 6 h after bombardment. Scale bar in (A) = 500 μm; others = 10 μm.

AtROP-GEF1, which has been shown to regulate pollen tube growth in transformed pollen tubes (Gu et al., 2006). Rac/Rop GTPases are known to play critical roles in regulating the actin cytoskeleton in elongating pollen tubes and maintaining tip growth (Kost et al., 1999; Fu et al., 2001; Chen et al., 2003). ROP-GEFs, as guanine nucleotide exchange factors, would activate the Rac/Rop signaling pathway and, when overexpressed, induce pollen tube depolarization (Gu et al., 2006; Zhang and McCormick, 2007). AtROP-GEF1 is apparently a highly active GEF, as even bombardment by microprojectiles coated by less than 1 μg of a Lat52-AtROP-GEF1 results in deformed pollen tubes among almost 100% of all the transformed cells, exceeding the level achieved even in cultures that were transformed by 5 μg of Lat52-NtRac1 or constitutively active NtRac1 (Chen et al., 2002). The NtPLIM2b-GFP- and AtROP-GEF1-coexpressing tubes reveal evidently that the normally highly organized actin cytoskeleton revealed by NtPLIM2b-GFP in control tubes (Figure 5A) is totally disrupted in these depolarized cells (Figure 5B and 5D). A majority of the depolarized pollen tubes have long and thick actin bundles extending into the entire apical cytoplasm or transverse bands of actin circling the subapical cortex. Many of the transformed tubes are short, often having extended less than five grain-lengths, when control GFP-NtLIM1b-expressing pollen tubes in the same experiment have in general extended more than 10 grain-lengths of distance (Figure 5A). The occasional longer, SF3-NtPLIM2b-GFP and Lat52-AtROP-GEF1 cotransformed tubes usually maintain a long and slender tube shank that broadens in the proximal region of the tube, which terminates in an abruptly expanded tip (Figure 5B), suggesting an initial phase of relatively normal growth characteristics until AtROP1-GEF1 accumulates to a prohibitive level. Growth depolarization and actin deformation observed in these transiently transformed pollen tubes are considerably more

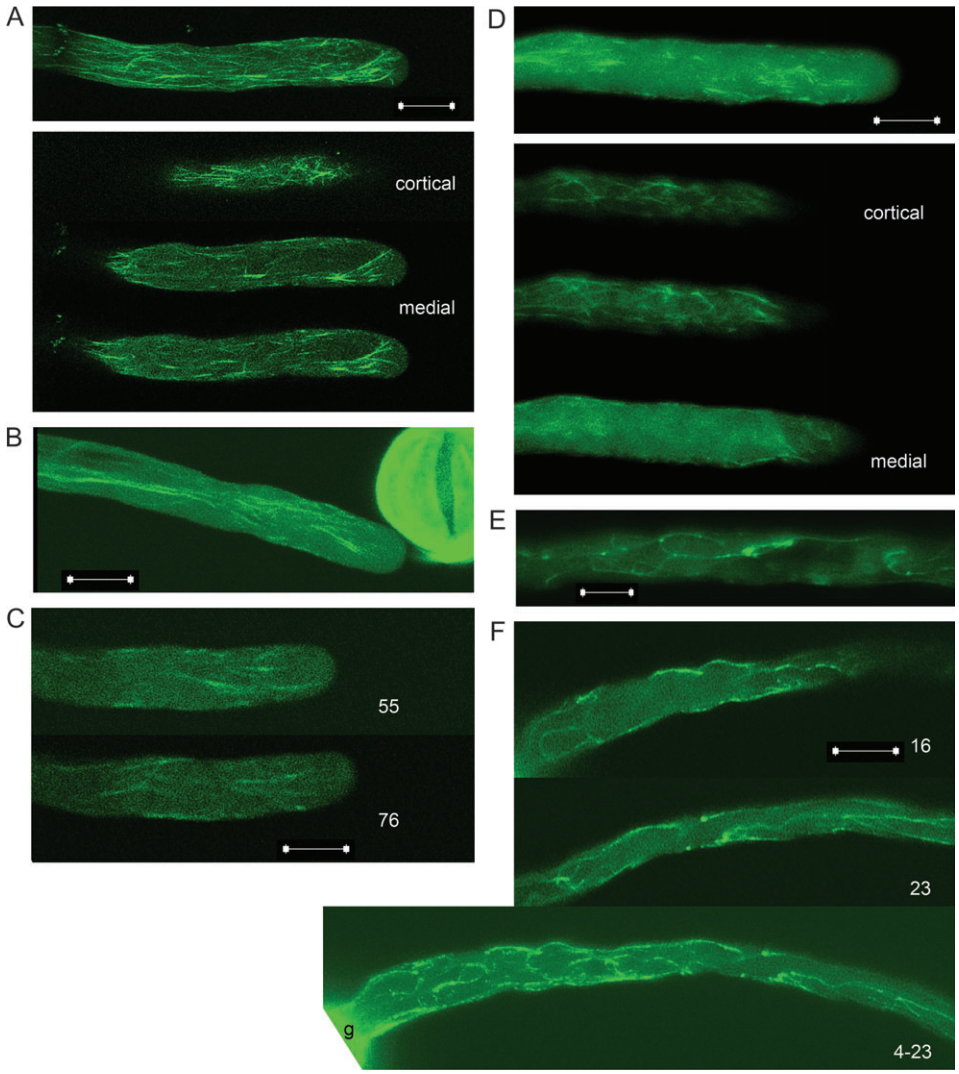

Figure 6. GFP-AtEB1 Reveals an Extensive and Dynamic Microtubule System in Elongating Tobacco Pollen Tubes.

(A) A whole tube projection (upper panel) and single optical sections (lower panel) from the cortical (upper) and medial region (middle and lower) of the proximal region of a pollen tube showing an extensive network of GFP-AtEB1-labeled microtubules throughout the pollen tube cytoplasm. A movie for the Z-stack is shown in Supplemental Movie 6A.

(B) Another example of a GFP-AtEB1-expressing pollen tube.

(C) (Top panel) The 55th and 76th images (upper and lower) of a time series taken from the medial plane at 5-s intervals of a GFP-AtEB1-expressing pollen tube. A movie of this time series is shown in Supplemental Movie 6C.

(D) A whole tube projection (upper panel) and images (lower panel) of single cortical sections (upper and middle) and a medial section (lower) of the proximal region of a pollen tube revealing net-like GFP-AtEB1-labeled cortex-associated microtubules but few microtubules spanning the central cytoplasm. A movie for the Z-stack is shown in Supplemental Movie 6D.

(E) A single optical section from the distal region showing a GFP-AtEB1-labeled net-like structure.

(F) (Top two panels) Single optical sections (16th and 23rd of a stack) from contiguous regions from the distal region a GFP-AtEB1-expressing pollen tube. (Lower panel) Maximum projection from the 4th to the 23rd optical sections from this stack showing an extensive cortical net-like microtubule structure.

Pollen was transformed with 2.5 (A) or 5 μg (B–F) of Lat52-GFP-AtEB1 and cultured for 2–4 h after bombardment. Pollen tubes shown in (B–F) were cultured in GM(6%S). Z-sections were taken at 1-μm steps; the time series in (C) was taken at 5-s intervals for 94 frames. Scale bars = 10 μm.

pronounced than those revealed in phalloidin-stained stable transformed pollen tubes that overexpressed a tomato ROP-GEF homolog (Kaothien et al., 2005), suggesting perhaps a modulating effect on these regulators in stably transformed pollen in order to preserve their viability.

The more established actin marker, GFP-mTalin (Kost et al., 1998; Fu et al., 2001), is also used in combination with AtROP-GEF1. In control cultures transformed by 1 μg of Lat52-GFP-mTalin, a third to half of the transformed tubes routinely can maintain a relatively normal morphology and show an

extensively decorated actin cytoskeleton comprising long actin bundles (Figure 5E) when observed between 3 and 5 h after bombardment. GFP-mTalin-labeled actin microfilaments are relatively sparse, or not observed, in the apical cytoplasm of these transformed tubes (Figure 5E). Coexpression of AtROP-GEF1 with GFP-mTalin induces severe depolarization and deformation of the actin cytoskeleton structure (Figure 5F), similar to those seen when NtPLIM2b-GFP is used as a marker. Pollen tubes with transverse actin bands encircling the bulbous subapical cortex (Figure 5D and 5F, upper panel) are the most prevalent among AtROP1-GEF1-induced anomaly. Transverse actin bands are also prevalent among Rac/Rop overexpressing and tip depolarized pollen tubes (Kost et al., 1999; Fu et al., 2001). In our hands, expression of GFP-mTalin alone is actually often associated with transformation of longitudinally aligned actins in the subapical region into transverse bands (Figure 5G; Wilsen et al., 2006). However, severe tip depolarization as those induced by ROP-GEFs or by Rac/Rops rarely occurs in pollen tubes that are transformed only by Lat52-GFP-mTalin, permitting unambiguous identification of phenotypes induced by regulators such as those in the Rac/Rop signaling pathways or other actin regulatory proteins (Cheung and Wu, 2004).

GFP-AtEB1 as a Reporter Protein for Microtubules in Elongating Pollen Tubes

Probably because of a lack of evidence for an essential role in the polar cell growth process in angiosperm pollen tubes, the dynamics of its extensive microtubule system have not received the same level of attention as the actin cytoskeleton. The plus end tracking microtubule binding protein AtEB1 has been shown to associate with what is believed to be microtubule plus ends as well as with stabilized microtubules in *Arabidopsis* epidermal cells (Mathur et al., 2003). When expressed in transiently transformed pollen tubes, GFP-AtEB1 reveals an extensive network of cables in patterns that, on casual observation, look very similar to that for the actin cytoskeleton (Figure 6A and 6B), namely long axially oriented cables in the shank and shorter cables that gather into a basket-shaped structure in the core cytoplasm in the subapical region. However, close inspection of serial images in time series (Figure 6C; Supplemental Movie 6C) suggests microtubules in the core cytoplasm and along the cortex have distinguishable dynamic properties. In particular, microtubules that concentrate in the subapical region up till about 50–60 μm from the tube apex are dynamic, parallel to the growth axis and enter the apical dome in discrete episodes, as can be seen in the time series. On the other hand, in cortical microtubules, while also undergoing dynamic changes in that their lengths increase and decrease with time, their association with the cortex is relatively stable, as they remain in approximately the same location over time (see Supplemental Movie 6C). In the more posterior part of the tube, GFP-AtEB1-labeled cables are often not seen in the core cytoplasm. Instead, a net-like structure is prevalent along the cortex and appears immobile, suggesting possible attachment of cortical microtubules to the cell membrane (Figure 6B, 6E and 6F). A cortical net-like network along the subapical cortex is also evident in tubes that have fewer GFP-AtEB1-labeled cables in the anterior core cytoplasm (Figure 6D). It is also evident, especially in single optical sections, that GFP-AtEB1 also labels punctuate structures (Figure 6E and 6F), perhaps marking the microtubule plus ends. Moreover, GFP-AtEB1 also labels a prominent structure in longer pollen tubes, suggestive of the generative cell (not shown), whose signal often overwhelms observation of the cytoplasmic microtubules. Thus, the GFP-AtEB1-labeled microtubules are best observed in an early period, prior to 4 h, after bombardment.

DISCUSSION

GFP-Labeled Actin Reporter Proteins Reveal an Actin Cytoskeleton in the Shank of Elongating Pollen Tubes Compatible with a Role for Long-Range Intracellular Trafficking

That the actin cytoskeleton in elongating pollen tubes comprises an extensive network of polymerized actins with different degrees of architectural complexity is indisputable among the numerous studies that have examined this important cellular structure (Derksen et al., 1995; Hepler et al., 2001; Cheung and Wu, 2008). The extraordinary density and organized alignment of apparently long actin cables in the shank of the tube are best exemplified in the recent re-analysis of the pollen tube actin cytoskeleton by an improved rapid freeze-whole mount procedure, followed by immuno-staining and observation by CLSM (Lovy-Wheeler et al., 2005). Each of the previously reported GFP-labeled actin-binding proteins, GFP-mTalin (Kost et al., 1999; Fu et al., 2001), GFP-NtADF1, GFP-LlADF1 (Chen et al., 2002) and GFP-fimbrin (Wilsen et al., 2006), and NtPLIM2b-GFP described here reveals to different extents the shank collection of actin cables, with GFP-NtADF1 and NtPLIM2b-GFP displaying the dynamics of the system most dramatically and a reverse-fountain motility pattern for these GFP-labeled actin cables is readily observed. Together with studies that demonstrate the effect of inhibitors on actin dynamics on cytoplasmic streaming (see Hepler et al., 2001), the evidence supporting a dynamic network of actin cables generally paralleling to the long axis of the tube as the underlying structural elements that support the reverse fountain intracellular organelle trafficking activity is unequivocal. Contrary to the GFP-ADFs, the dynamics of the network distal to the subapical region revealed by GFP-mTalin appears to be rather subdued (Kost et al., 1999). While GFP-fimbrin reveals a shank actin density most similar to that seen in fixed pollen tubes, its strong propensity to induce growth and actin abnormality renders it the least useful (Wilsen et al., 2006). Thus, the GFP-ADFs and NtPLIM2b-GFP are probably actin markers described to date that may best facilitate analysis of organelle and actin interactions in live elongating pollen tubes.

A Subapical Actin Structure with Varying Morphology in Elongating Pollen Tubes

Prevalence of an actin mesh or basket-like structure in the subapical region of elongating pollen tubes (see Hepler et al.,

2001; and, e.g. Fossiner et al., 2002; Chen et al., 2002; Lovy-Wheeler et al., 2005), and an intimate relationship between the integrity of this structure and the tip growth process (Gibbon et al., 1999; Vidali et al., 2001), implicate important functional roles in how this actin microfilament collection contributes to the polar cell growth process. Moreover, multiple interlinking cellular features important to the tip growth process converge at the subapical region. The reversal of the intracellular trafficking pattern at the subapical region implies a high level of actin reconfiguration in that cytoplasmic domain. A fluctuating apical [Ca^{2+}] gradient and a subapical alkaline region (see Robinson and Messerli, 2002; Feijo et al., 2001; Holdaway-Clarke and Hepler, 2003) imply constant changes in ionic conditions that are known to impact on actin dynamics (Ren and Xiang, 2007). Vesicular trafficking activities are, in turn, impacted on to modulate the deposition of membrane-associated regulators of ion transport. Furthermore, nascent actin microfilaments assemble from the cell membrane around the apical domain (Cheung and Wu, 2004) and ADF is predominantly localized to the subapical cytoplasm, where pH conditions favor their activity (Chen et al., 2002; Lovy-Wheeler et al., 2007). These together support the notion of continuous and high levels of actin dynamics, assembly, disassembly, and modifications into higher-order configurations occurring in the apical and subapical cytoplasmic domain of elongating pollen tubes. The question is then whether one specific actin morphological structure, even undergoing continuous dynamic changes within, is adequate and responsible for driving the rapid tip growth process. Or, is the tip growth process rather supported by a collection of dynamic actin microfilaments that are constantly being assembled into and disassembled from different structural configurations during the growth process to provide the plasticity needed for rapid growth and directional orientation?

The fact that the subapical actin structure in pollen tubes has been described as structures that seem to be variations of a similar blueprint, a ring or collar (Kost et al., 1998; Gibbon et al., 1999; Fu et al., 2001), a mesh (Geitmann et al., 2000; Chen et al., 2002), a funnel- or basket-like structure (Vidali et al., 2001; Hormanseder et al., 2005), and a fringe or collar of microfilaments (Lovy-Wheeler et al., 2005), suggests perhaps a continuum of related structures each existing part of the time or interconverting from one to another during pollen tube growth. The most prevalent structures observed in live cells, particularly pollen tubes expressing GFP-ADFs and NtPLIM2b-GFP, range from being mesh-like and spanning across the subpical cytoplasm, to funnel- or basket-like with actin cables emanating from the apical flank and converging towards the subapical cytoplasm (Figures 1–4; Chen et al., 2002; Feijo and Moreno, 2004; Wilsen et al., 2006; Moreno et al., 2006; Cheung and Wu, 2008). These structures have varying densities of actin cables and often appear as spanning across the subapical cytoplasm, as suggested by their prominence in medial optical sections of elongating pollen tubes, and presence of short actin cables in the central cytoplasm in images of whole tube projections (Figure 1B and 1D). The density of actin cables in these varying structures is usually highest around the cortical region, declining progressively towards, but rarely entirely absent from, the core cytoplasm, as long as a subapical structure can be revealed by the expressed actin markers (Figure 2C). Thus, the notion of a subapical core of cytoplasm devoid of actin microfilaments (Lovy-Wheeler et al., 2005) is apparently not suggested by these live cell observations. The exclusive accumulation of transport vesicles in the apical clear zone and differential transport of different classes of organelles into different depth of the apical dome (Lovy-Wheeler et al., 2005; Cheung and Wu, 2007) almost provoke a visual image whereby sieves with varying pore sizes provide some kind of a filtration mechanism, permitting size differentiation for passage into the apical cytoplasm. A subapical actin structure comprising actin filaments across the subapical cytoplasm and subtending the apical clear zone provides an attractive model for a biological sieve (Kost et al., 1998). The fluctuating ionic conditions in the apical and subapical cytoplasm will permit rapid remodeling of the actin organization within this subapical actin structure during pollen tube elongation.

A cortical actin fringe, or a dense collar of actin filaments, has been revealed by immuno-staining at the subapical region of lily, and, to a lesser extent, tobacco pollen tubes that have been fixed by improved fixation procedures (Lovy-Wheeler et al., 2005). In lily pollen tubes, starting between 1 and 5 μm distal from the tube apex, the actin fringe spans between 5 and 10 μm along the apical flank. In tobacco, a similar but probably proportionally reduced and less consistently detected structure spanning 3–5 μm was also observed (Lovy-Wheeler et al., 2005; Fossiner et al., 2002; Hormanseder et al., 2005; and I. Fossiner, personal communication). Since the actin fringe is observed in virtually every pollen tube fixed under the described conditions, it is proposed that the actin fringe is a fundamental attribute in growing pollen tubes (Lovy-Wheeler et al., 2005). It is, however, difficult to envisage an actin network that, on the one hand, has to be highly dynamic to account for the cellular activities observed in the subapical region, yet can be universally captured as one uniform structure by flash fixation in virtually every pollen tube observed. To account for the apparent constant presence of what ought to be a highly dynamic structure that is maintained at a finite distance from the migrating tube tip, the actin fringe in an elongating lily pollen tube is speculated to completely turn over every 16–33 s (Lovy-Wheeler et al., 2005), reconciling the notion of a highly dynamic cortical structure that is yet also observed as a permanent fixture at the subapical region.

However, absent from live cell observations is a subapical actin structure that maintains association with the cortex to an extent suggested by the actin fringe in fixed pollen tubes. Membrane-anchored actin filaments have been observed emanating from the apical and subapical membrane domains when the level of a membrane-anchored formin is augmented (Cheung and Wu, 2004). Thus, presence of

membrane-anchored actin filaments around the subapical cortex is not unexpected. However, a detectable level of polymerized actin along the apical and subapical membrane domain is always associated with severely retarded or arrested growth during live cell observations (Cheung and Wu, 2004; unpublished observations). These then suggest rapidly elongating pollen tubes may not tolerate the accumulation of apical and subapical membrane-anchored actin microfilaments to readily detectable levels or with a stability that allowed them to be readily imaged. GFP-labeled ADFs have been referred to best identify the actin fringe (Lovy-Wheeler, 2005; Wilsen et al., 2006). Indeed, in pollen tubes in which a relatively broad actin mesh that extends edge to edge is revealed by GFP-NtADF1 or GFP-LlADF1 (e.g. Figure 1E; Figure 5 in Chen et al., 2002; Figure 3 in Wilsen et al., 2006), actin filaments close to the cortex would most likely be among the actin that constitutes the actin fringe upon fixation. However, in instances in which actin cables are observed close to the cortex in GFP-ADF-expressing pollen tubes, apparent dynamic association followed by detachment from the cortex is evident (see, e.g. Figure 2C; Supplemental Movie 2C; Figure 3D; Supplemental Movie 3D; Chen et al., 2002).

Increasing knowledge about the pollen tube growth process and improving technology have already allowed us to advance from previous convictions that claims of a dense actin meshwork in the tube apical region are erroneous (e.g. Miller et al., 1996) to a general consensus that such a collection of dynamic actin microfilaments indeed exists. The picture that emerges in the last decade for the actin status at the subapical region of pollen tube appears to be a collection of actin microfilaments that undergoes constant remodeling to assume a continuum of structures that span between a mesh and a deep funnel across the tube. Some of the time, these structures would have their edges extend to and immediately peel away from the cortex. These pollen tubes, when flash-fixed, would be the most likely candidates of having a dense subapical cortical network approaching that of an actin fringe. However, to refer to the subapical actin structure in live elongating pollen tubes by any single morphological descriptive would be conceptually too confining for a structure that comprises highly dynamic constituent microfilaments and, at the same time, maintains a high level of morphological plasticity. The combined use of 'subapical actin structure' as a general description for the collection of actin in the subapical cytosplasm of an elongating pollen tube and specific morphological terminology that most closely reflects the structure at the time of observation would reflect more accurately the constantly changing actin status at this critical region in these polar growth cells.

Using GFP-Labeled Actin-Binding Proteins as Actin Markers in Functional Studies

An obvious goal of the development of actin reporter proteins by different groups is for adoption in functional studies that examine regulatory components of the polar cell growth process and how the actin cytoskeleton underlies or mediates their functions. This has been amply demonstrated, especially in microprojectile bombardment-mediated transient transformation experiments that permit a wide range of gene delivery and expression levels, allowing subtle as well as prominent structures to be observed (Fu et al., 2001; Chen et al., 2002; Cheung and Wu, 2004; Gu et al., 2005). We find that a productive use of these actin markers in transient expression studies is dependent on careful establishment of the best combination of input transgene dosage that least compromises growth yet allows a level of gene expression so that observations within the most robust pollen tube growth period are possible. For tobacco and lily, that would be between 2 and 8, and, at the most, 10 h after bombardment. In the case illustrated here for NtPLIM2b-GFP, for instance, we have found it most efficient to bombard two pollen samples, one by microprojectiles coated with lower amounts of transgene DNA (e.g. closer to 1.25 μg) and another with more DNA (e.g. 2.5 μg). This would achieve a supply of appropriately labeled transformed pollen tubes spanning the most robust pollen tube growth period in one experimental setting. The authenticity of the revealed actin cytoskeleton is most likely reflected in pollen tubes whose growth rates fall within the observed range for control pollen tubes at the time of observation. Ideally, biological insights should be derived from observations made in transformed tubes that show relatively normal growth rates under the culture conditions used. However, from tobacco pollen tubes growing at depressed growth rates due to GFP-mTalin expression, fine spatial and temporal information can apparently be adequately resolved to reveal a phase relationship between actin polymerization and oscillatory growth (Hwang et al., 2005).

The efficiency of microprojectile delivery and gene expression from the delivered transgenes vary for each transformed pollen grain. Therefore, even well tolerated actin marker proteins, such as GFP-NtADF1 and NtPLIM2b-GFP, may induce actin abnormalities and compromise growth in transformed tubes that highly express these marker proteins (e.g. Figure 4D; Wilsen et al., 2006). The aberrant actin cytoskeleton observed in two NtPLIM2b-GFP-expressing pollen tubes shown here (Figure 4D) underscores the occurrence of these anomalies. Low-frequency occurrence of GFP-ADFs and NtPLIM2b-GFP-induced growth and actin abnormalities like those shown here (Figure 4D; Figure 5G) and previously described (Wilsen et al., 2006) are readily identified and thus do not easily lead to mistaken interpretation that they are consequences of experimental manipulations that explore functional participation of different regulatory molecules in the tip growth process.

An obvious deficiency of the actin reporter proteins described thus far is none of them decorates the entire complement of the actin cytoskeleton as revealed in fixed cells. Given the sensitivity of the polar cell growth process to perturbations of actin dynamics, and the sensitivity in turn of actin structures and dynamics to structural and to environmental perturbation in the cytoplasm, it is probably unrealistic to expect any

GFP-based actin marker to be associated with microfilaments to the extent that it reveals the entire complement of polymerized actin without negatively impacting on the growth process. That an actin-based live cell reporter protein has not been reported thus far in plant further underscores the sensitivity of actin polymerization to structural perturbation. As GFP-ADFs, NtPLIM2b-GFP, and GFP-mTalin do show overlapping actin-labeling ability, it would be possible to corroborate observations made by using one marker system by another to an adequate extent, further ensuring the authenticity of these live cell observations.

Towards Studying the Interaction between Actin and Microtubules in Elongating Pollen Tubes

In pollen tubes, an extensive network of microtubules almost paralleling the actin cytoskeleton can be observed throughout its shank cytoplasm (Fossiner et al., 2002; Gossot and Geitmann, 2007; Poulter et al., 2008). Coordination between microtubules and the actin cytoskeleton is known to be important for cytoplasmic streaming and the tip growth process in gymnosperm pollen tubes (Anderhag et al., 2000). However, in angiosperm pollen tubes, the microtubules have only been attributed a clear role in maintaining the shape of the generative cell and transport of the tube cell nucleus and the generative cell (Astrom et al., 1995; Raudaskoski et al., 2001). Tip growth and cytoplasmic streaming appear not to be affected even under conditions in which microtubules are entirely disassembled by polymerization inhibitors (Gossot and Geitmann, 2007; Poulter et al., 2008). However, evidence suggesting the integrity of the microtubule system is linked to a properly organized actin cytoskeleton in angiosperm pollen tubes is beginning to emerge. Immuno-detection of microtubules in poppy pollen tubes treated with latrunculin B at concentrations that disintegrate the shank actin cables showed that the normally long and axially oriented microtubules are also disintegrated and replaced by a severely fragmented labeling pattern (Gossot and Geitmann, 2007; Poulter et al., 2008). On the other hand, although the integrity of the actin cytoskeleton does not appear to depend on an intact microtubule system, pollen germination and tube growth are synergistically inhibited when sub-inhibitory concentration of latrunculin B and the microtubule polymerization inhibitors are used in combination (Gossot and Geitmann, 2007; S. Costa, Ph.D. thesis, University of Lisbon). Moreover, in-vitro motility assays showed that pollen tube organelles, including Golgi bodies and mitochondria, are capable of moving along microtubules and do so at rates different from trafficking along actin filaments, leading to the suggestion that the microtubules may contribute to short-range trafficking in these polar growth cells (Romagnoli et al., 2003, 2007).

The GFP-AtEB1-revealed microtubules in pollen tubes shown here (Figure 6) suggest a relatively stable cortical microtubule network, while those in the core cytoplasm of the proximal region of the tube are more dynamic and appear to gather at slightly higher density around the subapical region than in the more distal cytoplasm. Additional microtubule reporter proteins will need to be developed to provide corroborative support for the GFP-AtEB1-revealed structure and dynamic. Like the different actin marker proteins, different microtubule reporters may allow more pronounced views on certain subpopulations of this cytoskeleton. Revealing the microtubule network under live cell conditions would be a first step towards exploring what must be more subtle but significant aspects of how the overall cytoskeleton act together, such as how the microtubules and actin cytoskeleton interact, to achieve the highly regulated tip growth process.

Conclusion

The most precise description of the cytoskeleton in elongating pollen tubes will likely be from perfectly preserved flash-fixed samples and detection methods that provide a global view of various discernable structural elements in these polar growth cells. Reconstruction from snapshots from fixed samples, each capturing some of the predominant features of the cytoskeleton organization in elongating pollen tubes, will likely reflect more accurately the reality of the intracellular dynamics that underlie this rapid polarized cell growth process rather than any single view may suggest. While unlikely to reveal the entire complement of the pollen tube cytoskeleton, fluorescent protein probes in live cell studies reveal discernable cytoskeleton structures consistently and show that they are sensitive to factors known to regulate the tip growth process. The opportunities afforded by live cell studies to advance our views on intracellular dynamics and rapidly elucidate our understanding of functional and signaling relationships in cellular process are unparalleled by studies in fixed cells. With rapidly advancing technology to achieve high temporal and spatial resolution of live cell observations, superimposition of these live cell observations with ultrastructural information (e.g. Betzig et al., 2006) may ultimately be attainable to provide both structural and dynamic precision that accurately describes the intracellular organization and activity within these amazing polar growth cells.

METHODS

Chimeric Gene Constructs

All fusion genes were constructed using standard recombinant DNA methodology. NtPLIM2b (Genbank accession # EU496813) was identified as highly and specifically expressed in tobacco pollen in a differential display (Liang and Pardee, 1997) effort. A full-length clone was isolated from a *Nicotiana tabacum* pollen cDNA library. The deduced amino acid sequence for NtPLIM2b is shown in Supplemental Figure 1. AtROP-GEF1 (At4g38430) and AtEB1 (At5g67270) cDNA was isolated by RT–PCR of *Arabidopsis* seedling mRNA. SF3-GFP-NtPLIM2b, Lat52-GFP-AtEB1, and the previously described constructs Lat52-GFP, Lat52-GFP-NtADF1, Zmc13-GFP-LlADF1, and Lat52-GFP-mTalin (Chen et al., 2002) are derived from the Bluescript pKS vector (Stratagene). SF3-GFP-NtPLIM2b was introduced

into a Ti plasmid vector for *Agrobacterium*-mediated tobacco (*N. tabacum* petit Havana SR1) leaf disc transformation. SF3 (Baltz et al., 1992) and Lat52 (Twell et al., 1990) are pollen-predominant promoters from sunflower and tomato, respectively.

Pollen Transient Transformation and Plant Transformation

Agrobacterium-mediated tobacco leaf disc transformation followed standard procedures (Delebrese et al., 1986). Microprojectile bombardment was used for transient pollen transformation. The overall procedure is as described in Chen et al. (2002). DNA ranging from 0.5 to 10 μg was used per microprojectile coating. The amount of DNA used for each experiment is indicated in the text or figure legend. Between 5 and 10 mg of tobacco and 10 mg of lily (*Lilium longiflorum*) pollen was used for each transformation. A single bombardment was used for tobacco pollen transformation and double bombardments were used for lily pollen.

In-Vitro Pollen Germination and Tube Growth Culture and Pollen Tube Observation Regimes

Tobacco pollen germination medium with minor composition variations was used over the years that spanned the studies reported here. All of them supported robust tobacco pollen tube growth with indistinguishable pollen tube morphology. The basic germination medium (GM) for tobacco pollen was 0.8 μM $MgSO_4.7H_2O$, 1.6 μM H_3BO_3, 3 mM $Ca(NO_3)_2.4H_2O$, 1 mM KNO_3, 10 mM MES, pH 6.0, 8% sucrose; if solidified, agarose was added to 0.7%. When supplemented by polyethylene glycol, PEG-4000, as in Chen et al. (2002), 8% sucrose was substituted by 15% PEG 2% sucrose (GM(P2%S)), or by 12% PEG and 5% sucrose (GM(P5%S)). If solidified, 0.35% phytagel (Sigma) was added. GM(6%S) was a slight modification from GM, the major difference being 6% sucrose and 50 μM $CaCl_2$ are used. In general, the PEG-supplemented media, all variations from Read et al. (1993), provided faster growth rates, but the cytoskeleton patterns revealed in the tobacco pollen tubes grown in these media are not obviously distinguishable. When not indicated, GM was used. Lily pollen germination medium (LGM) was as described (Cardenas et al., 2006) and is composed of 1.6 mM H_3BO_3, 1 mM KCl, 0.1 mM $CaCl_2$, 7% sucrose, and 15 mM MES, pH 5.5.

After bombardment, pollen grains were cultured either on solidified medium or in liquid cultures with gentle rotation. Expression of marker proteins was examined at about 3 h after bombardment. Microscopic observations were most frequently made between 3 and 8 h after bombardment. Observations rarely continued beyond 8 h after bombardment, but occasionally observations made in pollen tubes from cultures as old as 10 h may still be usable. Where relevant, culture time for individual samples or experiments is indicated in the text or figure legend. Transformation efficiency varied between bombardments. In general, as culture time increased, the number of observable transformants increased, since expression of marker proteins increased over time. The number of observable transformants usually exceeded that which realistically could be analyzed microscopically within the duration of an experiment, especially when using confocal laser scanning microscopy (CSLM).

Chemical fixation of pollen tubes followed that described in Vidali et al. (2001) and Chen et al. (2002). In latrunculin treatments, a relatively high concentration of 250 nM Latruculin B in growth medium was used.

Microscopic Observation of Pollen Tubes

In CSLM, GFP was excited by the 488-nm line of argon lasers. All the data presented here are obtained from CLSM (Biorad MRC600 or Zeiss Meta 510), except for data on bulk growth rates analysis shown in Figure 4A and 4B, which were obtained by imaging pollen tubes by widefield fluorescence carried out on a Nikon E800. Measurement of distance elongated by pollen tubes was carried by measurement software for the SPOT camera system on the Nikon E800, the Zeiss LSM browser, or Image J.

GenBank accession number for NtPLIM2b is EU496813.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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