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Overexpression of an Arabidopsis Formin Stimulates Supernumerary Actin Cable Formation from Pollen Tube Cell Membrane[W]

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Formins, actin-nucleating proteins that stimulate the de novo polymerization of actin filaments, are important for diverse cellular and developmental processes, especially those dependent on polarity establishment. A subset of plant formins, referred to as group I, is distinct from formins from other species in having evolved a unique N-terminal structure with a signal peptide, a Pro-rich, potentially glycosylated extracellular domain, and a transmembrane domain. We show here that overexpression of the Arabidopsis formin AFH1 in pollen tubes induces the formation of arrays of actin cables that project into the cytoplasm from the cell membrane and that its N-terminal structure targets AFH1 to the cell membrane. Pollen tube elongation is a polar cell growth process dependent on an active and tightly regulated actin cytoskeleton. Slight increases in AFH1 stimulate growth, but its overexpression induces tube broadening, growth depolarization, and growth arrest in transformed pollen tubes. These results suggest that AFH1-regulated actin polymerization is important for the polar pollen cell growth process. Moreover, severe membrane deformation was observed in the apical region of tip-expanded, AFH1-overexpressing pollen tubes in which an abundance of AFH1-induced membrane-associated actin cables was evident. These observations suggest that regulated AFH1 activity at the cell surface is important for maintaining tip-focused cell membrane expansion for the polar extension of pollen tubes. The cell surface–located group-I formins may play the integrin-analogous role as mediators of external stimuli to the actin cytoskeleton, and AFH1 could be important for mediating extracellular signals from female tissues to elicit the proper pollen tube growth response during pollination.

INTRODUCTION

The pollen tube cell relies on its actin cytoskeleton to migrate over long distances in the female extracellular matrix and transport its two resident sperm cells to the ovules for fertilization. Pollen tubes achieve this by a polar cell growth process whereby the protoplast is confined to the most proximal region of the long tubular structure and growth is restricted to the tube apex (Derksen et al., 1995; Hepler et al., 2001). The polarized pollen tube morphology is accompanied by an asymmetric distribution of its cellular constituents. Cellular asymmetry is maintained largely by actomyosin-driven intracellular trafficking activities whereby larger organelles, such as the Golgi bodies, are transported to the subapical region along the cell cortex and recycled to the rear of the cell in the reverse direction in the core of the tube. The apical zone of elongating pollen tubes is packed with a high density of vesicles that abut the tip membrane, where fusion occurs, delivering cell wall materials to the growing front. The apical cytoplasm has a smooth morphology relative to the more granular cytoplasm seen in the rest of the tube and is referred to as the "clear zone" of the pollen tube. Forward- and backward-moving long actin cables are abundant in the shank of the tube, but they do not invade the clear zone. The subapical region is marked by a funnel-shaped actin mesh subtending and seemingly feeding its relatively shorter and finer actin cables to the thicker and longer grain-ward-moving actin bundles in elongating cells (Geitman et al., 2000; Vidali et al., 2001; Chen et al., 2002). Actin-disrupting drugs arrest pollen tube growth, dissociate the elaborate actin structures, stop cytoplasmic streaming, and permit the invasion of larger organelles into the apical zone (Gibbon et al., 1999; Vidali et al., 2001). Therefore, pollen tubes are efficient actin-based molecular machines that support growth at rates of, e.g., ~1 cm/h for maize pollen tubes elongating in the female tissues. Much remains to be learned regarding how actin polymerization in pollen tubes is regulated to achieve the necessary organization and dynamics to support the rapid and directionally guided polar cell growth process.

The Arp2/3 complex and formins are two major protein families known to have actin-nucleating activity, although other actin binding proteins, such as the barbed-end binding gelsolin and capping protein, also stabilize actin oligomers and thus also may participate in the regulation of actin nucleation, the rate-limiting step for actin polymerization (Higgs and Pollard, 2001; Pruyne et al., 2002; Sagot et al., 2002b; Kovar et al., 2003; Pollard and Borisy, 2003; Pring et al., 2003). Formins are morphoregulatory proteins important to a broad spectrum of

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cellular and developmental processes in a wide range of organisms (Wasserman, 1998; Zeller et al., 1999; Sawin, 2002). They regulate multiple actin-related processes and may coordinate microtubules and the actin cytoskeleton (Fujiwara et al., 1999; Heil-Chapdelaine et al., 1999; Palazzo et al., 2001; Ishizaki et al., 2001). Mutations in formins induce defects in polarized growth, such as budding and the formation of mating protrusion in yeast. They also are essential for cytokinesis in yeast and animal cells (Kato et al., 2000; Feierbach and Chang, 2001). Yeast mutants with loss of formin function rapidly lose their actin cables, whereas overexpressing formins induce supernumerary actin cable formation and growth arrest (Evangelista et al., 2002; Sagot et al., 2002a; Kovar et al., 2003). Overexpressed animal formins also increase the level of stress fibers in cultured cells (Tominaga et al., 2000; Copeland and Treisman, 2002) and enhance cell migration (Koda et al., 2003). In vitro, the FH1 and FH2 domains (Figure 1) from budding and fission yeast formins stimulate actin nucleation (Pruyne et al., 2002; Sagot et al., 2002b; Kovar et al., 2003; Pring et al., 2003). Contrary to the pointed-end binding Arp2/3 complex that initiates new actin filaments off the side of preexisting filaments to form a branched actin network (Higgs and Pollard, 2001; Welch and Mullins, 2002; Pollard and Borisy, 2003), the yeast formins bind to barbed ends, permit only pointed-end growth, and induce actin filament formation de novo. Profilin apparently interacts with the poly-Pro-containing FH1 domain in the yeast formins and enhances their actin-nucleating activity (Sagot et al., 2002b; Kovar et al., 2003). Some animal and yeast formins are autoinhibited, activated upon binding by activated Rho GTPases to the G protein binding domain (GBD) (Evangelista et al., 1997; Watanabe et al., 1997; Alberts, 2001; Palazzo et al., 2001; Gasman et al., 2003). A less conserved FH3 domain has been identified between GBD and FH1 to confer a localization property for some formins (Petersen et al., 1998; Kato et al., 2000).

In contrast to formins from other species, plants have evolved two classes of formins, referred to as groups I and II (Arabidopsis Genome Initiative, 2000; Banno and Chua, 2000; Cvrckova, 2000; Deeks et al., 2002). Plant formins are similar to formins from other species in having a Pro-rich FH1 region and a FH2-homologous domain in their C-terminal halves (Figure 1). A stretch of variable sequences is located in the N-terminal half of these proteins. However, regions homologous with domains involved in autoinhibition and Rho GTPase activation in animal and fungal formins have not been identified among plant formins. Group-I formins are unique among the formin family in having evolved an N-terminal extension composed of a Pro-rich domain and a hydrophobic domain adequate for a transmembrane span. Putative signal peptides could be identified for these formins and presumably would target them to the secretory pathway. Therefore, group-I formins are potentially cell surface–associated proteins that may function as molecular links connecting the extracellular environment to the actin cytoskeleton.

AFH1 is a group-I formin from the Arabidopsis 20-member formin gene family (Banno and Chua, 2000). We show here that this pollen-expressed formin induces actin cables from the pollen tube cell membrane and that the pollen polar cell growth process is sensitive to increases in AFH1, resulting in growth depolarization and ultimately growth arrest.

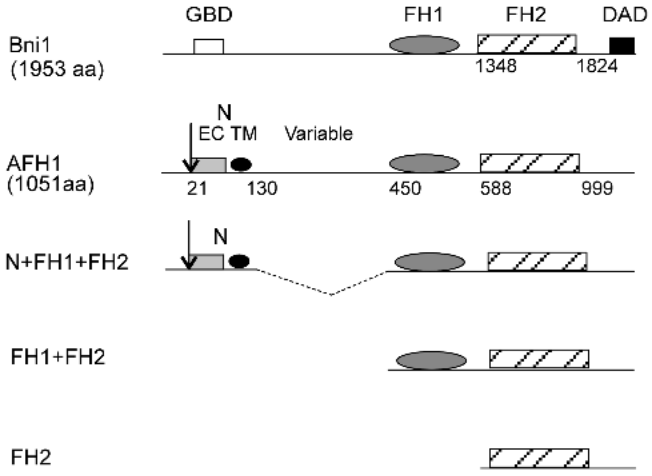

Figure 1. Domain Maps for Wild-Type and Mutant AFH1.

The yeast Bni1 domain map is shown as a representative formin. The FH1 and FH2 domains are aligned. The N-terminal extracellular domain (EC) and transmembrane domain (TM) in AFH1 are collectively referred to as the N domain. The region between N and the FH1 and FH2 domains in AFH1 shows little identity with sequences from other AFHs or formins from other organisms and is referred to as its variable domain. The arrows indicate the putative signal peptide cleavage site in AFH1. Numbers beneath Bni1 and AFH1 indicate the number of amino acid residues (aa) in these proteins. Numbers underneath the domain maps indicate amino acid positions that define the various domains. The maps are not drawn to scale. GBD denotes the Rho-GTPase binding domain, and DAD denotes the Diaphanous domain. Sequences homologous with GBD and DAD cannot be identified in plant formins. [N+FH1+FH2] is a variable domain–deleted AFH1 mutant, and [FH1+FH2] is an N-terminal half–deleted mutant. GFP fusion protein constructs with AFH1, [N+FH1+FH2], and [FH1+FH2] (not shown) have the GFP coding sequence inserted in frame at the 3′ end of the AFH1 coding sequence. The pollen promoter Lat52 (Twell et al., 1990) was used to express these chimeric genes in pollen tubes.

RESULTS

The Formin-Homology Domains in AFH1 Induce Supernumerary Actin Cable Formation in Pollen Tubes

The promoter of the ubiquitously expressed AFH1 (Banno and Chua, 2000) was active in elongating pollen tubes (Figure 2A), and full-length AFH1 cDNAs were recovered readily by reverse transcriptase–mediated PCR from Arabidopsis pollen mRNA (data not shown), suggesting the relative prevalence of AFH1 transcripts in the male gametophyte. The FH2 domain in AFH1 is ~40% similar to the same domain in formins from yeast (38%) and human (46%). Its FH1 domain is similar to other formins in being Pro rich, including a stretch of nine Pro residues. To determine if the FH1 and FH2 domains in AFH1 are similar to their analogous domains in yeast formins in stimulating actin cable formation, the AFH1 N-terminal half–deleted

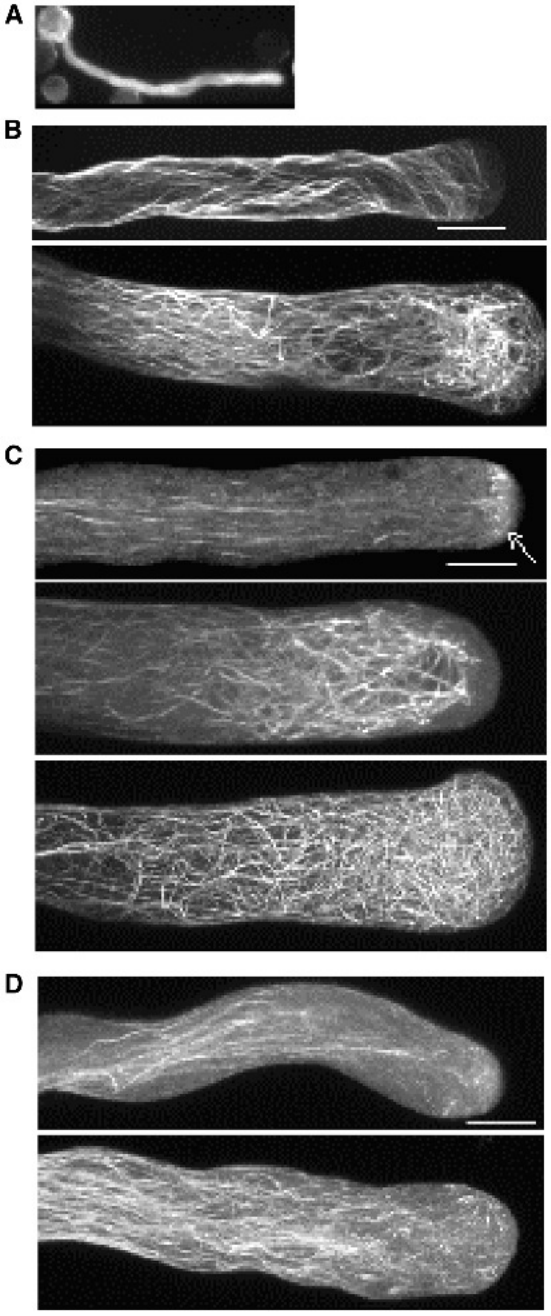

Figure 2. The Formin Domains in AFH1 Are Active in Inducing Supernumerary Actin Cable Formation.

(A) A pollen tube transformed by AFH1 promoter–GFP showing a strong GFP signal.

(B) At top, a control pollen tube transformed by Lat52:GFP-mTalin (1 μg). At bottom, a pollen tube cotransformed by Lat52:GFP-mTalin (1 μg) and Lat52:[FH1+FH2] (5 μg). Typically, ~30 to 35% of all cotransformed pollen tubes displayed a phenotype similar to that shown.

(C) At top, a control pollen tube transformed by Lat52:GFP-NtADF1 (1 μg). The arrow indicates the subapical actin mesh (Chen et al., 2002). The middle and bottom images show pollen tubes cotransformed by Lat52:GFP-NtADF1 (1 μg) and Lat52:[FH1+FH2] (2.5 and 5 μg, respectively). Typically ~50% of pollen tubes cotransformed by 2.5 μg of Lat52:[FH1+FH2] developed phenotypes similar to that shown in the middle image at ~5 h after culturing. Less than 10% of the transformed tubes showed more severe phenotypes, similar to that seen in the bottom image. The number of transformed tubes that developed more severe phenotypes increased with culturing time. For pollen tube samples cotransformed by 5 μg of Lat52:[FH1+FH2], a majority (60 to 75%) of all transformed tubes routinely developed phenotypes comparable to that shown here by ~5 to 6 h after culturing. A higher dose of Lat52:[FH1+FH2] consistently induced more severe actin cytoskeleton and growth defects in a larger population of transformed pollen tubes.

(D) At top, a control pollen tube transformed by Lat52:GFP-NtADF1. At bottom, a pollen tube transformed by Lat52:GFP-NtADF1 and Lat52:FH2 (10 μg). A majority of the cotransformed pollen tubes showed a phenotype comparable to or less severe than that exhibited by the tube shown here. At comparable culturing time, <10% of all transformed pollen tubes examined developed a phenotype comparable to those shown in **(C)**, middle and bottom images.

Pollen tubes in **(B)** to **(D)** were imaged between 5 and 6 h after culturing in germination medium. Notably, the pattern of actin cables induced by [FH1+FH2] had not been seen in control pollen tubes expressing either of the two actin markers in our previous studies. The lower percentage of pollen tubes expressing GFP-mTalin as the actin marker that developed supernumerary actin cables was attributable to the fact that at least half of the pollen tubes developed GFP-mTalin–induced defects (K. Wilsen, A.Y. Cheung, and P. Hepler, unpublished results) before the [FH1+FH2]-induced phenotype was attained. Bars in **(B)** to **(D)** = ~10 μm, the approximate subapical width in control pollen tubes.

mutant [FH1+FH2] (Figure 1) was expressed in transformed tobacco pollen tubes (Figures 2B and 2C). Because a functional green fluorescent protein (GFP)–labeled actin protein is not available for imaging the actin cytoskeleton in live plant cells, the pollen tube actin cytoskeleton was imaged by cotransformation with one of two actin binding protein genes: the pollen-expressed Lat52:GFP-mTalin (Kost et al., 1998) and Lat52:GFP-NtADF1 (for Nicotiana tabacum actin-depolymerizing factor1) (Chen et al., 2002). GFP-mTalin typically reveals an extensive array of long actin cables in the tube shank that terminate in the subapical region (Figure 2B, top image) (Kost et al., 1998; Fu et al., 2001). GFP-NtADF1 most prominently decorates a subapical actin mesh and, when expression level is higher, also binds to long actin cables in the shank (Figures 2C and 2D, top images) (Chen et al., 2002). GFP-NtADF1 has little effect on pollen tube growth when expressed at moderate levels (Chen et al., 2002) (see Figures 5A to 5C) and serves as a good alternative actin marker to GFP-mTalin, for which the pollen tube actin cytoskeleton and growth have less tolerance (K. Wilsen, A.Y. Cheung, and P. Hepler, unpublished results).

Both GFP-mTalin and GFP-NtADF1 revealed increased levels of actin cables in pollen tubes that coexpressed [FH1+FH2] (Figures 2B, bottom image, and 2C, middle and bottom images). The supernumerary actin cables were especially evident in the apical and subapical regions. Control pollen tubes expressing either of the actin marker proteins never displayed actin cytoskeleton phenotypes like those induced by [FH1+FH2]. The number of actin cables seen in single optical sections of [FH1+FH2]-overexpressing tubes was ~3 to 5 times to >10 times that observed in control actin marker–expressing pollen tubes (e.g.,

in tubes similar to those shown in Figures 2C, middle image, and 2B and 2C, bottom images, respectively). Overexpression of [FH1+FH2] also induced growth-related phenotypes in the form of tube broadening, tip expansion, and, ultimately, growth arrest, indicating that regulated formin activity is important for the polar cell growth process. Overexpression of the [FH1+FH2] domains of yeast formins also resulted in defects in polarity, increased levels of actin cables, and growth arrest (Evangelista et al., 2002; Sagot et al., 2002b; Kovar et al., 2003). Therefore, our observations in pollen tubes suggest that [FH1+FH2] in AFH1 is functionally analogous to its counterparts in yeast formins. Moreover, the FH2 domain alone (Figure 1) was considerably less active than [FH1+FH2] in stimulating actin cable formation (cf. the bottom image in Figure 2D with the middle and bottom images in Figure 2C). This finding suggests that the FH1 domain positively regulates the activity of AFH1, probably via interactions with endogenous profilin, similar to what has been observed for the yeast formins (Sagot et al., 2002b; Kovar et al., 2003).

The N-Terminal Domains Target AFH1 to the Pollen Tube Cell Membrane

An actin cable–inducing activity coupled with cell membrane localization would have significant implications for the biological role of AFH1. To explore this possibility, the coding regions for full-length AFH1, its variable domain–deleted mutant [N+FH1+FH2], and the N-terminal half–deleted [FH1+FH2] (Figure 1) were fused at their C termini with GFP. Transformed pollen tubes expressing AFH1:GFP or [N+FH1+FH2]:GFP fusion proteins showed strong green fluorescence signals along the cell surface (Figures 3A and 3B). By contrast, pollen tubes expressing the N-terminal half–deleted [FH1+FH2]:GFP

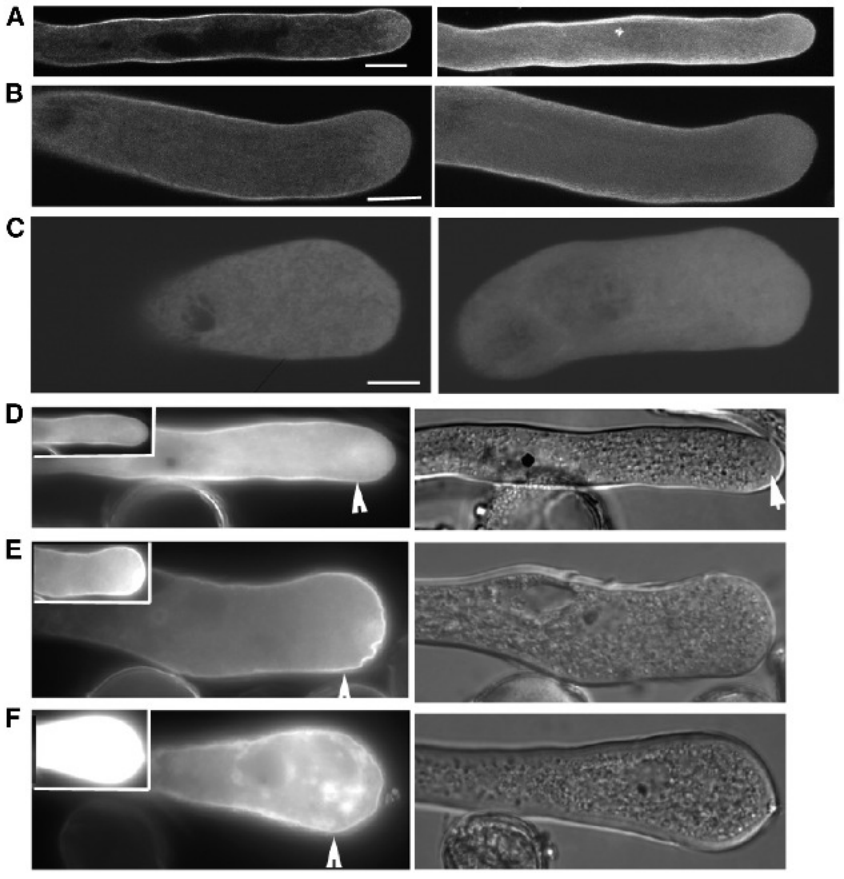

Figure 3. The N-Terminal Domains of AFH1 Are Important for Its Targeting to the Pollen Tube Cell Surface.

(A) to **(C)** Confocal images of pollen tubes transformed by Lat52:AFH1:GFP (2.5 μg) **(A)**, Lat52:[N+FH1+FH2]:GFP (2.5 μg) **(B)**, and Lat52:[FH1+FH2]:GFP (5 μg) **(C)**. The images at left are single optical sections from the medial region. The images at right show projections for the entire tubes. Bars = 10 μm.

(D) to **(F)** The photographs at left show epifluorescent images of pollen tubes expressing different levels of AFH1:GFP. The insets are of images captured under identical conditions to show the relative fluorescence levels in the corresponding tubes in each figure. With higher levels of AFH1:GFP, pollen tubes assumed a broader shank **(E)**, and those expressing very high levels of this protein developed a ballooned tip **(F)**. All images are shown at the same magnification. Pollen tube widths (at the arrowheads) are 12.47, 22.37, and 23.5 μm, respectively, for the tubes in **(D)**, **(E)**, and **(F)**. The photographs at right show differential interference contrast images for the corresponding pollen tubes shown at left. The clear zone (arrow), typical of actively elongating pollen tubes, is evident only in **(D)**.

showed an even distribution of green fluorescence throughout the transformed tube (Figure 3C), consistent with its being a cytosolic protein. Together, these observations indicate that the N-terminal region preceding the variable domain is necessary to target AFH1 to the secretory pathway and localize it to the cell membrane. These GFP fusion proteins were biologically active and induced tube broadening, tip expansion, and other defects (Figures 3C, 3E, and 3F; see below), as did their nontagged counterparts (see below). Moreover, the degree of induced tube broadening correlated with the fluorescence levels in the transformed cells (Figures 3D to 3F), suggesting a correlation between expression levels from the transgenes and the induced pollen tube phenotype.

To determine if AFH1 induces actin cables from the cell membrane, AFH1 and the variable domain–deleted [N+FH1+FH2] were overexpressed in pollen tubes. Both Lat52:AFH1 and Lat52:[N+FH1+FH2] induced an abundance of GFP-NtADF1–labeled or GFP-mTalin–labeled actin along the periphery of cotransformed pollen tubes (Figures 4A, 4B, and 4E to 4G, 5D, and 6A to 6C). When images from the entire tube were projected, cytosolic actin cables often were more randomly organized (Figures 4A and 4E, stack; see also Figure 6B, stack) or they were not as evident as in control tubes (Figures 4F, stack, and 4G; see also Figures 6A and 6C, stack), but their presence was evident in single optical sections (see supplemental data online). Serial optical sectioning of pollen tubes cotransformed by the actin marker genes, in particular Lat52:GFP-NtADF1 and Lat52:AFH1 or Lat52:[N+FH1+FH2], revealed striking arrays of punctate actin structures (see the 1- to 5-μm sections in Figure 4A, the 1- to 2.5-μm sections in Figure 4B, and the 2-μm section in Figure 4F). The punctate actin structures in AFH1- or [N+FH1+FH2]-overexpressing pollen tubes were detected first in tangential sections across the lower surface of these tubes (see the 1- and 3.5-μm sections in Figure 4A and the 1-μm section in Figure 4B). They appeared to extend ~1.5 to 2 μm from the cell membrane, because they faded as imaging approached the internal cytoplasm (e.g., in the 2.5- and 3.5-μm and the 5- and 5.5-μm sections in Figure 4A and the 2.5- and 3-μm sections in Figure 4B). They reappeared as imaging reached the top surface of these tubes (e.g., in the 20-μm section in Figure 4A and the 19.5- and 20-μm sections in Figure 4B). These punctate structures most likely represented actin cables that project from the cell membrane into the cytoplasm, almost perpendicular to the long axis of the tube. In some tubes, the GFP-labeled punctate actin structures remained obvious along the cell periphery even in the median sections (e.g., in the 9- and 12.5-μm sections in Figure 4A, the 8- and 11.5-μm sections in Figure 4B, and the 10.5-μm section in Figure 4F; see also Figures 6A and 6C). In others, short actin cables could be seen from the cell periphery projecting into the cytoplasm in sections that were farther from the tube surface (see the 3- to 6.5-μm sections in Figure 4B and the 3- and 4-μm sections in Figure 4E; see also Figure 5D). Similar tube surface–associated punctate structures were not observed in control pollen tubes expressing only the actin marker proteins (Figure 4D) or along with [FH1+FH2] (Figure 4C). These observations suggest that AFH1 stimulates the assembly of actin filaments from the cell membrane and that its N-terminal extension is necessary to target this protein to the pollen tube cell surface.

AFH1 Levels Affect Pollen Tube Growth

As is evident from the images shown in Figures 2 to 4, a majority of transformed pollen tubes that overexpressed AFH1, its mutant variants, or their GFP-tagged counterparts developed a broader shank (Figure 5B; see also Figures 3E and 3F). Some, especially those in which supernumerary cables were evident, developed a ballooned tip (Figures 2B and 2C, middle and bottom images, and 4A, 4E, and 4F). When individual pollen tubes were examined, those with a broader girth usually were growth retarded relative to control tubes, and those with a ballooned tip were growth arrested. However, cumulative growth over a period of time among populations of pollen tubes transformed by increasing amounts of Lat52:AFH1 revealed that low levels of transgene input actually stimulated growth, especially during the early hours (3 to 5 h) after germination (Figure 5A, left). When pollen tubes were transformed by <1 μg of Lat52:AFH1, the pollen tube morphology and observable actin cytoskeleton structure remained comparable to those in control pollen tubes transformed only by an actin marker gene (Figure 5C) (Chen et al., 2002). Many of them, especially those cotransformed by 0.25 or 0.5 μg of Lat52:AFH1, were longer than pollen tubes in control cultures (Figure 5A, 4 h of growth). When examined individually, many of them elongated at rates slightly higher than in control tubes (Figure 5C). Pollen tubes transformed by 1 or 1.5 μg of Lat52:AFH1 began to show the growth-inhibitory effect of overexpressed AFH1 (Figures 5A and 5D) toward 4 to 5 h after culturing. Although supernumerary actin cables were not yet evident in most of these tubes, tip membrane–associated actin filaments became prevalent in some (Figure 5D), and these tubes elongated at rates slower than that in control tubes when monitored individually (Figure 5D). In some images, the tip-arisen actin cables were intimately linked to the actin cables in the subapical region (Figure 5D, 60 s; see also supplemental data online). Although some individuals among pollen tubes transformed by 2.5 or 5 μg of Lat52:AFH1 began to show AFH1-induced actin cytoskeleton and morphological defects as early as 4 to 5 h after culturing, the cumulative growth among these transformed tubes was reduced only moderately even over more prolonged culturing times (Figure 5A, 8 h of growth). By 8 h after culturing, most of these tubes had developed supernumerary actin cables, ballooned, and were growth arrested. Therefore, it is likely that growth stimulation by low levels of AFH1 expressed from the input transgene had occurred during the initial hours of growth, possibly even before 2 to 3 h after culturing, when marker gene expression was still too low for the detection of these transformed tubes. Growth then was inhibited during the later hours, when AFH1 levels became too high for normal polar tube growth to continue.

Overexpressing AFH1 Induces Cell Membrane Deformation

AFH1:GFP-expressing pollen tubes often showed uneven edges along the pollen tube apical zone (Figures 3E, 3F, 6D, and 6E), as did many of the transformed pollen tubes overexpressing

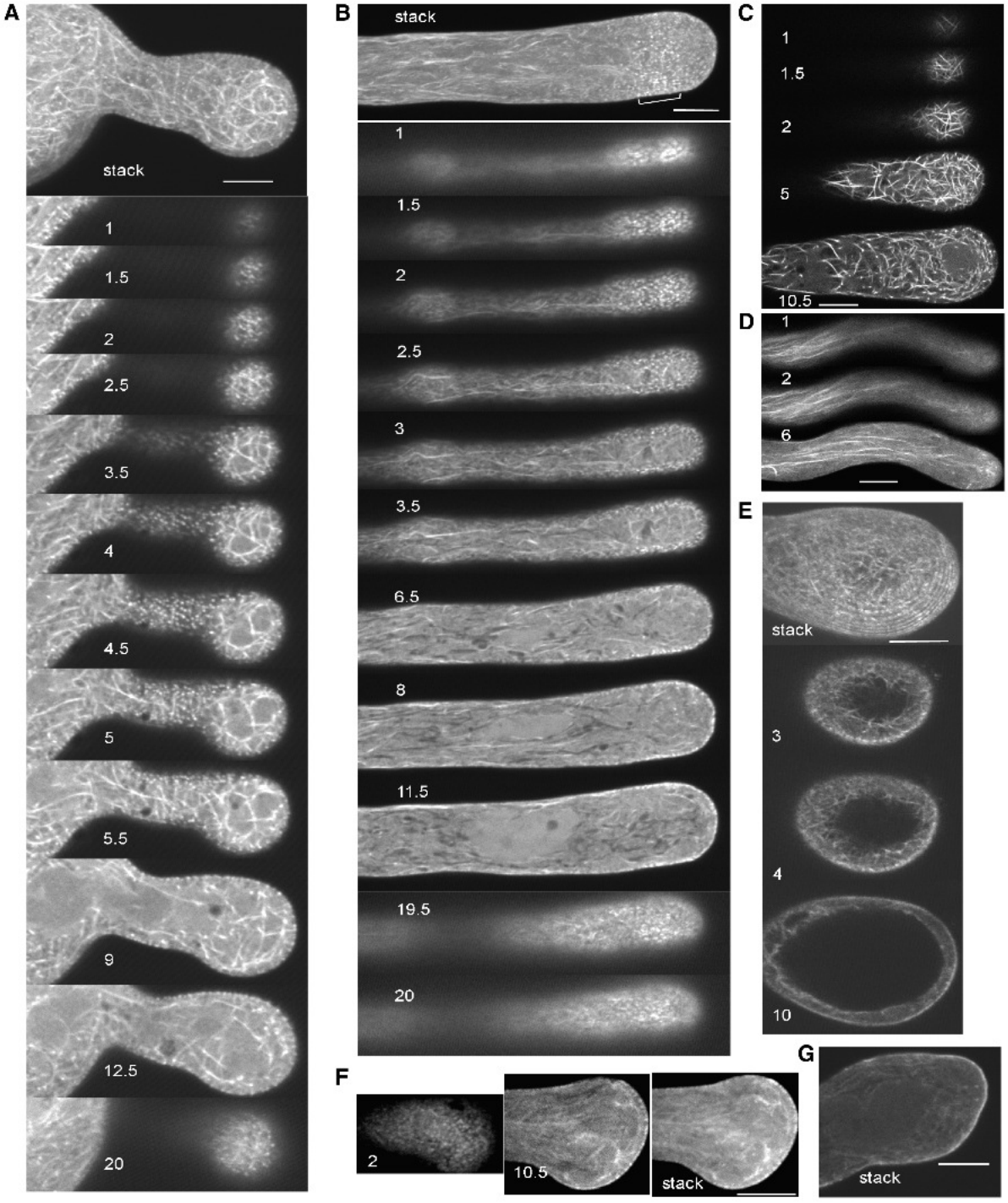

Figure 4. AFH1 and [N+FH1+FH2] Induce Actin Cables from the Cell Surface.

(A) and **(B)** Pollen tubes were cotransformed by Lat52:GFP-NtADF1 (1 μg) and Lat52:[N+FH1+FH2] (5 μg) **(A)** or Lat52:AFH1 (5 μg) **(B)**. The top image in each column shows a stack of all optical sections across these transformed pollen tubes. Selected single optical sections (at 0.5-μm steps) at various distances from the bottom surface are shown. In **(A)**, the 1-μm optical section was closest to the bottom surface of the ballooned apical region, whereas the 2.5-μm optical section was closest to the bottom surface of the narrower shank region. The bracket in the top image in **(B)** denotes the subapical region with the most prominent AFH1-induced short actin bundles along the tube cortex. See the supplemental data online for animation of the projection of these Z-series images.

(C) A pollen tube transformed by Lat52:GFP-NtADF1 (1 μg) and Lat52:[FH1+FH2] (5 μg) (the same tube shown in Figure 2C, bottom). Selected single optical sections from the bottom surface (top, 1 μm) to the median region (bottom) are shown. The occasional brighter spots seen in these sections represent intersecting points of actin cables or cross-sectioned cables.

AFH1 or [N+FH1+FH2] and an actin marker protein (Figures 6A to 6C). The surface undulations seen in AFH1:GFP-expressing tubes, which were not observed in any control pollen tubes (Figure 4D), already suggested cell membrane deformation. To confirm this, GFP-labeled cell membrane proteins (e.g., a pollen receptor kinase, Ntprk:GFP) (Cheung et al., 2002), were coexpressed with unlabeled AFH1 to mark the pollen tube plasmalemma. The normal cell membrane labeling pattern and the tip concentration of green fluorescence (Figure 6F, inset) were obliterated in these tubes. Instead, severe cell surface invaginations occurred, often with large rings observed in tangential sections across the bottom of the tube and uneven edges in midplane images (Figure 6F). In actin marker protein and AFH1- or [N+FH1+FH2]-coexpressing pollen tubes, the accumulation of actin cables was evident along the convoluted cell periphery (Figures 6A to 6C). Only mild membrane deformation was observed, and that very rarely, among pollen tubes overexpressing [FH1+FH2], consistent with membrane-associated actin cable assembly being a contributing factor to the membrane invagination observed in AFH1- and [N+FH1+FH2]-overexpressing tubes.

DISCUSSION

Nucleation of a new actin filament by assembly from monomers is the rate-limiting step in actin polymerization (Pollard et al., 2000). Although proteins homologous with the two known families of actin-nucleating proteins, the Arp2/3 complex and formins, are encoded in plant genomes (Arabidopsis Genome Initiative, 2000), little is known about this critical stage of actin regulation in plant cells. Recent studies in Arabidopsis mutants defective in putative Arp2/3 components revealed only subtle growth-related phenotypes and altered actin cytoskeleton structure in restricted vegetative cell types in the absence of these proteins (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a, 2003b). The activity of the putative Arabidopsis Arp2/3 complex in stimulating actin polymerization remains to be demonstrated. The findings reported here show that a member of the Arabidopsis formin family, AFH1, is active in inducing supernumerary actin cable formation in pollen tubes, mimicking activities reported for formins from yeast and animals (Tominaga et al., 2000; Copeland and Treisman, 2002; Evangelista et al., 2002; Sagot et al., 2002a). Moreover, almost all of the 20-member Arabidopsis AFH gene family (Deeks et al., 2002) are expressed in various organs, and in addition to AFH1, the ability to stimulate actin cable formation is shared by several other group-I and -II AFHs that have been examined to date (our unpublished observations). Thus, the plant formin family is by far the most complex among this conserved family of actin regulatory proteins in different organisms. Formins are known to play key roles in actin-related cellular processes, in particular those that involve polarity establishment (Wasserman, 1998; Sawin, 2002). The results presented here represent our initial efforts to explore the functional participation of plant formins in pollen tubes, tip-growing cells that depend critically on the actin cytoskeleton for their growth and polarity.

In addition to the N-terminal extracellular and transmembrane domains characteristic of group-I formins, our results show several other aspects of AFH1 that further distinguish it from formins from other organisms. As mentioned above, domains homologous with GBD and DAD on formins from other organisms (Evangelista et al., 1997; Alberts, 2001; Palazzo et al., 2001) have not been identified in plant formins (Figure 1). For animal and yeast formins, activated Rho GTPases bind to GBD and disrupt the GBD–DAD interaction, releasing autoinhibition, and probably relocate the activated, cytosolic formins to stimulate actin nucleation along the cell membrane. The full-length AFH1 and variable domain–deleted [N+FH1+FH2] and [FH1+FH2] were comparably active when expressed in transformed pollen tubes, suggesting that autoinhibition probably is not a major regulatory mechanism for its activity to stimulate actin polymerization. The association of AFH1:GFP with the cell membrane and the ability of AFH1 to induce cell membrane–associated actin cables indicate that this formin is self-sufficient in targeting to the cell membrane to stimulate actin cable formation. Whether its activity at the cell membrane is regulated by direct interactions with signal molecules at its extracellular domain or via other signaling molecules, such as Rho GTPases, which are known to be important for regulating the pollen tube actin cytoskeleton and its polar growth, remains to be explored. The reduced activity in the FH2 domain of AFH1 relative to that of the poly-Pro-containing [FH1+FH2] to stimulate actin cable formation (Figures 1 and 2) suggests that intracellularly, as for the yeast formins, profilin is likely to be an important regulator for AFH1 activity. On the other hand, a putative A-type

Figure 4. (continued).

(D) A pollen tube transformed by the marker Lat52:GFP-NtADF1 (1 μg) alone. Selected single optical sections from the bottom surface around the proximal region of the tube (top, 1 μm) to the median section (bottom) are shown. These sections are from the tube shown in Figure 2D, top, which displayed a relatively high level of GFP-NtADF1–labeled actin cables among similarly transformed pollen tubes. No punctate actin structures similar to those seen in AFH1- or [N+FH1+FH2]-overexpressing pollen tubes were seen in these tubes.

(E) to **(G)** Pollen tubes transformed by the actin marker gene Lat52:GFP-mTalin (1 μg) and Lat52:[N+FH1+FH2] **(E)** or Lat52:AFH1 (**[F]** and **[G]**) (5 μg each). The top image in **(E)** and the right image in **(F)** show projections of all optical images for the entire tube. Other images show single sections at the indicated distances (micrometers) from the bottom surface. The actin cytoskeleton and growth phenotype in tubes that expressed the GFP-mTalin marker were more variable than those in tubes that expressed GFP-NtADF1 as the actin marker. Nevertheless, a surface concentration of short actin cables was evident in these tubes, and punctate actin structures were observed in tangential sections and along the periphery of median sections of some of these tubes (**[F]**, 2 and 10.5 μm). In **(E)**, the pollen tube developed a large vacuole at the swollen tip region. Actin cables in the middle of the tube seen in the whole-tube projection reflect actins detected primarily in the tangential sections across the tube surface. The concentric rings of GFP-mTalin label seen in the whole-tube projection resulted from stacking of all of the cell periphery actin captured in each optical section.
Bars = ~10 μm.

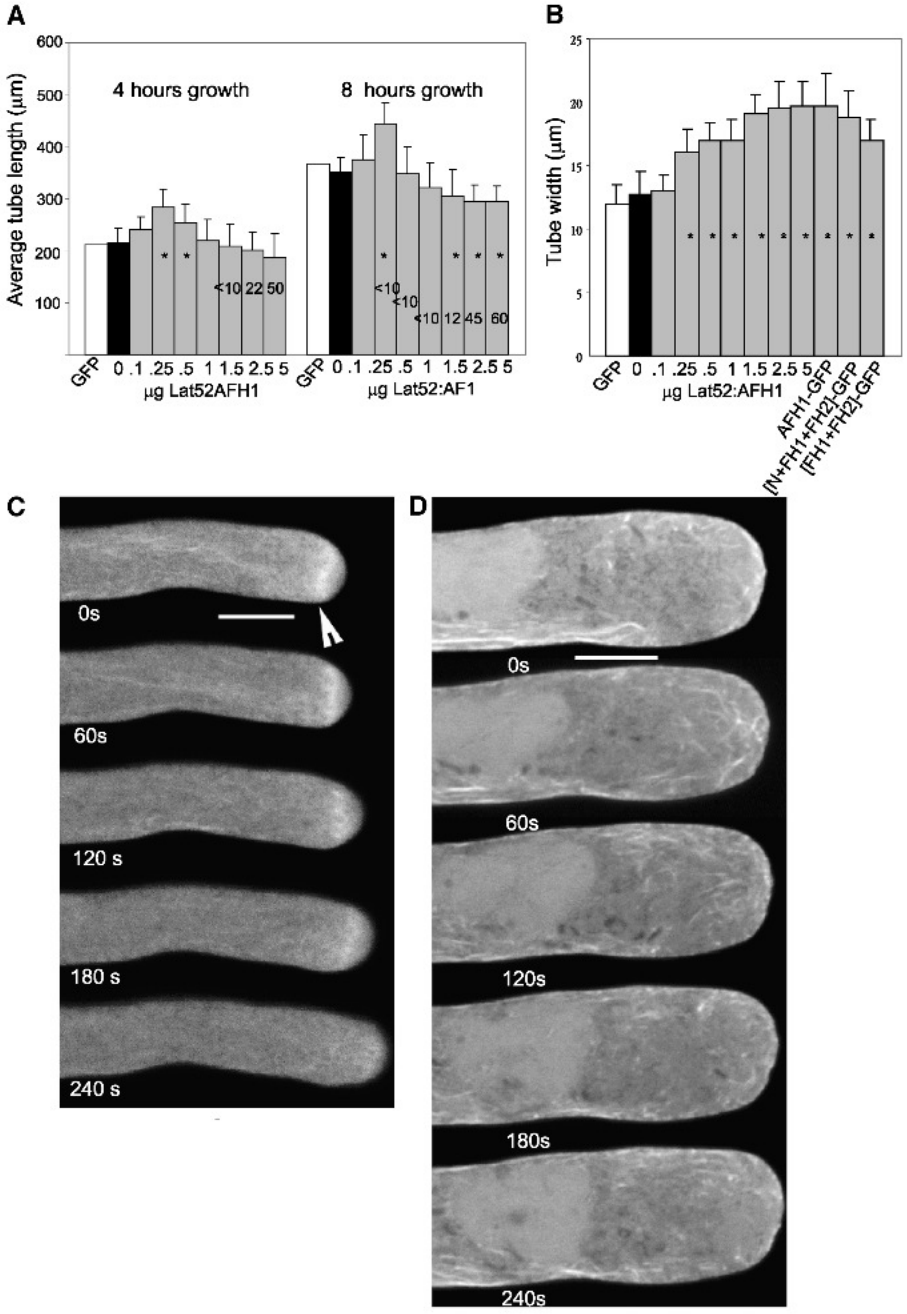

Figure 5. AFH1 Affects Pollen Tube Growth.

(A) Comparison of cumulative growth among populations of pollen tubes transformed by increasing amounts of Lat52:AFH1 and control tubes transformed by the marker gene Lat52:GFP-NtADF1. All closed bars show results from pollen tubes that had been transformed by the same amount of Lat52:GFP-NtADF1 (1 μg) and increasing amounts of Lat52:AFH1 (0 to 5 μg). Average pollen tube lengths for 35 transformed tubes from each sample at 4 and 8 h after culturing in germination medium are shown. All closed bars were averaged from three experiments. The open bars (GFP) show averages from pollen tubes transformed by Lat52:GFP as an additional control. This was included in only one of the three experiments, but comparable growth rates for tubes transformed by Lat52:GFP and Lat52:GFP-NtADF1 (1 μg each) were observed consistently in different sets of experiments (Chen et al., 2002; K. Wilsen, A.Y. Cheung, and P. Hepler, unpublished results). The number in each bar indicates the approximate percentage of transformed tubes that had developed supernumerary actin cables. Asterisks indicate that data in these samples were significantly different (Student's t test, $P < 0.05$) from that in control pollen cultures that received no Lat52:AFH1.

(B) Comparison of pollen tube diameter. All closed bars, except the three at right, show data from pollen tubes cotransformed by Lat52:GFP-NtADF1 (1 μg) and increasing amounts of Lat52:AFH1 (0 to 5 μg). The three data bars at right show data from pollen tubes transformed by Lat52:GFP-AFH1 or its mutant variants (5 μg each) as indicated. The open bar (GFP) shows data from pollen tubes transformed by Lat52:GFP (1 μg) as an additional

K^+ channel protein, FIP2, interacts with the C-terminal region of the FH1 domain in yeast two-hybrid studies and in vitro (Banno and Chua, 2000), suggesting at least another possible intracellular regulatory mechanism for AFH1.

Overexpressed AFH1 and its cytosolic variant [FH1+FH2] induce dramatic actin phenotypes (Figures 2 and 4) that are unsurpassed by similar actin cable formation–stimulating activities from animal and yeast formins when overexpressed in their corresponding cell types. The relatively uniform fluorescent signal along the surface of AFH1:GFP- and [N+FH1+FH2]:GFP-expressing tubes (Figures 3A, 3B, and 3D) suggests an even distribution of these proteins along the pollen tube cell membrane. However, AFH1 and [N+FH1+FH2] often induced punctate actin structures along the pollen tube surface. How these arrays of discrete actin cables arose remains to be resolved. Given the structure of group-I formins, it is tempting to propose that interactions between the extracellular domain of AFH1 and certain pollen tube wall components, or between its cytosolic region and pollen tube cortical components, have induced clustering of AFH1 activity, giving rise to the observed punctate actin arrays. On the other hand, rapid restructuring and cross-linking of AFH1-induced nascent actin filaments (e.g., by ADF, fimbrim, and villin), similar to those proposed for the Arp2/3-generated actin filaments (Pollard et al., 2000; Pollard and Borisy, 2003), also could have given rise to bundles of actin filaments that abut the cell membrane. Determination of the protein components in these cell membrane–associated actin bundles will be needed to understand how the AFH1-induced actin arrays were formed.

The energetics of actin polymerization and the actin organization at mammalian migratory cell fronts led to suggestions that membrane-associated actin polymerization may provide protrusive force at the leading edge of migratory cells (Mogilner and Oster, 1996; Svitkina and Borisy, 1999; Pollard et al., 2000; Pollard and Borisy, 2003). Tip extension in live pollen tubes and the process of actin polymerization itself are tightly coupled (Gibbon et al., 1999; Vidali et al., 2001). Under normal pollen tube growth conditions, properly regulated membrane-associated actin polymerization induced by formins could contribute to protrusive growth at the tip. The biological significance of the overexpressed AFH1-induced membrane deformation (Figure 6) remains to be determined. However, actin polymerization–induced membrane deformation in giant liposomes has been interpreted to reflect protrusive force generated by the polymerization process (Miyata et al., 1999; Pollard et al., 2000). Therefore, it is possible that the cell membrane deformation observed in AFH1-overexpressing pollen tubes (Figure 6) has resulted from uneven forces generated by differential levels of actin polymerization along the cell membrane. On the other hand, increased levels of actin cables around the cell periphery could have affected the efficiency and location of secretory vesicle fusion with the cell membrane. This could result in the increased or ectopic accumulation of secretory materials not properly accommodated by or incorporated into the cell wall. These excess secretory materials would press against the cell membrane, leading to the observed invagination and aberrant tube morphology (Roy et al., 1998). Active membrane retrieval is believed to occur at the apical and subapical membranes of elongating pollen tubes (Hepler et al., 2001). Given the role of actin in regulating endocytosis and coupling endocytosis to exocytosis (Taunton, 2001; Gundelfinger et al., 2003; Sokac et al., 2003) and the emerging understanding of a signaling relationship between formins and endosomes (Tominaga et al., 2000; Gasman et al., 2003), it also is possible that the AFH1-induced membrane invaginations have resulted from excessive or aberrant endocytic activity.

Pollen tube growth is highly sensitive to perturbations in the actin cytoskeleton. Its elaborate actin organization, with opposing streams of rapidly moving actin cables reversing direction in the subapical region, where a dynamic actin mesh is located, is believed to require coordinated activities from multiple actin-regulatory proteins (Hepler et al., 2001). Altering the levels of profilin or ADF, or signaling molecules such as Rho GTPases that regulate actin polymerization, severely inhibits the polar tube growth process (Kost et al., 1999; Fu et al., 2001; Chen et al., 2003). The disrupted pollen tube actin cytoskeleton resulting from overexpressed AFH1 (Figures 2, 4, and 6) indicates that a proper level of actin cable formation is as critical as actin-remodeling activities to produce the normal pollen tube cytoskeleton structure.

Figure 5. (continued).

control. Width was measured at 10 μm behind the apex for all pollen tubes. Data were averaged from 15 transformed pollen tubes in each sample at 8 h after culturing from one experiment only. Observations made from numerous experiments using wild-type and mutant AFH1, as shown in the other figures, conformed to the broadening trend induced by AFH1 in this data set. Noticeable pollen tube broadening occurred even in tubes that had not accumulated enough AFH1 to induce the supernumerary actin cable phenotypes (e.g., in the samples transformed by Lat52:GFP-NtADF1 and 0.5 and 1 μg of Lat52:AFH1). Asterisks indicate that data in these samples were significantly different (Student's t test, $P < 0.05$) from that in control pollen cultures that received no Lat52:AFH1.

(C) A pollen tube typical of those transformed by Lat52:GFP-NtADF1 (1 μg) and Lat52:AFH1 (0.25 μg). Images from a time series over 4 min of growth at ~4 h after culturing are shown. The tube was elongating at a growth rate slightly faster than that of average control pollen tubes (~5 μm in 4 min) and showed the GFP-NtADF1–labeled subapical actin mesh (arrowhead) and some actin cables in the shank, typical of normally elongating pollen tubes (Chen et al., 2002). A movie for this time series is available in the supplemental data online.

(D) A pollen tube transformed by Lat52:GFP-NtADF1 (1 μg) and Lat52:AFH1 (1 μg). Images of a time series over 4 min of growth at ~4 h after culturing are shown. The pollen tube had extended only ~2 μm during this period. The subapical actin mesh was no longer prominent, but concentrations of actin cables could be seen in some individual images at the subapical region (60- and 120-s images). A movie for this time series is available in the supplemental data online.

The time series in **(C)** and **(D)** were taken at 10-s intervals. Bars in **(C)** and **(D)** = ~10 μm.

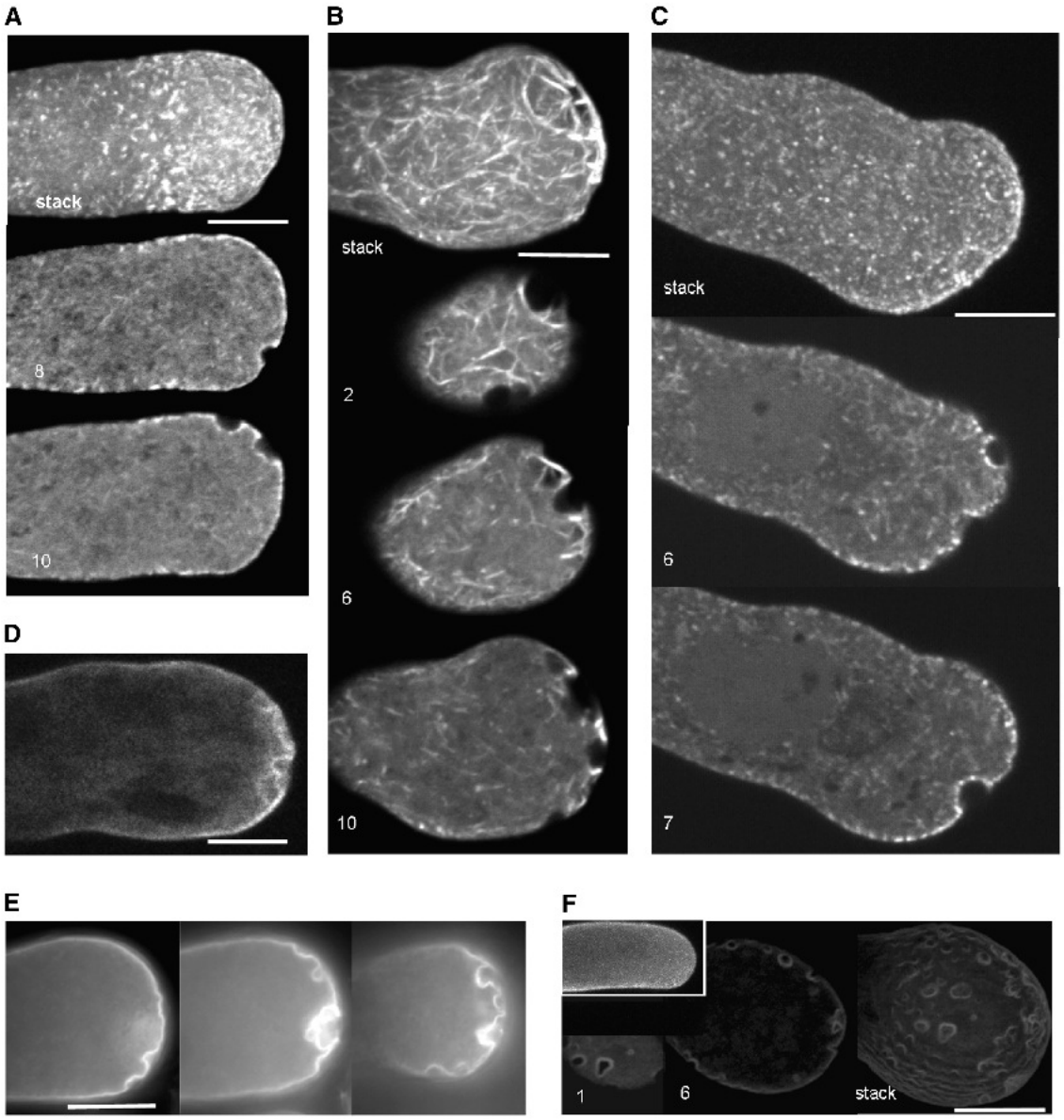

Figure 6. AFH1 Induces Cell Membrane Deformation.

(A) and **(B)** Pollen tubes cotransformed by the marker gene Lat52:GFP-NtADF1 (1 μg) and Lat52:[N+FH1+FH2] **(A)** or Lat52:AFH1 **(B)** (5 μg each).
(C) A pollen tube cotransformed by Lat52:GFP-mTalin (1 μg) and Lat52:AFH1 (5 μg).
Top images in **(A)** to **(C)** show the entire stack of images across these tubes. The lower images show single optical sections in which membrane invaginations were most prominent. Numbers in each panel indicate distances (micrometers) from the bottom surface.
(D) A single confocal section from the medial region of a pollen tube transformed by Lat52:AFH1:GFP (5 μg).
(E) Epifluorescent images of a single pollen tube transformed by Lat52:AFH1:GFP (5 μg) taken at different focal planes to highlight the severe membrane deformation along the surface. The same tube also is shown in Figure 3E.
(F) A pollen tube cotransformed by the cell membrane marker gene Lat52:Ntprk:GFP (Cheung et al., 2002) (5 μg) and Lat52:AFH1 (5 μg). Selected single optical sections at the indicated distances from the bottom surface and a stack for the entire tube are shown. The concentric rings of fluorescence seen in the stack reflect the surface labeling captured in individual sections. The inset shows a control pollen tube transformed by Lat52:Ntprk:GFP.
Bars = ~10 μm.

The growth-stimulatory effect from slight increases in AFH1 activity and the negative impact overexpressed AFH1 had on pollen tube morphological and growth properties (Figure 5) suggest that properly regulated actin filament formation is important for the pollen tube growth process. In pollen tubes cotransformed by an actin marker gene and low doses of Lat52:AFH1 in which supernumerary actin cables were not yet evident, actin-remodeling activities must have been able to keep pace with the modest increase in nascent actin filaments and incorporate them into the overall actin cytoskeleton, fueling even higher cellular activities that rely on actin dynamics. The moderately increased growth rates among these pollen tubes could have been a consequence of more efficient secretion and membrane recycling at the tube tip caused by the increased level of actin cables. Alternatively, the enhanced growth could have resulted directly from the increased protrusive force generated by the increased membrane-associated actin polymerization activity. In pollen tubes transformed by high doses of Lat52:AFH1 or its variants, the association of reduced or arrested growth with an abundance of cell periphery or cytosolic actin cables would be the consequence of actin-remodeling and cycling activities not capable of keeping pace with the significantly increased levels of nascent actin filaments, and so disrupting the normal balance in actin dynamics, the overall actin cytoskeleton structure, and inhibiting growth.

The growth-related phenotypes associated with altered levels of AFH1 shown here clearly indicate that regulated AFH1 level, or its activity, is critical for normal pollen tube growth in culture. However, where nascent actin filaments are assembled in elongating pollen tubes remains a mystery. In general, short actin cables have been difficult to observe at the apical region in rapidly elongating pollen tubes (Kost et al., 1999; Hepler et al., 2001; Chen et al., 2002). The presence of tip membrane–associated actin in growth-retarded, AFH1-overexpressing pollen tubes (Figure 5D) suggests that tip-arisen short actin cables could be a normal component in elongating pollen tubes. That they are not observed easily in rapidly elongating pollen tubes could be attributable to their rapid incorporation into the subapical (Figure 5D, 1 min; see also supplemental data online) and shank actin structures. On the other hand, the density of actin cables induced by the overexpressed AFH1 often was highest at the subapical region (Figures 2C, middle image, and 4B, stack, bracketed region). Possibly, endogenous mechanisms exist that preferentially enhance AFH1 activity in the subapical region where rapid actin reorganization is believed to occur (Gibbon et al., 1999; Geitman et al., 2000; Hepler et al., 2001; Vidali et al., 2001; Chen et al., 2002). The subapical membrane-arisen actin cables would be spatially optimal for active remodeling and rapid incorporation into the actin mesh located in the vicinity. Ongoing efforts are focused on determining the localization of the endogenous AFH1 and other pollen-expressed formins. This knowledge, together with the understanding of the spatial regulation of the activity of these formins, will provide clues to where the assembly of actin filaments is initiated and explore how nascent actin filaments are incorporated into the overall pollen tube actin organization.

Given that molecules homologous with integrins have not been identified in plants (Arabidopsis Genome Initiative, 2000), group-I formins could have evolved to serve the analogous function of mediating extracellular stimuli to the actin cytoskeleton. Pollen tube growth is regulated and guided directionally by factors from the female tissues (Cheung and Wu, 2001; Hepler et al., 2001; Palanivelu et al., 2003). Group-I formins, with their diverged and potentially highly glycosylated extracellular domains, could serve important signaling roles for these male–female interactions. How alterations in pollen tube formin activity affect pollen tube performance in vivo remains to be determined. Besides AFH1, at least two other group-I formins are expressed in Arabidopsis pollen (our unpublished observations). Analysis of pollen tube performance in tagged Arabidopsis insertional mutants defective in individual or multiple pollen-expressed formins and in transformed plants that upregulate pollen formin activity will provide insight into the biological role of formins in pollination.

METHODS

cDNA Isolation and Recombinant DNA Strategies

Oligonucleotide primers to the 5′ and 3′ ends of the coding region of AFH1 (At3g2550) were used in reverse transcriptase–mediated PCR from Arabidopsis thaliana pollen mRNA to obtain the full-length AFH1 cDNA used in this study. The AFH1 promoter (2142 bp upstream of the translational start site) was isolated by PCR from Arabidopsis genomic DNA. PCR-based strategies were used to generate variants of AFH1 and to construct their respective GFP fusion chimeric genes. The pollen promoter Lat52 (Twell et al., 1990) was used to express all chimeric genes described here, except that the AFH1 promoter was fused with GFP for the analysis of AFH1 promoter activity in pollen.

Microprojectile Transformation of Pollen, Pollen Tube Growth, and Observation

Transient transformation of tobacco pollen by microprojectile bombardment is used commonly in studies examining signaling and cytoskeletal proteins encoded by genes from different plant species, including Arabidopsis (Kost et al., 1999; Fu et al., 2001; Chen et al., 2002, 2003). Frozen pollen grains (stored at −20°C after isolation from freshly dehisced anthers and 2 h of dehydration at room temperature) were used. Microprojectile bombardment of pollen was performed as described previously (Chen et al., 2002). The previously described actin marker genes Lat52:GFP-mTalin (Kost et al., 1998) and Lat52:GFP-NtADF1 (Chen et al., 2002) were used for actin imaging. In each bombardment, 1 μg of DNA was used to coat the microprojectiles. The amount of Lat52:AFH1 transgene or its variants used in each experiment is indicated in the figure legends. For control pollen used in growth comparison experiments, different amounts of a mock plasmid, Lat52:GUS, were included so that every pollen grain sample was bombarded with microprojectiles that were coated with the same amount of total DNA. Bombarded pollen grains were cultured as described previously (Chen et al., 2003). Growth-related measurements and imaging were made between 4 and 8 h after plating. The cumulative growth of pollen tubes was determined by measuring a population of transformed pollen tubes in each culture at specific times after the initiation of culturing. Growth rates for individual pollen tubes were monitored over a span of 3 to 5 min between 4 and 6 h after culturing, when fluorescence was detectable and growth in control pollen tubes was still vigorous. Epifluorescence microscopy was performed with a Nikon Eclipse E800 microscope (Tokyo, Japan). Confocal imaging was performed with a Bio-Rad 600 (Richmond, CA) or a Zeiss 510 (Jena, Germany) system at the University of Massachusetts Central Microscopy Facility.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact A.Y. Cheung, acheung@biochem.umass.edu.

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