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Actin-Depolymerizing Factor Mediates Rac/Rop GTPase–Regulated Pollen Tube Growth

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Pollen tube elongation is a rapid tip growth process that is driven by a dynamic actin cytoskeleton. A ubiquitous family of actin binding proteins, actin-depolymerizing factors (ADFs)/cofilins, bind to actin filaments, induce severing, enhance depolymerization from their slow-growing end, and are important for maintaining actin dynamics in vivo. ADFs/cofilins are regulated by multiple mechanisms, among which Rho small GTPase–activated phosphorylation at a terminal region Ser residue plays an important role in regulating their actin binding and depolymerizing activity, affecting actin reorganization. We have shown previously that a tobacco pollen-specific ADF, NtADF1, is important for maintaining normal pollen tube actin cytoskeleton organization and growth. Here, we show that tobacco pollen grains accumulate phosphorylated and nonphosphorylated forms of ADFs, suggesting that phosphorylation could be a regulatory mechanism for their activity. In plants, Rho-related Rac/Rop GTPases have been shown to be important regulators for pollen tube growth. Overexpression of Rac/Rop GTPases converts polar growth into isotropic growth, resulting in pollen tubes with ballooned tips and a disrupted actin cytoskeleton. Using the Rac/Rop GTPase–induced defective pollen tube phenotype as a functional assay, we show that overexpression of NtADF1 suppresses the ability of NtRac1, a tobacco Rac/Rop GTPase, to convert pollen tube tip growth to isotropic growth. This finding suggests that NtADF1 acts in a common pathway with NtRac1 to regulate pollen tube growth. A mutant form of NtADF1 with a nonphosphorylatable Ala substitution at its Ser-6 position [NtADF1(S6A)] shows increased activity, whereas the mutant NtADF1(S6D), which has a phospho-mimicking Asp substitution at the same position, shows reduced ability to counteract the effect of NtRac1. These observations suggest that phosphorylation at Ser-6 of NtADF1 could be important for its integration into the NtRac1 signaling pathway. Moreover, overexpression of NtRac1 diminishes the actin binding activity of green fluorescent protein (GFP)–NtADF1 but has little effect on the association of GFP-NtADF1(S6A) with actin cables in pollen tubes. Together, these observations suggest that NtRac1-activated activity regulates the actin binding and depolymerizing activity of NtADF1, probably via phosphorylation at Ser-6. This notion is further supported by the observation that overexpressing a constitutively active NtRac1 in transformed pollen grains significantly increases the ratio of phosphorylated to nonphosphorylated ADFs. Together, the observations reported here strongly support the idea that NtRac1 modulates NtADF1 activity through phosphorylation at Ser-6 to regulate actin dynamics.

INTRODUCTION

Pollen tube growth is a central process in sexual reproduction in plants. Interactions between pollen and the stigma surface initiate pollen germination, which involves an asymmetric extrusion of the pollen cytoplasm through a germination pore to initiate the outgrowth of a pollen tube. Pollen tubes elongate by tip growth, and the actin cytoskeleton supports the intracellular trafficking of organelles and secretory vesicles along axially oriented actin cables throughout the shank of elongating pollen tubes (Hepler et al., 2001; Cheung et al., 2002). The secretory vesicles deliver new membrane and cell wall materials at the tip to support growth. The tip-ward flow of these organelles and vesicles moves along the edge of the tube, reaches the subapical region, reverses direction, and flows back toward the grain through the center of the tube, giving rise to a reverse fountain cytoplasmic streaming pattern (Hepler et al., 2001). The long actin filaments also extend to the subapical region but do not invade the apical region, referred to as the clear zone, where short actin bundles have been observed (Kost et al., 1999; Fu et al., 2001). The base of the clear zone is

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marked by the presence of a dense mesh of randomly oriented, short actin filaments (Geitmann et al., 2000; Vidali et al., 2001; Chen et al., 2002). Rapid actin remodeling at this subapical region is believed to be important in reversing cytoplasmic flows and supporting pollen tube elongation (Hepler et al., 2001).

Actin dynamics is regulated by a number of actin binding proteins (Staiger et al., 1997; Ayscough, 1998; McGough, 1998; Bamburg, 1999; Bamburg et al., 1999; Hepler et al., 2001; Higgs and Pollard, 2001). These include the G-actin binding protein profilin, the G- and F-actin binding proteins of the actin-depolymerizing factors (ADFs)/cofilins, and others that affect different aspects of actin polymerization and higher order organization. In pollen tubes, increasing the level of profilin or ADF results in the disruption of the normal actin cytoskeleton organization and the inhibition of pollen tube growth (Vidali et al., 2001; Chen et al., 2002). ADFs/cofilins are ubiquitous low molecular mass actin binding proteins important for regulating actin dynamics (Lappalainen and Drubin, 1997; Carlier, 1998; Bamburg, 1999). They enhance actin depolymerization by binding preferentially to the minus, slow-growing end of actin filaments, increasing the off rate of actin monomers, and by inducing filament severing (Carlier et al., 1997; McGough et al., 1997; Bamburg et al., 1999; McGough and Chiu, 1999). We have shown that green fluorescent protein (GFP)–NtADF1 (a tobacco pollen-specific ADF) and GFP-LIADF (a lily pollen ADF) associate prominently with the subapical actin mesh in elongating tobacco and lily pollen tubes, respectively, and with long actin cables in the shank as well (Chen et al., 2002). The ADF-rich actin mesh is located in a region in which the cytoplasm is slightly more alkaline than in the apex (Feijo et al., 1999; Hepler et al., 2001). A slightly alkaline pH is favorable for the actin-depolymerizing activity of some ADFs/cofilins (Hawkins et al., 1993; Maciver, 1998; Maciver et al., 1998; Bamburg, 1999), including NtADF1 (Chen et al., 2002). Therefore, it is plausible that ADFs play an important role in maintaining a high level of actin cycling activity at the subapical region to maintain an actin organization that is optimum for pollen tube growth and reorientation.

Pollen tubes are believed to respond to female signals and to regulate their growth characteristics as they navigate the female tissues, which sometimes involves pronounced reorientation of their growth trajectories (Cheung and Wu, 2001; Higashiyama et al., 2001; Johnson and Preuss, 2002). Understanding of the signal transduction pathways that transmit external cues to maintain a pollen tube actin cytoskeleton optimum for the pollen tube growth process is just emerging (Franklin-Tong, 1999; Hepler et al., 2001). In plants, the Rho subfamily of the Ras-related small GTPase superfamily is represented solely by a large number of Rac-like GTPases referred to as Racs (Winge et al., 1997, 2000; Kost et al., 1999) or Rops (Rhos in plants) (Yang, 2002). Among their multiple functional roles, Rho-GTPases are known to be important regulators for the actin cytoskeleton at the leading edge of motile cells, such as in the lamellipodia of migrating mammalian cells, in axon outgrowth, and in budding yeast (Hall, 1998; Mackey and Hall, 1998). They are activated by extracellular stimuli, such as growth factors in fibroblast cells, chemoattractants and chemorepellants in neural cells, and pheromones in yeast, and link membrane receptors via a signaling pathway to regulate the organization of the actin cytoskeleton as one of their downstream effects.

Several Rac/Rop GTPases have been shown to be important regulators of the polar pollen tube growth process. Overexpression of wild-type or constitutively active Atrac2 (Kost et al., 1999) or Rop1At (Li et al., 1999; Fu et al., 2001) from Arabidopsis converts polar tube growth into isotropic growth in that the transformed pollen tubes assume a spoon- or balloon-shaped morphology at the tip. The actin cytoskeleton in the expanded tip region becomes more extensive and highly disorganized. Overexpression of a tobacco pollen-expressed Rac/Rop GTPase, NtRac1, resulted in similar pollen tube defects (see below). When their activities are overexpressed in root hairs, another tip growth cell type, Rac/Rop GTPases also induce depolarization and altered actin cytoskeleton organization (Molendijk et al., 2001; Jones et al., 2002; Tao et al., 2002). Therefore, Rac/Rop GTPases appear to be similar to their counterpart Rho GTPases in animal and yeast in regulating actin organization, especially in polar growth cells. These Rac/Rop GTPases could activate a signaling pathway that acts directly on the actin cytoskeleton by regulating the activity of actin binding proteins (Bamburg, 1999; Lawler, 1999). These small GTPases also may signal changes in intracellular phospholipid conditions (Kost et al., 1999). This in turn would influence the activity of several actin binding proteins (Bamburg, 1999; Franklin-Tong, 1999; Staiger, 2000; Hepler et al., 2001) and intracellular Ca^{2+} conditions (Li et al., 1999), which, along with Ca^{2+} fluxes at the apex, are critical for pollen tube growth (Hepler et al., 2001). Therefore, as molecular switches that regulate multiple downstream pathways, Rac/Rop GTPases may simultaneously alter several cellular conditions that are important to the polar pollen tube growth process.

Recent studies have shown that the ability of mammalian ADFs/cofilins to depolymerize actin is regulated by Rho GTPase–activated phosphorylation at the Ser-3 position (Moriyama et al., 1996; Arber et al., 1998; Yang et al., 1998; Lawler, 1999; Maekawa et al., 1999; Sumi et al., 1999). Therefore, mammalian ADFs/cofilins play a key role in mediating one of the Rho GTPase–activated signaling pathways that ultimately lead to actin reorganization. Dephosphorylation would reactivate their activity (Agnew et al., 1995; Bamburg, 1999; Baum, 2002; Niwa et al., 2002). Although phosphorylation of an N-terminal region Ser residue may not necessarily be a universal regulatory mechanism for ADFs/cofilins (Lappalainen et al., 1997; Bamburg, 1999), plant ADFs characterized to date show a conserved Ser-6 (Lopez et al., 1996; Carlier et al., 1997; Dong et al., 2001a; Chen et al., 2002). The Ser-6 residue in the maize vegetative

tissue–expressed ZmADF3 can be phosphorylated in vitro by a Ca^{2+}-stimulated calmodulin domain–containing kinase activity present in French bean suspension cell extracts (Smertenko et al., 1998; Allwood et al., 2001). A substitution by Asp at Ser-6 (S6D) of ZmADF3 obliterates its in vitro actin-depolymerizing activity (Smertenko et al., 1998). In elongating pollen tubes, the actin binding ability of GFP-NtADF1 is enhanced by an Ala substitution at Ser-6 (S6A), which renders this amino acid position nonphosphorylatable, but it is abolished by the phospho-mimicking S6D substitution. This finding also correlates with the increasing ability of overproduced NtADF1(S6D), wild-type NtADF1, and NtADF1(S6A) to affect pollen tube growth (Chen et al., 2002). These observations are consistent with the phosphorylation of Ser-6 in NtADF1 as playing an important role in its in vivo actin binding and depolymerizing activity.

Here, we show that tobacco pollen ADFs exist in phosphorylated and nonphosphorylated forms, suggesting that phosphorylation could be a mechanism that regulates their activity in vivo. The impact of Rac/Rop GTPases on the plant cell actin cytoskeleton (Yang, 2002) and the importance of the charge property at the conserved Ser-6 residue among plant ADFs toward their actin-interacting ability suggest that a Rac/Rop GTPase and ADF functional linkage is possible. Using the Rac/Rop-induced pollen tube tip ballooning as a functional assay, we show that overexpression of tobacco pollen NtADF1 counteracts the isotropic growth phenotype induced by the tobacco pollen-expressed NtRac1. Furthermore, this ability is dependent on the charge property at Ser-6 in that NtADF1(S6A) enhances but NtADF1(S6D) diminishes the ability of the mutant proteins to counteract the effect of NtRac1 on pollen tube growth. Moreover, overexpression of NtRac1 in pollen tubes reduces the ability of GFP-NtADF1 to bind to actin cables, but it has no effect on the association of GFP-NtADF1(S6A) with the actin cytoskeleton. Together with the increase in the ratio of phosphorylated to nonphosphorylated forms of ADF in pollen grains that overexpress a constitutively active NtRac1, these findings suggest that NtRac1 mediates the phosphorylation of NtADF1 at Ser-6, modulating its actin binding and depolymerizing activity and thereby affecting actin reorganization during polar pollen tube growth.

RESULTS

Pollen ADFs Exist in Nonphosphorylated and Phosphorylated Forms

We observed previously that the actin binding and pollen tube growth-regulating activities of NtADF1 strongly depend on the charge property at Ser-6, with the S6A substitution enhancing and the S6D substitution inhibiting both of these activities (Chen et al., 2002). These observations strongly suggest that phosphorylation at Ser-6 plays an important role in regulating the actin-depolymerizing activity of NtADF1. However, in plants, a phosphorylated form of ADF has been observed only for the vegetative tissue–expressed AtADF1 in transformed Arabidopsis when it was overproduced to 30 to 50 times the endogenous ADF level (Dong et al., 2001b). Pollen protein blots reacted with anti-NtADF1 antibodies revealed protein species at z 17 kD (Figure 1A), approximating the predicted molecular mass of these proteins (Kim et al., 1993; Chen et al., 2002). When pollen proteins were resolved by two-dimensional SDS-PAGE, reaction with anti-NtADF1 antibodies on protein blots revealed two predominant reactive spots of similar molecular mass but different pI values (Figure 1B, top). Upon phosphatase treatment, only the more alkaline NtADF spot was preserved (Figure 1B, bottom), indicating that phosphorylation was responsible for the more acidic NtADF species. The presence of comparable levels of phosphorylated and nonphosphorylated forms of ADFs in mature pollen grains suggests that phosphorylation may be an important mechanism in regulating the activity of these actin binding proteins.

Overexpression of NtRac1 Induces Isotropic Pollen Tube Growth

We wanted to use the Rac/Rop GTPase–induced defective pollen tube phenotype (Kost et al., 1999; Li et al., 1999) as a biological assay to examine the functional relationship between NtRac1 and ADF in the NtRac1-regulated pathways that culminate in pollen isotropic growth. Figure 2A shows the phenotypes of pollen tubes transformed by a pollen-specific Lat52-GFP-NtRac1 chimeric gene. In a culture of microprojectile bombardment–transformed pollen tubes, the most severely defective pollen tubes assumed a balloon-tipped phenotype soon after germination, with little polar elongation (Figure 2A, first panel). Other pollen tubes elongated for 2 to 3 h, and their growth became isotropic and arrested when they were still relatively short (Figure 2A, second panel). The more moderately affected pollen tubes, which in general were less fluorescent, elongated at their tips for various distances before converting to ectopic growth and assuming a balloon-tipped morphology (Figure 2A, third and fourth panels). Tube elongation was aborted with the formation of the expanded pollen tube tip. Altogether, z 30 to 40% of 875 GFP-NtRac1–expressing pollen tubes assumed a balloon-tipped phenotype by 5 h after pollen germination. The level of the balloon-tipped phenotype increased with time, reaching z 60 and 80% of 1050 and 832 GFP-NtRac1–expressing tubes, respectively, by 7 and 9 h after germination. Untagged Lat52-NtRac1, when coexpressed with Lat52-GFP as a marker gene for transformation, induced similar defects, except that the time course and the level of balloon formation among transformed pollen tubes were shifted earlier and reached a higher level,

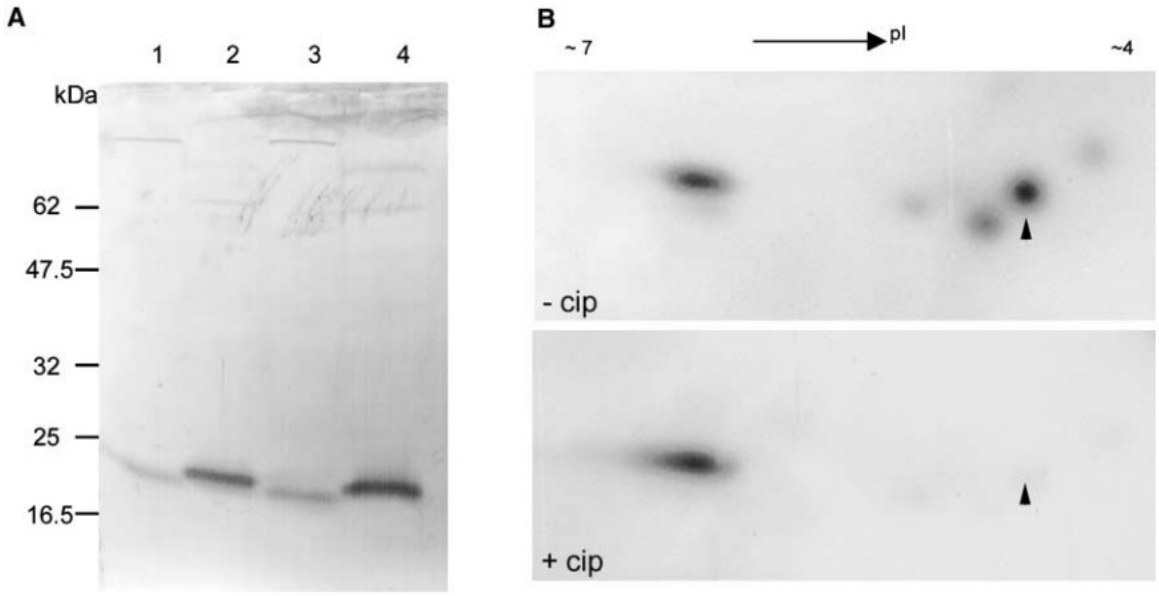

Figure 1. Tobacco Pollen ADFs Are Phosphorylated.

(A) An immunoblot of lily (lanes 1 and 2) and tobacco (lanes 3 and 4) pollen grain proteins after SDS-PAGE (17.5%). The blot was reacted with anti-NtADF1 antibodies and alkaline phosphatase–conjugated secondary antibodies. The molecular mass of the detected protein bands was z 17 kD, approximating the predicted molecular mass of pollen ADFs from these species (Kim et al., 1993; Chen et al., 2002). Lanes 2 and 4 contained fourfold concentrated versions of the samples loaded in lanes 1 and 3, respectively. The slightly retarded mobility of the NtADF protein bands in lanes 2 and 4 probably resulted from slightly higher salt concentrations in these concentrated protein samples.

(B) Immunoblots of tobacco pollen proteins after two-dimensional SDS-PAGE (17.5%). Both blots were reacted with anti-NtADF1 antibodies and horseradish peroxidase–conjugated secondary antibodies. NtADF spots were revealed by chemiluminescence detection. Both gels contained proteins that were not (−cip) or were (+cip) treated with alkaline phosphatase. Approximately 60 μg of total pollen protein was loaded in the −cip sample. The same amount of protein was treated with alkaline phosphatase in the +cip sample. The amount of protein loaded in the +cip sample was adjusted so that the intensity of the more alkaline NtADF species in the −cip and +cip samples was comparable to highlight the difference in the intensity of the more acidic NtADFs. Arrowheads point to the location of the more acidic NtADF spot. The minor protein spots detected by NtADF1 antibodies at the acidic range of the blot may represent minor pollen-expressed NtADFs.

respectively, suggesting higher signaling activity in the untagged protein (data not shown). Coexpression of the Rac/Rop GTPase negative regulator Rac-GDI (guanine nucleotide dissociation inhibitor), which maintains these small GTPases in their GDP-bound inactive form (Mackey and Hall, 1998), significantly reduced the level of NtRac1-induced balloon tip formation to z 6.5% of all transformed tubes at 3 h after germination. This finding confirms the notion that the isotropic pollen tube growth defect was induced by enhanced NtRac1 activity derived from the overexpressed NtRac1 or GFP-NtRac1.

To date, GFP-mouse talin (GFP-mTalin) (Kost et al., 1998) is the most commonly used marker for the actin cytoskeleton in live plant cells. Coexpression of GFP-mTalin with untagged NtRac1 showed that the actin cytoskeleton still was arranged primarily in a long axially oriented organization in pollen tubes that were just beginning to convert from polar to isotropic growth (Figure 2C). In fully ballooned pollen tubes, the GFP-mTalin–labeled actin cytoskeleton was very extensive and disorganized (Figure 2D). Relative to normal elongating pollen tubes (Figure 2B) (Chen et al., 2002), the actin cables in NtRac1-overexpressing pollen tubes were significantly more bundled along the shank, invaded, and occupied the entire expanded tube tip region (Figure 2D). The actin cytoskeleton revealed by GFP-mTalin appeared to be composed of mixed populations of thick bundles and finer cables, but a discrete pattern was not observed among many of these kinds of tubes.

NtADF1 Suppresses the NtRac1-Induced Isotropic Pollen Tube Growth Defect

NtADF1 is the most predominant pollen ADF (Chen et al., 2002). When pollen was cotransformed with Lat52-GFP-

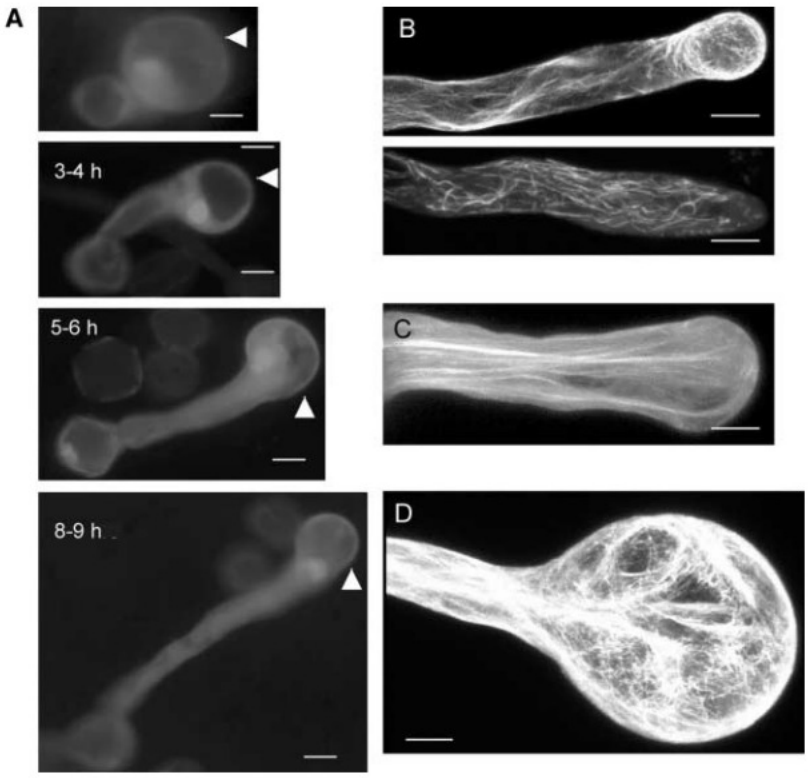

Figure 2. NtRac1 Induces Ectopic Pollen Tube Expansion and the Disruption of Normal Actin Organization.

(A) Epifluorescent micrographs of the GFP-NtRac1–induced pollen tube phenotype. The top panel shows a pollen tube that exhibited a balloon-tipped phenotype soon after germination. The other panels show pollen tubes that had elongated for various times before terminating in a balloon-tipped phenotype. Pollen tubes similar to those shown in the top two panels are most characteristic of those that have ballooned at early time points (3 to 4 h) after germination. The pollen tubes shown in the third and fourth panels are characteristic of those that have elongated farther and for longer periods of time before balloon formation and growth arrest. Arrowheads indicate the ballooned tip region. All images were taken by exposure time automatically set by the camera. In general, pollen tubes that ballooned early were more fluorescent than those that maintained a normal morphology at the same time point, suggesting a correlation of the phenotype with the level of GFP-NtRac1 expression (Cheung et al., 2003). Cotransformation using Lat52-GFP as a marker gene for transformation and Lat52-NtRac1 as the regulator gene resulted in qualitatively similar observations except that the more severe phenotypes, such as those shown in the top two panels, predominated even at the earliest time point. Bars = 10 μm.

(B) Confocal images of two pollen tubes transformed by Lat52-GFP-mTalin. GFP-mTalin revealed long actin cables aligned along the long axis of the pollen tube shank, which is characteristic of elongating pollen tubes (Kost et al., 1998, 1999; Vidali et al., 2001; Chen et al., 2002). The tip of the pollen tube shown in the top panel pointed upward slightly, leading to the impression of a bulge at the tip when sections across the entire tubes were projected. The actin filaments seen at the center of the bulge represent fine actin filaments close to the cortical region of the tube tip. Typically, long actin cables do not invade the apical region, as shown in the bottom panel. The pollen tubes shown here had the best tube morphology and actin cytoskeleton structure among transformed pollen tubes that expressed levels of GFP-mTalin detectable with our confocal microscope.

(C) and **(D)** Confocal images of pollen tubes cotransformed by Lat52-NtRac1 and Lat52-GFP-mTalin to visualize the actin cytoskeleton in tubes affected by the overexpression of NtRac1.

(C) This pollen tube was converting from tip growth to isotropic growth. The shank actin cables still were aligned axially, but they started to extend farther into the tip region than in normally tip-elongating tubes.

(D) A balloon-tipped pollen tube showing highly bundled actin cables in the shank and an extensive network of intertwining actin cables in the ballooned tip.

NtRac1 and Lat52-NtADF1, the level of balloon-tipped pollen tubes was negligible at 3 h after bombardment and germination, when z 30% of the control pollen tubes transformed by Lat52-GFP-NtRac1 and the mock Lat52 β-glucuronidase had already developed ballooned tips (Figure 3A). The more extensive actin cytoskeleton network observed in NtRac1-overexpressing, balloon-tipped pollen tubes suggests an increased level of actin polymerization in these tubes (Figure 2D). Therefore, suppression of the GFP-NtRac1–induced pollen tube defects by coexpressed NtADF1 is likely to be

the consequence of the increased actin-depolymerizing activities derived from the overexpressed NtADF1.

Ser-6 in NtADF1 Is Important for Its Ability to Suppress the Effect of NtRac1

The ability of NtADF1 to counteract the effect of NtRac1 suggests that they act in a common signaling pathway to regulate actin polymerization and polar pollen tube growth. To gain insight into how they interact, we took advantage of the stronger activity of a constitutively active form of NtRac1 resulting from a G15V substitution [referred to as NtRac1(G15V) below] or of GFP-NtRac1(G15V) in inducing pollen tube isotropic growth. Overexpression of GFP-NtRac1(G15V) affected >70% of all transformed pollen tubes by 3 h after germination (Figure 3B). This higher level of phenotypic effects allowed the differential NtRac1-counteracting activities derived from different cotransforming genes to be resolved unambiguously (see below).

When pollen tubes were cotransformed with Lat52-GFP-NtRac1(G15V) and Lat52-NtADF1, the level of balloon-tipped pollen tubes was reduced to z 30% of the total transformed pollen tube population at 3 h after germination (Figure 3B). Because Ser-6 in NtADF1 has been suggested to be important for its actin binding and depolymerizing activities (Chen et al., 2002), we examined whether it is important for its ability to suppress the effect of NtRac1 in growing pollen tubes. When the nonphosphorylatable NtADF1(S6A), which binds actin very strongly (Chen et al., 2002), was coexpressed with GFP-NtRac1(G15V), the level of the balloon-tipped tubes declined to z 20% of all transformed pollen tubes at 3 h after germination. On the other hand, when the phosphomimicking NtADF1(S6D), which does not bind to actin (Chen et al., 2002), was coexpressed with GFP-NtRac1(G15V), the level of balloon-tipped pollen tubes remained at z 65% (Figure 3B), not significantly different from the level observed in the control GFP-NtRac1(G15V)–expressing tubes.

In microprojectile bombardment–transformed pollen tube cultures, gene expression levels from GFP-tagged transgenes, as reflected by green fluorescence intensity, or from the biological effect that nontagged transgenes have on pollen tube growth correlate directly with the input amount of the transgenes (Chen et al., 2002; Chen, 2002). Although GFP-NtADF1(S6A) and GFP-NtADF1 were biologically weaker than their nontagged counterparts (Chen et al., 2002), they nevertheless attenuated the balloon-tipped phenotype induced by NtRac1 (expressed from 1.25 μg of transforming Lat52-NtRac1), whereas GFP-NtADF1(S6D) failed to do so (data not shown). The green fluorescence in pollen transformed by each of these three GFP-labeled NtADF1 transgenes was comparable. These observations further demonstrated that the differential effects of suppressing the GFP-NtRac1–induced phenotype by the three forms of NtADF1 shown in Figure 3B had resulted not from differential gene expression but from their different biological activities.

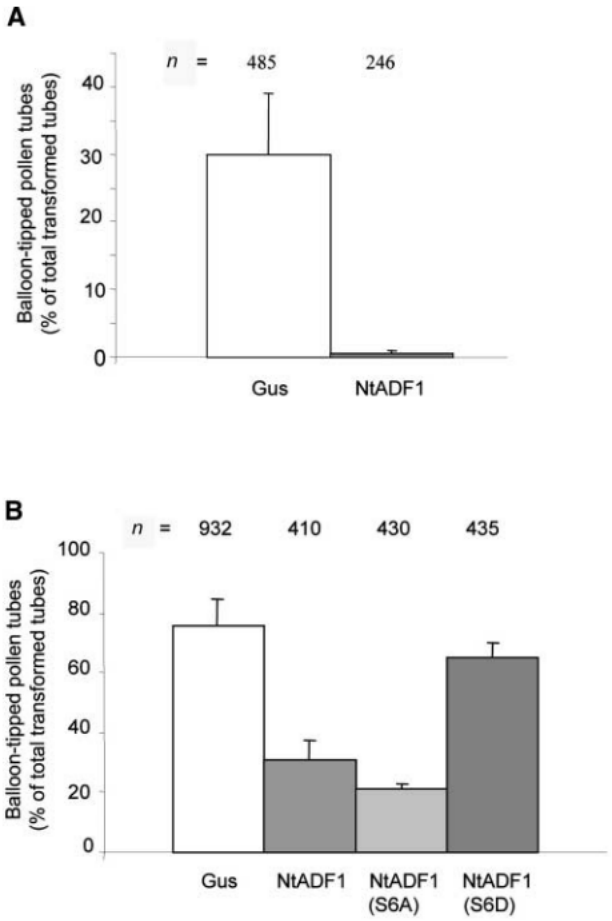

Figure 3. NtADF1 Counteracts the Ability of NtRac1 to Induce Isotropic Pollen Tube Growth, and Ser-6 in NtADF1 Is Important for This Activity.

Pollen grains were cotransformed with Lat52-GFP-NtRac1 **(A)** or Lat52-GFP-NtRac1(G15V) and Lat52-NtADF1, Lat52-NtADF1(S6A), or Lat52-NtADF(S6D), as indicated **(B)**. Lat52-GUS was included in the control samples to ensure that comparable amounts of DNA were introduced into the transformed pollen grains. Pollen tubes were scored at 3 h after germination. Pollen tube tips that were equal to or larger than the diameter of the pollen grains were scored as balloon tipped. Data were obtained from three independent experiments. In each experiment, every condition was tested three times. The total number of pollen tubes scored (n) for each condition is indicated above the data bars. Error bars indicate standard deviations. t test analysis of the data shown in **(B)** indicates the level of significance to be P = 0.000023, 0.000016, and 0.499 for the NtADF1, NtADF1(S6A), and NtADF1(S6D) data, respectively, relative to the control data. The level of significance between the NtADF1 data and the NtADF1(S6A) and NtADF1(S6D) data was P = 0.08588 and 0.00013, respectively.

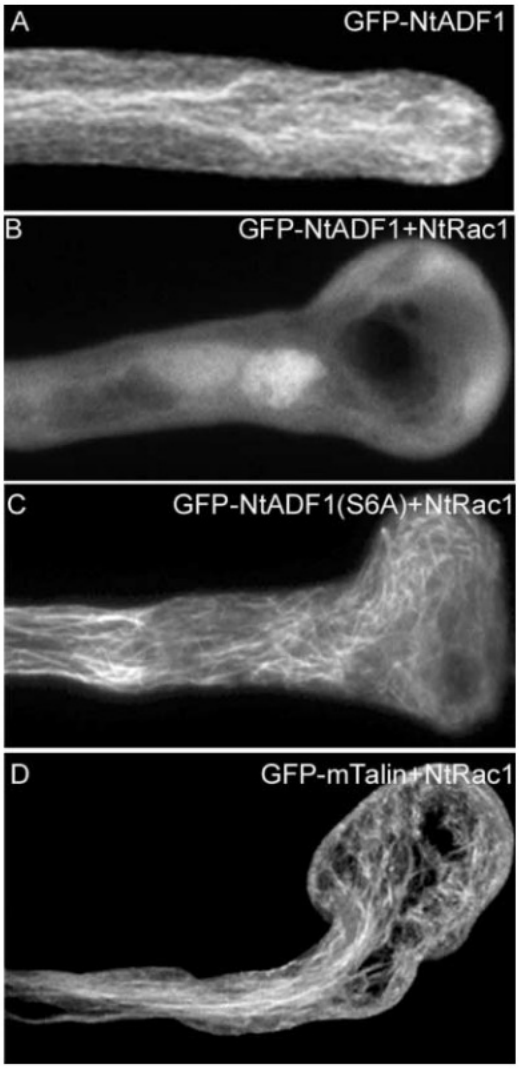

Figure 4. NtRac1 Reduces Interactions between NtADF1 and Actin in Transformed Pollen Tubes.

The recombinant protein expressed in each of the transformed pollen tubes shown is indicated.

(A) A normally elongating transformed pollen tube showing the GFP-NtADF1 association with long actin cables in the shank and an actin mesh at the subapical region, as reported previously (Chen et al., 2002).

(B) A balloon-tipped pollen tube that was cotransformed with Lat52-NtRac1 and Lat52-GFP-NtADF1 showing green fluorescence throughout the tube but no discrete association with filamentous structures. By contrast, some thin GFP-labeled actin cables were observed amid a strong cytosolic green fluorescent signal, reflecting the effect of the overexpressed NtRac1, in tubes that still maintained the normal tube morphology at the same time point (data not shown).

(C) A balloon-tipped pollen tube that was transformed with Lat52-NtRac1 and Lat52-GFP-NtADF1(S6A) showing that the association between the GFP-tagged mutant NtADF1 and the actin cables was preserved, indicating that the actin binding activity of GFP-NtADF1(S6A) is resistant to the effect of NtRac1.

(D) A highly disorganized actin network in the ballooned tip region, similar to but less bundled than the GFP-mTalin, revealed actin cytoskeleton in NtRac1-coexpressing balloon-tipped pollen tubes.

NtRac1 Reduces the Ability of GFP-NtADF1 to Associate with F-Actin

The suppression of the NtRac1-induced phenotype by NtADF1 (Figure 3) suggests a functional linkage between this small GTPase and the actin binding protein. To determine if NtRac1 regulates the interaction between NtADF1 and actin filaments, pollen tubes were cotransformed with Lat52-NtRac1 and Lat52-GFP-NtADF1. In transformed pollen tubes whose expanded pollen tube tip phenotype had not been suppressed effectively, GFP-NtADF1 did not associate with actin cables (Figure 4B). This differs from their behavior in control pollen tubes that expressed GFP-NtADF1 alone (Figure 4A) (Chen et al., 2002). On the other hand, when the nonphosphorylatable GFP-NtADF1(S6A) was coexpressed with NtRac1 in transformed pollen tubes, the GFP-tagged protein still associated efficiently with actin cables (Figure 4C), similar to its behavior in normally elongating pollen tubes (Chen et al., 2002). Contrary to normal pollen tubes, the GFP-NtADF1(S6A) localization pattern revealed an actin network that was highly disorganized at the expanded tip region of the NtRac1-coexpressing balloon-tipped tubes (Figure 4C). These observations strongly suggest that the NtRac1-mediated phosphorylation at Ser-6 of NtADF1 weakened its interaction with actin but that the activity of NtADF1(S6A) was unaffected because of the nonphosphorylatable Ala substitution.

Overexpression of NtRac1(G15V) in Transformed Pollen Grains Alters the Distribution of Phosphorylated and Nonphosphorylated Forms of ADF

Pollen grains from Agrobacterium-transformed plants that overexpressed the constitutively active NtRac1(G15V) were highly defective in their ability to develop normal pollen tubes in vitro (Figure 5B). In pollen germination cultures, these pollen grains were competent to hydrate, and the percentage of grains that initiated a polar outgrowth was comparable to that observed in control pollen. However, none of the extruded buds could assume polar elongation. Ectopic growth predominated almost immediately upon tube emergence, and growth was aborted without apparent tube elongation (Figure 5B). Protein blot analysis showed that the level of total pollen ADFs in these transformed pollen grains was comparable to that in control wild-type grains (data not shown). However, immunoblots from two-dimensional SDS-PAGE of proteins from these NtRac1(G15V)-overexpressing pollen grains showed at least a 3.5-fold increase in the ratio of phosphorylated to nonphosphorylated NtADFs relative to that seen in control wild-type pollen (cf. Figures 1B, top, and 5A). The vast

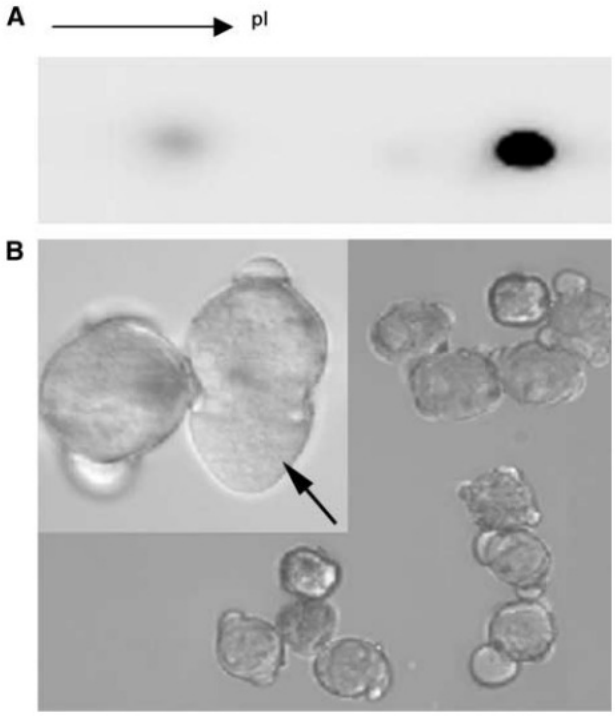

Figure 5. Overexpressing NtRac1(G15V) Increases the Ratio of Phosphorylated to Nonphosphorylated Forms of Pollen ADF.

(A) Immunoblot of proteins from pollen grains isolated from a representative Lat52-NtRac1(G15V)-transformed plant after two-dimensional SDS-PAGE. Densitometric scanning of immunoblots prepared from three different preparations of pollen proteins indicates a ratio of at least 3.5 for the phosphorylated ADF to the nonphosphorylated form, more than a threefold increase from that observed among ADFs from control wild-type pollen. Approximately 60 μg of pollen proteins was loaded.

(B) Germination cultures of pollen grains from a representative Lat52-NtRac1(G15V)-transformed plant. The arrow in the inset points to the expanded tip of a just-emerged pollen tube, which is characteristic of most of the germinating pollen grains.

increase in the ratio of phosphorylated to nonphosphorylated forms of NtADFs observed in these transformed pollen grains provides direct evidence that increasing NtRac1 signaling activity upregulates pollen ADF phosphorylation.

DISCUSSION

The importance of ADFs/cofilins in regulating actin dynamics in other organisms (Bamburg, 1999) and their prominent association with actin at a pollen tube region where cytosolic conditions may favor its actin-depolymerizing activity (Chen et al., 2002) strongly suggest that pollen ADF plays an important role in regulating pollen tube tip growth. By having its activity modulated by a Rac/Rop GTPase–activated activity, as demonstrated here, pollen ADF serves as an intermediate in a signaling pathway activated by these small GTPases that regulates actin dynamics in pollen tube polar outgrowth. Pollen Rac/Rop GTPases could mediate multiple regulatory signals from the female tissues to regulate the pollen tube growth process. Pollen ADF then would be responsive to the changing activity of Rac/Rop GTPases as affected by these extracellular signals, translating them into levels of actin-depolymerizing activity that support the optimum level of actin cycling for pollen tube growth through different female tissue environments.

The Actin-Interacting Activity of NtADF1 Is Dependent on Ser-6 and Is Likely to Be Regulated by Phosphorylation

Ser-6 phosphorylation has been suggested as a possible mechanism to modulate plant ADF activity (Smertenko et al., 1998; Allwood et al., 2001). However, the presence of phosphorylated ADFs under normal cellular conditions has not been firmly established before this study. We have shown previously that the relative strength of actin binding and depolymerizing activity increases from the phosphomimicking NtADF1(S6D) to wild-type NtADF1 whose Ser-6 may cycle between a phosphorylated and a nonphosphorylated state to the nonphosphorylatable NtADF1(S6A) (Chen et al., 2002). The presence of both phosphorylated and nonphosphorylated ADFs in tobacco pollen grains (Figure 1B) indicates that phosphorylation occurs normally in these proteins. Therefore, all observations to date on NtADF1 and total pollen ADFs strongly support a regulatory mechanism for pollen ADF via phosphorylation at Ser-6.

The approximately equal distribution between phosphorylated and nonphosphorylated NtADFs in mature pollen grains (Figure 1B) is significantly higher than that observed in plant vegetative cells (Dong et al., 2001b) but is more similar to that observed in cultured mammalian neuronal cells (Meberg et al., 1998). Pollen grains and neuronal cells share the ability to initiate the polar outgrowth of cytoplasmic extensions. Therefore, different cell types may regulate their actin-depolymerizing activity differentially to meet the levels of actin cycling needed to sustain different growth characteristics. The identification of enzymes that phosphorylate and dephosphorylate pollen and vegetative ADFs and how they are regulated will help advance our understanding of how the optimum equilibrium between the two forms of ADFs is maintained in different cell types.

NtRac1 Regulates the Activity of NtADF1, Which Directly Regulates Actin Dynamics in Pollen Tubes

NtADF1(S6A), NtADF1, and NtADF1(S6D) differentially counteract the ability of GFP-NtRac1(G15V) to induce isotropic

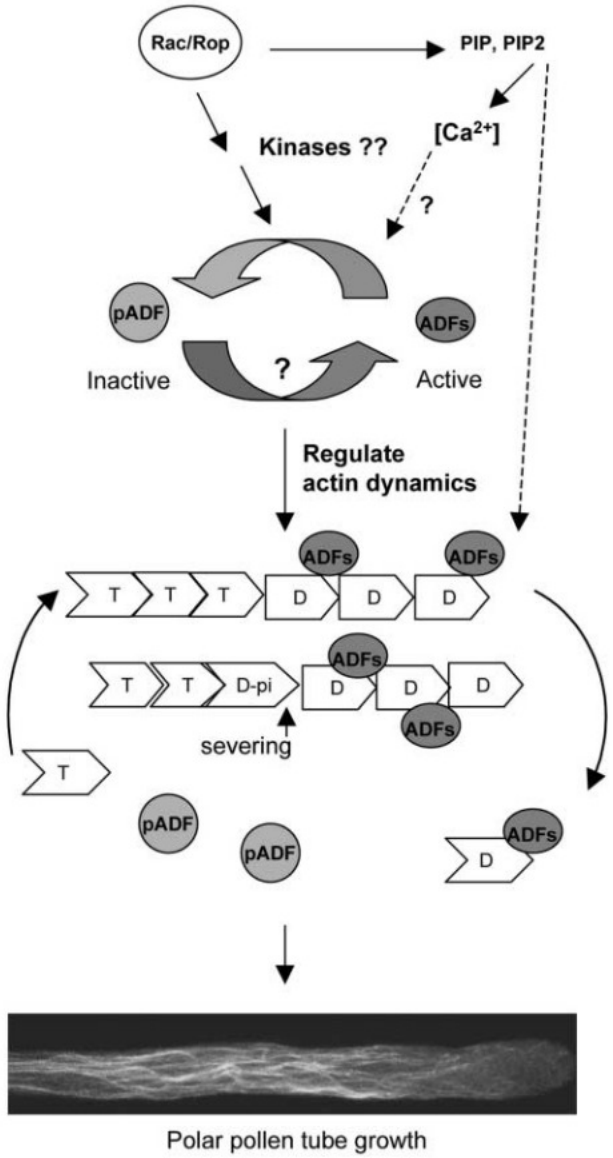

Figure 6. A Model for Rac/Rop GTPase Signaling to the Pollen Tube Actin Cytoskeleton via ADF.

The cycling of actin polymerization and depolymerization is shown in the middle of the figure. Arrow blocks with T or D embedded within represent actin monomers. T indicates ATP-bound, D indicates ADP-bound, and D-pi indicates ADF- and Pi-bound actin monomers (Bamburg, 1999). The minus (pointed) and plus (barbed) ends of the actin filaments are shown accordingly. The equilibrium of nonphosphorylated ADF and phosphorylated ADF (pADF) and their activity is depicted as regulated by Rac/Rop GTPase–activated, yet to be identified enzyme(s). ADF dephosphorylation and its regulatory mechanisms also remain to be revealed. The possible effect of Rac/Rop-regulated phospholipid PIP and PIP2 conditions on ADF activity is indicated by the long dotted arrow. The phosphoinositide-mediated changes in intracellular Ca^{2+} conditions also may affect the phosphorylation or dephosphorylation of ADFs (indicated by the short dotted arrow) if the enzymes involved are regulated by this ion. Active ADFs bind to actin and have higher affinity for ADP actin; thus, they preferentially bind to and enhance depolymerization from the pointed end (Carlier et al., 1997; Bamburg, 1999). ADF also binds to actin cooperatively, so multiple ADF molecules may be bound to an actin filament, inducing filament severing (McGough et al., 1997; McGough and Chiu, 1999). pADF does not associate with actin cables. The typical actin cytoskeleton structure as revealed by GFP-NtADF1 labeling in elongating pollen tubes (Chen et al., 2002) is shown at bottom. The level of actin cycling that maintains the actin cytoskeleton organization in elongating pollen tubes would depend on the combined activities from regulated levels of ADF, pADF, and other actin binding proteins to affect polar tube growth (Hepler et al., 2001).

pollen tube growth (Figure 3B). Based on the relative actin binding activity of their respective GFP-tagged proteins (Chen et al., 2002), the observations reported here are consistent with the conclusion that the overexpressed wild-type and S6A forms of NtADF1 have increased the level of actin-depolymerizing activities in these pollen tubes to overcome the effect of GFP-NtRac1(G15V) to increase actin polymerization. The failure of NtADF1(S6D) to bind to actin filaments would have precluded its ability to depolymerize actin, resulting in its depressed ability to counteract the GFP-NtRac1(G15V)-induced phenotype (Figure 3B).

In Lat52-GFP-NtADF1– and Lat52-NtRac1–cotransformed pollen tubes, the absence of a GFP-NtADF1 association with filamentous or cable structures (Figure 4B) suggests that one of the effects of overexpressing NtRac1 is a reduction in the actin binding and depolymerizing activity of NtADF1. Thus, the insensitivity of the nonphosphorylatable GFP-NtADF1(S6A) to the coexpressed NtRac1 (Figure 4C), together with the failure of the phospho-mimicking GFP-NtADF1(S6D) to bind to actin cables (Chen et al., 2002), suggest that overexpressing NtRac1 most likely affected NtADF phosphorylation or dephosphorylation activity in these transformed pollen tubes, resulting in increased NtADF phosphorylation and inactivation of their actin binding and depolymerizing activities. The intermediate strength of NtADF1 in binding to actin (Chen et al., 2002) and suppressing the NtRac1-induced phenotype (Figure 3B) is consistent with a cycling of Ser-6 in an uncharged actin binding form and a phosphorylated non-actin binding form in vivo. These observations strongly suggest an NtRac1-activated pathway involving the GTPase-regulated phosphorylation of ADFs/cofilins (Bamburg, 1999; Lawler, 1999).

However, ADFs/cofilins also are inhibited by phosphatidylinositol-4-monophosphate (PIP) and PIP2 binding (Gungabissoon et al., 1998; Bamburg, 1999). Although the binding site for PIP and PIP2 has not been determined for any plant ADF, studies in animal cells suggests that both the N and C termini of cofilins can bind these phospholipids

(Yonezawa et al., 1991; Kusano et al., 1999). Overexpression of Atrac2 in pollen tubes induces the accumulation of PIP2, leading to the suggestion that it acts in a common pathway with this small GTPase, as in mammalian Rho GTPase signaling (Kost et al., 1999). Therefore, the possibility that the NtRac1-induced inhibition of the NtADF1–actin interaction could have resulted, at least partially, from changes in intracellular phospholipid concentration or distribution cannot be excluded. However, the inability of NtRac1 to affect the actin binding activity of the nonphosphorylatable NtADF1(S6A) (Figure 4C) and the vast increase in the ratio of phosphorylated to nonphosphorylated ADFs in NtRac1(G15V)-overexpressing pollen grains (Figure 5A) provide strong support for the idea that NtRac1-induced phosphorylation plays a major role in directly regulating the actin binding and depolymerizing activity of NtADF1 to affect actin reorganization.

The plant enzymes involved most likely have diverged from those used by animal cells, because sequences homologous with the animal ADF/cofilin kinases and phosphatases have not been detected among plant genome databases (Arabidopsis Genome Initiative, 2000). Calcium-stimulated calmodulin domain kinases form a large family of enzymes unique to plants and protists that mediate a broad range of cellular processes (Harmon et al., 2000). The in vitro phosphorylation of Ser-6 in the maize vegetative ZmADF3 by a calcium-stimulated calmodulin domain kinase activity from French bean has led to the conclusion that plant ADFs are phosphorylated by this type of enzyme (Smertenko et al., 1998; Allwood et al., 2001). In pollen, NtADF1 phosphorylation activity has not been detected in extracts prepared according to the conditions described for the French bean protein extract (Smertenko et al., 1998; Allwood et al., 2001). However, pollen-expressed and vegetative tissue–expressed ADFs are known to be quite diverged in their sequences (Lopez et al., 1996; Chen et al., 2002), to the extent that their antibodies do not cross-react with proteins from the reciprocal source (Smertenko et al., 2001; our unpublished observations). It is possible that pollen ADF phosphorylation enzymes may be different from those in vegetative tissues or suspension cultured cells. Therefore, for NtADF1, it remains to be determined whether the NtRac1-stimulated phosphorylation strongly implicated in these studies involves an enzyme, or enzymes, similar or dissimilar to the calcium-stimulated calmodulin domain–containing enzyme found in French bean. Moreover, how the dephosphorylation of phosphorylated NtADFs is regulated and whether the system is responsive to Rac/Rop GTPase signaling remain to be explored.

A Proposed Model of a Rac/Rop GTPase–Activated Pathway to Regulate Actin Dynamics by Regulating the Activity of ADFs

The experiments described here have focused on the regulation of pollen ADF by a Rac/Rop GTPase–activated activity in a signaling pathway that has major impact on pollen tube growth (Figure 6). A signaling linkage between phosphoinositides and cytosolic Ca^{2+} conditions (Figure 6) is well established in animal cells (Clapman, 1995), and it also may be an important aspect of the regulation of intracellular Ca^{2+} conditions in pollen tubes (Franklin-Tong, 1999). Ca^{2+} fluxes at the pollen tube tip and a tip-focused Ca^{2+} concentration gradient are critical to pollen tube growth (Hepler et al., 2001). The downregulation of Rop1At has been said to affect both of these regulatory aspects involving Ca^{2+} (Li et al., 1999). Therefore, besides the potential that Rac/Rop GTPase–regulated phospholipid levels may directly affect the activity of pollen ADFs, phosphoinositides may indirectly affect ADF activity via their effect on intracellular Ca^{2+} (Figure 6). Furthermore, if pollen ADF phosphorylation involves a calcium-stimulated enzyme, changes in cytosolic Ca^{2+} concentration should affect ADF activity. Calcium-dependent and -independent dephosphorylation of ADFs/cofilins have been observed (Bamburg, 1999). Changes in Ca^{2+} concentration also could affect the balance between ADF and phosphorylated ADF if the plant phosphatase(s) involved is regulated by calcium.

Changes in the ratio between the active nonphosphorylated and the inactive phosphorylated forms of ADF will change the balance of actin depolymerization and polymerization, altering the rate of actin cycling and ultimately actin organization. Under the experimental condition of overexpressing Rac/Rop GTPases, the outcome is an actin network apparently overpolymerized to sustain normal, polar growth, thus resulting in ectopic expansion and, in the most severe case, isotropic growth (Figures 2 and 4). In normally elongating pollen tubes, the Rac/Rop GTPase activity must be regulated closely to avoid perturbing the balance of ADF activities and other downstream effectors that also affect actin polymerization so that an actin cytoskeleton most optimum for growth (Figure 6, bottom) can be maintained. As substrates for a Rac/Rop GTPase–activated phosphorylation that regulates their actin-depolymerizing activity, pollen ADFs can rapidly adjust their activity level in response to extracellular stimuli mediated by these small GTPases to contribute to the optimum level of actin cycling as pollen tubes elongate and change their orientation in the pistil.

METHODS

Recombinant DNA Methodology

Standard recombinant methodology (Ausubel et al., 2000) was followed. NtRac1, NtADF1, cDNA clones, constitutively active NtRac1(G15V), NtADF1(S6A), and NtADF1(S6D) mutations, green fluorescent protein (GFP) fusion to these sequences, and the Arabidopsis Rac-GDI (guanine nucleotide dissociation inhibitor) have been described (Chen et al., 2002; Tao et al., 2002). The pollen-specific Lat52 promoter (Twell et al., 1990) was used to express all chimeric genes. Chimeric genes were cloned in the Stratagene

pBluescript II SK+ vector. GFP–mouse talin was excised from a 35S-GFP–mouse talin (Kost et al., 1998) and placed behind the Lat52 promoter (Cheung et al., 2002).

Microprojectile Bombardment Transformation of Tobacco Pollen

Mature tobacco (Nicotiana tabacum) pollen was transformed by microprojectile bombardment as described (Chen et al., 2002; Cheung et al., 2002). Ten milligrams of pollen grains was used in each bombardment. For experiments that involved cotransformation, 5 μg of each of the transgenes was used unless indicated otherwise. In control samples, Lat52 β-glucuronidase was used as a mock transgene in place of the experimental transgenes to maintain comparable amounts of total DNA. Each pollen sample was bombarded twice to obtain a higher transformation frequency. After bombardment, pollen grains were cultured in a moist chamber. Pollen tube phenotypes were scored between 3 and 9 h after plating. Pollen tube tips that were equal to or larger than the diameter of the pollen grains were scored as balloon tipped. For experiments in which the NtRac1-induced phenotype was scored quantitatively, all conditions were tested three times in each experiment. All experiments were repeated at least three times with qualitatively similar results.

Plant Transformation

Agrobacterium tumefaciens Ti plasmid–mediated leaf disc transformation was performed as described (Chen et al., 2002) to obtain Lat52-NtRac1(G15V)–transformed tobacco plants. Pollen grains from the T2 generation of transformed plants were used in protein analysis.

Microscopic Observations of Elongating Pollen Tubes

Images of elongating pollen tubes and data processing for presentation were performed essentially as described (Chen et al., 2002; Cheung et al., 2002). Z-series images were collected in 1-μm steps and projected by Confocal Assistant imaging software (Bio-Rad).

Analysis of Pollen Proteins

Tobacco pollen grains were collected from greenhouse-grown plants, and lily (Lilium longiflorum) pollen grains were collected from plants maintained in growth chambers. For analysis of pollen actin-depolymerizing factors, proteins were extracted in 50 mM Tris-HCl, pH 7, 100 mM NaCl, 0.1% Triton X-100, 0.05% SDS, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1% proteinase inhibitor cocktail, and 0.05% casein. Calf intestinal alkaline phosphatase treatment, preparation of proteins for two-dimensional SDS-PAGE, and electrophoresis were performed as described (Minamide et al., 1997), except that the first dimension used a standard isoelectric focusing gel. Immunodetection of NtADFs used antibodies against recombinant His-tagged NtADF1 (Wong, 2001) followed by chemiluminescent detection according to the manufacturer's instructions (Perkin Elmer).

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

Accession Numbers

GenBank accession numbers for the proteins described in this article are as follows: AY029330 (NtRac1), AY029330 (NtADF1), and AAF21198 (AtRac-GDI).

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