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Control of Pollen Tube Tip Growth by a Rop GTPase–Dependent Pathway That Leads to Tip-Localized Calcium Influx

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We have shown that Rop1At, a pollen-specific Rop GTPase that is a member of the Rho family of small GTP binding proteins, acts as a key molecular switch controlling tip growth in Arabidopsis pollen tubes. Pollen-specific expression of constitutively active *rop1at* mutants induced isotropic growth of pollen tubes. Overexpression of wild-type Arabidopsis *Rop1At* led to ectopic accumulation of Rop1At in the plasma membrane at the tip and caused depolarization of pollen tube growth, which was less severe than that induced by the constitutively active *rop1at.* These results indicate that both Rop1At signaling and polar localization are critical for controlling the site of tip growth. Dominant negative *rop1at* mutants or antisense *rop1at* RNA inhibited tube growth at 0.5 mM extracellular Ca^{2+}, but growth inhibition was reversed by higher extracellular Ca^{2+}. Injection of anti-Rop antibodies disrupted the tip-focused intracellular Ca^{2+} gradient known to be crucial for tip growth. These studies provide strong evidence for a Rop GTPase–dependent tip growth pathway that couples the control of growth sites with the rate of tip growth through the regulation of tip-localized extracellular Ca^{2+} influxes and formation of the tip-high intracellular Ca^{2+} gradient in pollen tubes.

INTRODUCTION

Tip growth is an extreme form of polarized cell growth that is widespread in eukaryotic kingdoms and is associated with cells capable of directing their growth according to external guidance cues. Examples of tip growth include pollen tube and root hair growth in higher plants, nerve growth and axon guidance in animals, and hyphal growth in fungi. Tip growth requires the establishment of a defined growth site at the plasma membrane and the continuous propagation of this site to which Golgi-derived vesicles are targeted and fused (Yang, 1998). Thus, tip growth involves both spatial control (i.e., determination of growth sites) and temporal regulation (i.e., the rate of growth site propagation). However, the molecular mechanisms governing tip growth are poorly understood.

Male gametophyte pollen has been used as a model system to study tip growth mechanisms (Steer and Steer, 1989). Pollen tube growth is an essential developmental process that occurs during sexual reproduction in higher plants. On the stigma of the pistil, pollen grains germinate into a cylindrical tube, which penetrates the stigma, grows through the transmitting tract, and is guided toward the ovule to deliver the sperm cell for fertilization. Pollen tube elongation is extremely rapid (e.g., maize pollen tubes grow at 1 cm per hr), representing one of the most rapidly growing cells (Bedinger, 1992).

Overwhelming evidence suggests that Ca^{2+} plays a critical role in the regulation of tip growth (Miller et al., 1992; Pierson et al., 1994; Malhó and Trewavas, 1996; Hepler, 1997; Messerli and Robinson, 1997; Malhó, 1998b). A tip-focused intracellular Ca^{2+} gradient and a tip-localized influx of extracellular Ca^{2+} are required for pollen tube growth (Pierson et al., 1994; Malhó and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997). The oscillation of both gradients and influxes is associated with changes in growth rates (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). Furthermore, the site of Ca^{2+} influx and the high point of the Ca^{2+} gradient determine the orientation of pollen tube growth (i.e., the site of growth; Malhó et al., 1994, 1995; Malhó and Trewavas, 1996; Pierson et al., 1996). These observations suggest that the tip-focused Ca^{2+} gradient and the tip-localized influx may couple the spatial control of tip growth (i.e., the site of Golgi vesicle fusion) with temporal control (i.e., the rate of exocytosis and tube elongation). Thus, tip growth may involve a mechanism that couples the site of Ca^{2+} influx with the rate of influx. A tip-localized Ca^{2+}-dependent protein kinase activity has been suggested to regulate localized Ca^{2+} changes (Moutinho et al., 1998). However, how the tip-focused Ca^{2+} gradient is established and how the site and rate of extracellular Ca^{2+} influx are regulated are not known.

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Studies involving antibody microinjection and immunolocalization in pea pollen tubes have implied that Rop GTPases play a critical role in the control of pollen tube elongation and have raised the possibility that the Rop GTPases interact with Ca^{2+} signaling in pea pollen tubes (Lin et al., 1996; Lin and Yang, 1997). These GTPases belong to the plant-specific Rop subgroup of Rho GTPases (Yang and Watson, 1993; Delmer et al., 1995; Winge et al., 1997; Li et al., 1998). Rho GTPases are conserved signaling proteins that control a wide spectrum of cellular processes, including actin reorganization, actin-dependent cell polarization, membrane dynamics, gene expression, and cell cycle progression (Chant and Stowers, 1995; Ridley, 1996; Hall, 1998). Arabidopsis pollen expresses three *Rop* genes encoding proteins with nearly identical amino acid sequences, among which *Rop1At* is pollen specific and accounts for the majority of *Rop* transcripts in pollen (Li et al., 1998). Overexpression of Arabidopsis *Rop1At* in fission yeast was shown to cause isotropic growth, supporting a role for Rop1At in the spatial control of polar growth (Li et al., 1998). Based on these observations, we hypothesized that a Rop GTPase–dependent signaling pathway couples the specification of growth sites to the control of growth rate through the regulation of the tip-localized Ca^{2+} influx and gradient.

To test this hypothesis, we expressed constitutively active and dominant negative *rop1at* mutants and sense and antisense *Rop1At* genes under the control of pollen-specific promoters in Arabidopsis. Overexpression of both constitutively active *rop1at* and wild-type *Rop1At* resulted in delocalized cell growth in pollen tubes, whereas dominant negative and antisense *rop1at* genes caused inhibition of tube elongation under low extracellular Ca^{2+} levels, which was rescued by high levels of extracellular Ca^{2+}. Furthermore, blocking Rop signaling by using anti-Rop1Ps antibodies from pea dissipated the tip-high Ca^{2+} gradient. These results indicate that a Rop1At-dependent mechanism couples the spatial control (i.e., growth site specification) with the temporal control (i.e., growth rate) of tip growth through the regulation of tip-localized Ca^{2+} activities in pollen tubes. We propose that a positive feedback loop of Rop1At signaling and polar localization underlies the coupling of spatial and temporal controls of tip growth, as supported by the ectopic accumulation of Rop1At in the apical region of the plasma membrane when it is overexpressed in pollen tubes.

RESULTS

Constitutive Activation of Rop1At Causes Isotropic Growth of Pollen Tubes

To investigate the function of Rop1At in pollen, we generated transgenic Arabidopsis plants that express constitutively active mutant forms of rop1at (i.e., rop1G15V and rop1Q64L) under the control of the pollen-specific *LAT52* promoter (Twell et al., 1991). Mutations at these conserved residues are known to lock Rho GTPases in the GTP-bound form, leading to the permanent activation of Rho signaling (Ridley and Hall, 1992; Ridley et al., 1992). Among 60 independent primary transgenic plants examined, all showed the transformation of normal cylindrical pollen tubes (Figure 1A) into bulbous cells (Figure 1B) when pollen grains were germinated on an agar medium. The phenotypes were confirmed in subsequent generations of transgenic plants.

Isotropic growth begins at the time of germination and appears to occur in whole protoplasts of pollen grains. As such, the emergence of bulbous cells frequently breaks apart the cell wall of pollen grains, leaving the debris of pollen walls attached to the bulbous cell (Figure 1B, insert). The bulbous cells also lose the polarity of cytoplasmic organization. In wild-type tubes, the cytoplasm is organized into four distinct regions, the Golgi vesicle–rich organelle-free apex, the organelle-rich subapical zone, the vegetative nucleus- and sperm-containing region, and the vacuole-filled area located at the base of tubes. The cytoplasm of the bulbous cells frequently is occupied by a central large vacuole, which is typical of isotropic cultured plant cells. These phenotypes clearly indicate that Rop1At is involved in the establishment of a polar site for tip growth.

Rop1At Overexpression Induces Delocalized Pollen Tube Growth and Ectopic Localization of Rop1At in the Plasma Membrane

The loss-of-cell polarity phenotype in pollen tubes expressing constitutively active *rop1at* mutants may be explained by a critical role for Rop1At signaling in the interpretation of a localized spatial cue that determines the site of growth. In this case, Rop1At could be activated at the tip in normal pollen tubes, even if Rop1At might be distributed throughout the entire cell cortex. Thus, pollen tubes expressing constitutive active *rop1at* would behave as if the cue for growth is distributed evenly throughout the cell surface, and isodiametric growth would occur. Alternatively or additionally, proper localization of Rop1At to tip growth sites could play an important role in the regulation of polar growth in normal pollen tubes. Thus, excessive accumulation of the rop1at mutant protein resulting from the strong *LAT52* promoter could cause ectopic distribution of both mutant rop1at and wild-type Rop1At proteins, which in turn could lead to delocalized growth.

To assess which of these mechanisms are involved in the function of Rop1At, we increased the level of Rop1At proteins in pollen by overexpressing the wild-type *Rop1At* gene under the control of the *LAT52* promoter. As shown in Figures 1C and 1D, *Rop1At* overexpression caused various pollen tube phenotypes, including expanded tubes with swollen tips, highly expanded tubes, and bulbous tubes. The severity of these phenotypes correlated with the expression levels of the Rop1At protein. Among 25 indepen-

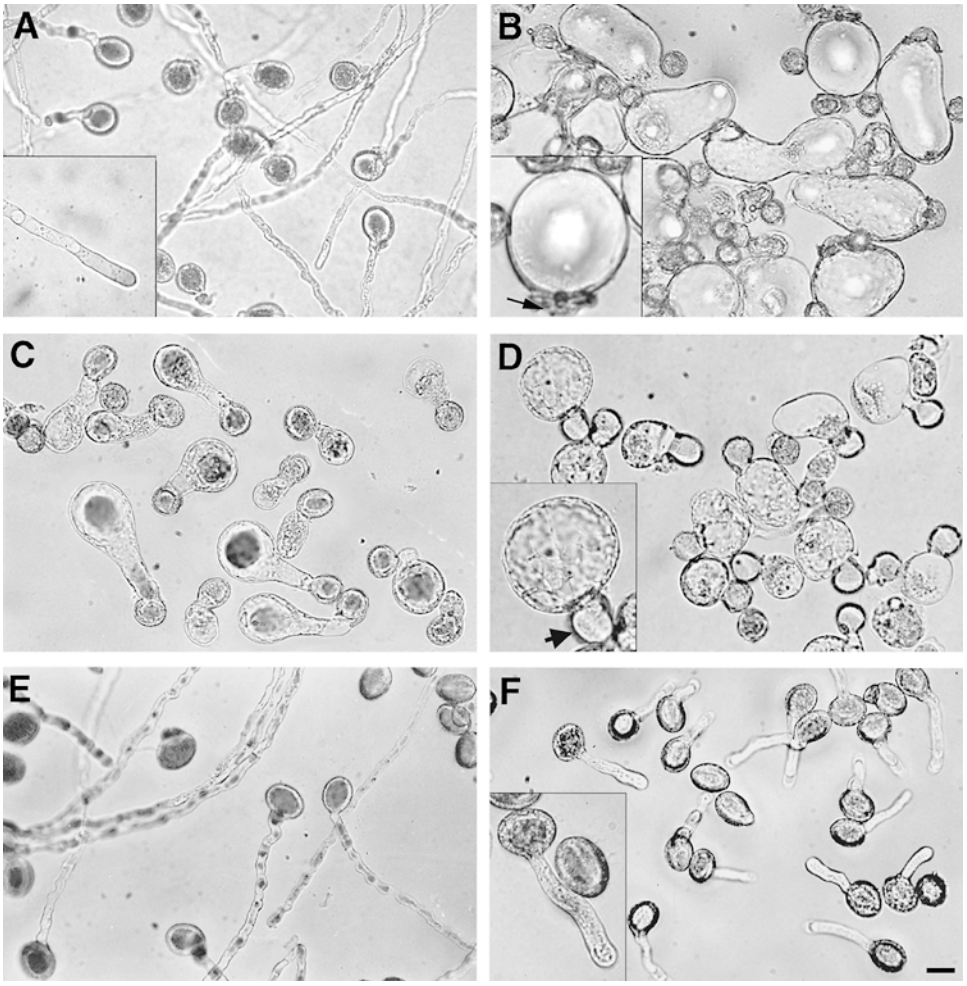

Figure 1. Spatial and Temporal Regulation of Arabidopsis Pollen Tube Tip Growth by Rop1At.

Genes encoding constitutively active *rop1at* mutants, wild-type *Rop1At*, and dominant *rop1at* mutant genes were constructed behind the pollen-specific *LAT52* promoter and transformed into Arabidopsis. Pollen from T_1 and T_2 plants was germinated on an agar medium containing 2 mM Ca^{2+} for 6 hr and examined under a light microscope.

(A) Pollen tubes from Columbia wild-type plants. The inset shows the morphology of a pollen tube.

(B) The constitutively active rop1at (G15V) transforms normal cylindrical tubes into bulbous cells. Another mutant, Q64L, causes identical phenotypes (data not shown). The inset shows a pollen tube that broke the cell wall of a pollen grain (arrow).

(C) Lower levels of wild-type *Rop1At* overexpression causes expanded tubes with swollen tips.

(D) High levels of *Rop1At* overexpression induce isotropic growth after pollen germination. The line shown is hemizygous and contains two copies of the transgene. The inset shows germinated pollen with an intact pollen grain (arrowhead).

(E) G15V/C194 mutation of Rop1At (the constitutively active mutation G15V and a second-site mutation that results in the change of the cysteine residue to serine at the C-terminal CSIL motif). Pollen tubes show normal growth and morphology.

(F) The dominant negative rop1at (D121A) mutant causes inhibition of pollen tube elongation. Another dominant negative mutant, T20N, causes identical phenotypes (data not shown). The inset shows a single slow-growing pollen tube.

Bar in **(F)** = 20 μm for **(A)** to **(F)**.

dent transgenic lines analyzed, 17 lines showed tubes with swollen tips or expanded tubes; only eight lines had bulbous tubes. In all cases, the most severe bulbous tubes were associated with high levels of Rop1At that resulted from multiple T-DNA insertions (Figure 2). These results further confirm a crucial role for Rop1At in the control of growth sites in pollen tubes.

However, *Rop1At* overexpression caused less severe delocalized growth phenotypes than did the expression of constitutively active *rop1at* mutants. As shown in Figure 2,

protein gel blot analyses demonstrated that these differences in phenotype did not result from lower levels of *Rop1At* expression in the *Rop1At*-overexpressing plants. This was further confirmed by the severe isodiametric growth induced by the constitutively active rop1at under the control of the *Rop1At* promoter, a much weaker pollen-specific promoter than is the *LAT52* promoter (data not shown). Furthermore, even in the most severely affected *Rop1At*-overexpressing pollen, we never observed the breakage of the cell wall of pollen grains seen in pollen expressing the constitutively active *rop1at* mutants (see Figures 1B and 1D, inserts). The difference in phenotypes induced by wild-type *Rop1At* overexpression versus the constitutively active *rop1at* genes indicates that the function of Rop1At in the specification of growth sites must involve the localized activation of Rop1At.

The fact that *Rop1At* overexpression also caused delocalized growth shows that the function of Rop1At in growth site specification involves further regulation that is dependent upon its cellular concentration, which may affect the stoichiometry of the cytosolic pool and the membrane-associated portion of Rop1At proteins. Studies in fungal and animal systems have shown that membrane association of Rho GTPases via an isoprenyl lipid is critical for their biological activity (Hancock and Marshall, 1993). Our previous biochemical and immunolocalization studies suggest that only a small fraction of Rop1Ps proteins appear to be localized to the apical plasma membrane of pea pollen tubes, implying a regulated recruitment of Rop1Ps to growth sites (Lin et al., 1996). To test whether membrane targeting of Rop1At is a prerequisite for its function, we mutated the Rop1At geranylgeranylation site. As shown in Figure 1E, this mutation (rop1C194S) rendered constitutively active rop1at proteins inactive, confirming the importance of membrane association of Rop1At for its biological function.

To determine whether *Rop1At* overexpression leads to ectopic membrane localization of Rop1At proteins, we expressed a chimeric gene encoding the jellyfish green fluorescence protein (GFP) fused to the N terminus of Rop1At (GFP–Rop1At; Li et al., 1998). As shown in Figure 3, we obtained transgenic plants with a wide range of pollen tube phenotypes, including normal tubes, different degrees of tube expansion, and bulbous tubes. Pollen-specific expression of the GFP alone did not cause any changes in tube morphology (Figure 3E). The severity of GFP–Rop1At—conferred phenotypes could be correlated with levels of the fusion protein, indicating that the fusion gene is fully functional.

Unlike fixed cells (Lin et al., 1996), the localization of Rop1At fusion proteins to the apical plasma membrane was barely detectable in living normal tubes with low levels of *GFP–Rop1At* expression (Figure 3A). This is most likely due to dynamic recycling or removal of Rop1At proteins from the apical plasma membrane in rapidly growing cells. However, a weak but defined fluorescence was observed in the apical region of the plasma membrane in expanded tubes with an intermediate level of the fusion protein (Figure 3B). Highly expanded tubes contained a greater amount of plasma membrane–localized fusion proteins, which were distributed beyond the apex of the tube (Figure 3C). In bulbous tubes, intense fluorescence was found all around the periphery of the cytoplasm (Figure 3D). The association of GFP–Rop1At fusion with the plasma membrane was confirmed by confocal imaging (Figures 3E and 3F). The GFP alone is totally cytosolic, whereas the GFP–Rop1At fusion clearly is associated with the plasma membrane, with the apical region containing more fusion protein. Because soluble Rop1At proteins have no biological activity, the correlation of *Rop1At* overexpression–induced delocalized growth with the ectopic plasma membrane localization of the GFP–Rop1At fusion protein suggests that dynamically regulated recruitment of Rop1At to the apical plasma membrane plays an important role in the maintenance of tip growth sites in pollen.

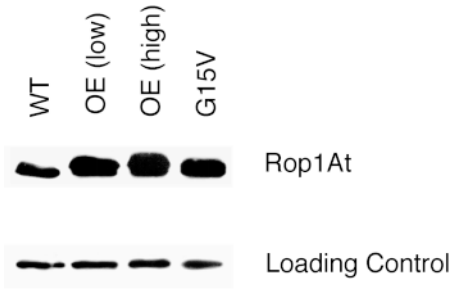

Figure 2. Protein Gel Blot Analyses of Rop Protein Levels in *Rop1At* Overexpressers and Transgenic Plants Expressing the Constitutively Active *rop1At* Mutant.

Open flowers from wild-type (WT) plants, two transgenic lines overexpressing different levels of *Rop1At* (OE), and one line overexpressing constitutively active *rop1at* (G15V) were harvested for protein gel blot analyses by using anti-Rop1Ps polyclonal antibodies (Lin et al., 1996). The antibodies react with different Rop GTPases and thus detected all Rop proteins expressed in open flowers. Under low-stringency conditions, the antibodies also react nonspecifically with a band of ~65 kD. Thus, this protein was used as a loading control. All transgenic lines used were those described in Figure 1. The low overexpresser line was homozygous, whereas the high overexpresser line was hemizygous with two transgenes (no homozygous plants were obtained). The constitutively active *rop1at* line was heterozygous with a single T-DNA insertion.

Dominant Negative *rop1at* Mutants Inhibit Pollen Tube Elongation

Rop1At could control tip growth by coupling the specification of growth sites to a mechanism that regulates growth or

exocytosis. In this case, the inactivation of Rop1At would cause growth inhibition. Alternatively, Rop1At might only be required for the specification of growth sites, and thus, its inactivation would lead to a default growth pathway, that is, isodiametric growth. The former possibility is supported by our previous studies with pea pollen tubes, which showed that injected anti-Rop1Ps antibodies inhibit tube elongation (Lin and Yang, 1997). To confirm this result, we introduced into Arabidopsis plants dominant negative *rop1at* mutants (i.e., rop1T20N or rop1D121A) under the control of the pollen-specific *LAT52* promoter. Similar mutations in other Rho GTPases lock them in inactive GDP-bound forms and thus inhibit their signaling activity (Ridley and Hall, 1992; Ridley et al., 1992). For all 12 independent transgenic lines examined, pollen tubes germinated and grew much more slowly than did the tubes of the wild-type plants (Figure 1F). The morphology of mutant tubes was largely normal, except that they were slightly wider and contained a denser cytoplasm than did wild-type tubes. The dense cytoplasm may be due to the accumulation of Golgi vesicles, which might be forced to fuse across a greater area at the tip, resulting in the wider tubes. Alternatively, slight tube expansion might be a consequence of the ectopic localization of wild-type Rop proteins due to the presence of dominant negative *rop1at* mutants.

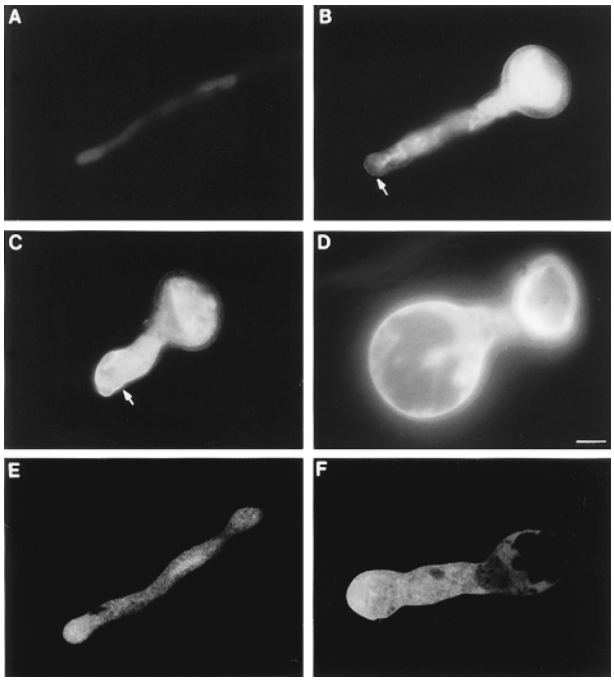

Figure 3. *GFP–Rop1At* Overexpression Leads to Its Ectopic Localization in the Plasma Membrane of Pollen Tubes.

GFP–Rop1At—expressing pollen tubes were examined under an epifluorescence microscope and photographed using a 35-mm camera. The same exposure times were used for the images shown in **(A)** and **(B)** (120 sec), and **(C)** and **(D)** (70 sec).

(A) Pollen tube morphology was normal when *GFP* expression was weak and barely detected on the plasma membrane.

(B) When the expression was stronger, tubes became expanded, and the localization of GFP to the apical region of the plasma membrane was observed. Arrow indicates GFP localization to the plasma membrane.

(C) Stronger GFP fluorescence is associated with more expanded tubes, in which membrane localization of GFP becomes more apparent and extends to the basal region of the tube. The arrow indicates GFP localization to the plasma membrane.

(D) In bulbous tubes, intense GFP fluorescence is localized to the area all around the plasma membrane.

(E) and **(F)** To confirm the membrane localization of *GFP–Rop1At,* we subjected pollen tubes to confocal imaging. A control pollen tube expressing *GFP* alone **(E)** was compared with a transgenic tube expressing similar levels of GFP–Rop1At **(F)**. The laser sections shown were 0.5 μm thick.

Bar in **(D)** = 10 μm for **(A)** to **(F)**.

Blocking of Rop1At Signaling Inhibits Tip-Localized Extracellular Ca^{2+} Influx

The above-mentioned results show that Rop1At is critical for both the specification of the growth site and the control of growth rate in pollen tubes. Interestingly, this dual function of Rop1At is analogous to the role of the tip-focused intracellular Ca^{2+} gradient and the tip-localized Ca^{2+} influx in pollen tube growth (Hepler et al., 1994; Malhó et al., 1994, 1995; Pierson et al., 1994; Malhó and Trewavas, 1996; Pierson et al., 1996). This observation raises an intriguing question: does Rop1At signaling regulate tip-localized Ca^{2+} activities?

To address this question, we first determined whether inhibition of Rop signaling alters the tip-focused intracellular Ca^{2+} gradient. Changes in intracellular Ca^{2+} in pea pollen tubes injected with anti-Rop1Ps antibody were visualized by using ratiometric Ca^{2+} imaging. We previously showed that injection of anti-Rop1Ps into pea pollen tubes specifically inhibited pollen tube growth (Lin and Yang, 1997). Pea pollen was used because the small size of Arabidopsis pollen tubes was not suitable for microinjection of Ca^{2+} indicator dyes. Figure 4 shows that the tip-focused Ca^{2+} gradient oscillated approximately every minute in control pea pollen tubes as found in other plant species (Pierson et al., 1996; Messerli and Robinson, 1997). When pea pollen tubes were cultured in the presence of 2 mM extracellular Ca^{2+} and injected with the amount of anti-Rop1Ps antibodies that arrested pollen tube growth (Lin and Yang, 1997), the tip-focused Ca^{2+} gradient disappeared within 1 to 2 min after injection (Figure 4). The dissipation of the Ca^{2+} gradients roughly coincided with the timing of antibody-induced growth inhibition. This effect was specific for anti-Rop1Ps antibodies, because injection of preimmune sera or coinjection of the anti-Rop1Ps antibody with purified recombinant Rop1At proteins did not dissipate the Ca^{2+} gradients.

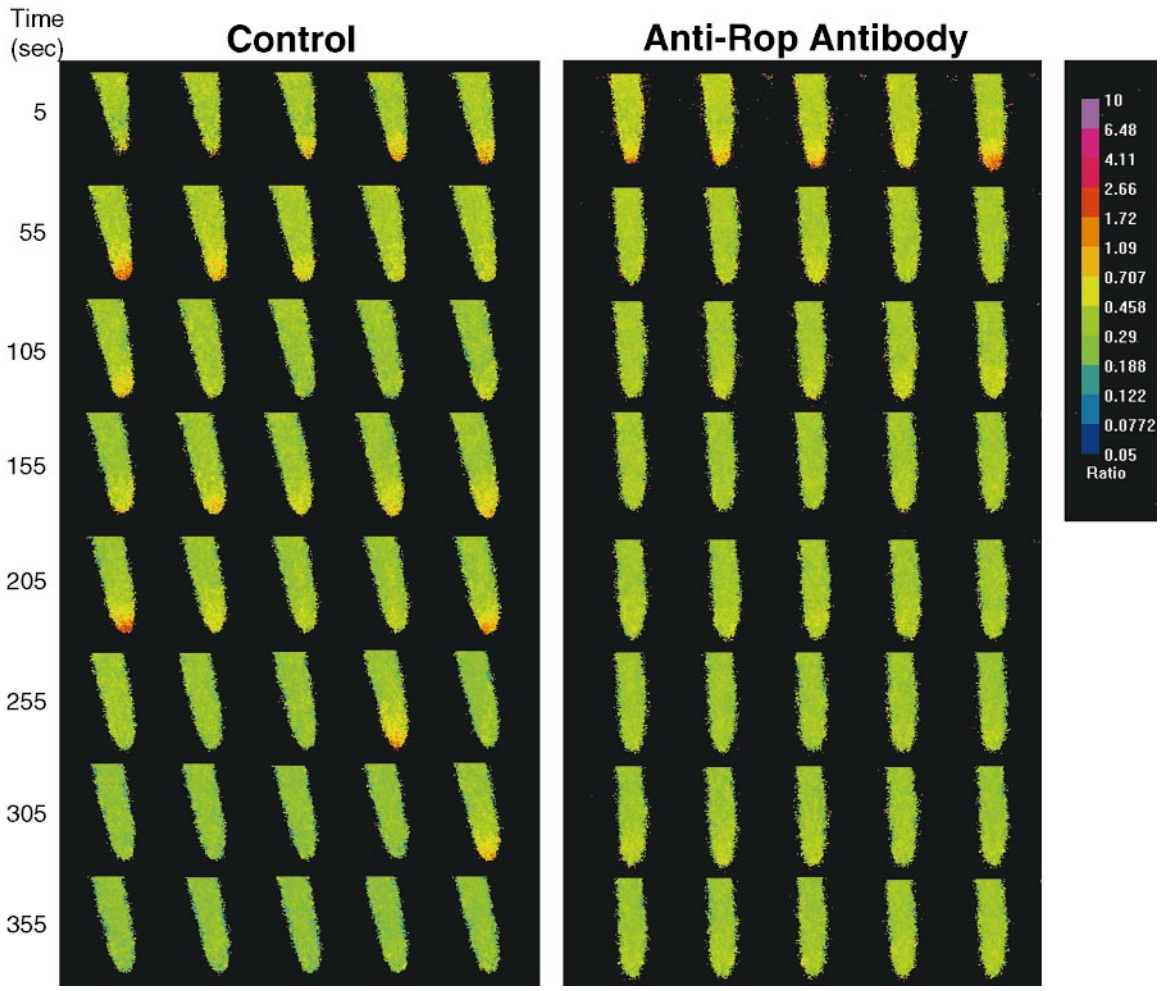

Figure 4. Injected Anti-Rop1Ps Antibodies Disrupt the Tip-Focused Ca^{2+} Gradient in Pea Pollen Tubes.

To visualize cytosolic Ca^{2+}, we injected in vitro–germinated pea pollen tubes with 5 mg/mL of fura-2 dextran (molecular weight of 10 kD) in 5 mM Hepes buffer, pH 7.0. To determine the effect of anti-Rop1Ps antibodies on cytosolic Ca^{2+}, we coinjected pollen tubes with fura-2 dextran and affinity-purified anti-Rop1Ps antibodies (Lin and Yang, 1997). To assure the specific effect of the antibodies, we also performed coinjection with preimmune antisera (Lin and Yang, 1997) or anti-Rop1Ps antibodies neutralized with recombinant Rop1At protein. Ca^{2+} ratio images (340:380 nm) were captured every 10 sec by using the Photon Technology International photon imaging system. The arbitrary color scale at right indicates relative levels of Ca^{2+}, with purple and blue representing the high and low ends of Ca^{2+} levels, respectively. In control tubes, eight of 11 tubes that were coinjected with preimmune preparation, seven of 10 tubes that were coinjected with antibodies plus Rop1At protein, and 10 of 15 tubes that were injected with buffer retained normal Ca^{2+} gradients. Those tubes that lost the Ca^{2+} gradient generally were associated with mechanical damage from the injection procedure. In contrast, the Ca^{2+} gradient was dissipated in 19 of 20 tubes that were coinjected with the antibodies alone.

We next examined the effect of altered Ca^{2+} influxes on Rop1At-dependent pollen tube growth. Pollen tubes were grown on media containing different concentrations of Ca^{2+}. Optimal growth of wild-type Arabidopsis pollen tubes required ~2 mM extracellular Ca^{2+} (Figure 5). Similar Ca^{2+} response growth curves have been shown in pollen tubes from other plant species (Steer and Steer, 1989). Suboptimal growth at low Ca^{2+} levels is most likely due to slower Ca^{2+} influx through the tip. However, it was not clear why high levels of extracellular Ca^{2+} (10 to 20 mM) inhibit tube elongation. It is unlikely that this phenomenon is due to the stiffening of the apical region of the cell wall by binding of

Ca^{2+} to pectin (Steer and Steer, 1989). Slower growth at high extracellular Ca^{2+} is most likely due to a negative feedback regulation of Ca^{2+} influx, as supported by our observation that intracellular Ca^{2+} levels were much lower when pea pollen tubes were grown at 10 mM extracellular Ca^{2+} than at 2 mM (Y. Lin, M.X. Zhu, and Z. Yang, unpublished data). Negative feedback regulation of Ca^{2+} channel activity is common in animal cells (Lee et al., 1999).

In contrast to wild-type Arabidopsis pollen tubes, high levels of extracellular Ca^{2+} partially rescued growth inhibition induced by dominant negative *rop1at* mutants (Figure 5). The rate of tube elongation increased in a linear manner as extracellular Ca^{2+} levels increased from 0.5 to 20 mM. The contrasting effects of extracellular Ca^{2+} on the growth of wild-type and dominant negative *rop1at* tubes further dispute the notion that high levels of extracellular Ca^{2+} inhibit tube growth by stiffening cell walls. Therefore, the rescue of dominant negative *rop1at* tubes by Ca^{2+} together with the antibody-induced dissipation of the intracellular Ca^{2+} gradient provide evidence that the tip-localized Ca^{2+} influx acts downstream of Rop1At.

A Rop1At-dependent signaling cascade may directly regulate Ca^{2+} influx. Alternatively, this cascade could regulate polar exocytosis and indirectly affect Ca^{2+} influx, because exocytosis has been proposed to stimulate tip-localized Ca^{2+} influx by inserting Ca^{2+} channels in the pollen tube tip, although no evidence for this hypothesis exists in the literature (Feijó et al., 1995). To determine whether the role of Rop1At in the regulation of Ca^{2+} influx is direct or indirect, we took advantages of antisense *rop1at* Arabidopsis lines that show weak phenotypes. In the two independent transgenic lines examined, pollen tubes expressing the *Rop1At* antisense gene exhibited slower elongation than did wild-type pollen tubes under 0.5 mM extracellular Ca^{2+} but elongated as well as wild-type tubes under 2 mM Ca^{2+}. Antisense expression resulted in a much weaker effect than did the dominant negative *rop1at* genes, presumably because of incomplete suppression of *Rop1At* expression and the presence of two other *Rop* genes that are almost identical to Rop1At (Li et al., 1998). Interestingly, pollen tubes expressing antisense *rop1at* elongated faster than did wild-type tubes under high levels of extracellular Ca^{2+} (10 to 20 mM). These results cannot be explained by an indirect effect of Rop1At on Ca^{2+} influx through the regulation of exocytosis. A simple explanation is that antisense *rop1at* expression causes a reduction in extracellular Ca^{2+} influx that uncouples the negative feedback regulation of Ca^{2+} influx.

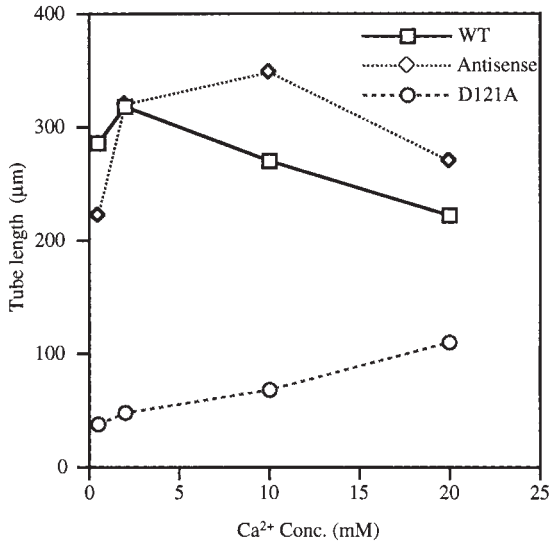

Figure 5. Increased Extracellular Ca^{2+} Levels Rescue the Inhibition of Pollen Tube Elongation Induced by the Dominant Negative *rop1at* Mutation and Antisense *rop1at* Expression.

Pollen from wild-type (WT) plants and transgenic plants homozygous for antisense *rop1at* or dominant negative *rop1at* genes was germinated on agar media containing 0.5, 2, 10, or 20 mM Ca^{2+}. The length of randomly chosen tubes was measured 6 hr after germination. Average lengths of >100 tubes from each line are shown. Statistical analyses (Student's *t* test) show that the differences between wild-type and dominant negative *rop1at*–expressing pollen tubes were highly significant among all treatments ($P = 0$). The differences between *rop1at* antisense plants and wild-type plants were also highly significant at 0.5, 10, and 20 mM Ca^{2+} ($P < 0.007$) but not at 2 mM Ca^{2+}. The experiments were repeated three times, and consistent results were obtained in all repeat experiments.

DISCUSSION

Results obtained during this study have helped to define a signaling mechanism that controls tip growth in pollen tubes. This mechanism involves the Rho family member Rop1At as a molecular switch that couples both temporal and spatial control of tip growth. The switch is most likely activated by an unknown localized cue for tip growth. Our results also provide evidence that Rop1At signaling regulates tip-localized Ca^{2+} influx and the formation of the tip-focused Ca^{2+} gradient.

Rop1At Controls the Site of Growth through Both Localized Activation and Polar Localization

Overexpression of either constitutively active *rop1at* or wild-type *Rop1At* in transgenic plants causes isotropic or depolarized cell growth in Arabidopsis pollen tubes, demonstrating that Rop1At plays a pivotal role in the control of growth sites in pollen tubes. While this manuscript was under review, N.-H. Chua's group reported similar depolarized growth induced by transient expression of Rop5At (At-Rac2)

in tobacco pollen tubes (Kost et al., 1999). Unlike the pollen-specific *Rop1At*, transcript levels for both *Rop5At* and *Rop3At* are much lower in pollen and the genes also are expressed in vegetative tissues; thus, they probably are functionally redundant to Rop1At in pollen (Li et al., 1998). Nonetheless, our results with transgenic plants clearly show that wild-type Rop1At and constitutively active *rop1at* cause distinct delocalized growth phenotypes when they are expressed at similar protein levels under the control of the pollen-specific *LAT52* promoter. First, depolarized growth of pollen tubes induced by *Rop1At* overexpression is less severe than that induced by constitutively active mutants. Second, constitutively active rop1at mutants but not wild-type Rop1At induce isotropic growth of the whole protoplast of pollen grains, causing the wall of pollen grains to break apart. Furthermore, the isotropic growth phenotypes are indistinguishable regardless of whether the constitutively active *rop1at* mutants are expressed under the control of the strong *LAT52* promoter or the weak *Rop1At* promoter (data not shown). Thus, we conclude that the function of Rop1At in polarity control must involve localized Rop1At signaling (i.e., the regulation of the GTP–GDP switch in a localized manner).

How is localized Rop1At signaling controlled? At least three possible models could explain localized Rop1At signaling: (1) Rop1At could be localized to the site of growth and activated by nonlocalized cues; (2) Rop1At could be randomly distributed and activated by a localized spatial cue; or (3) Rop1At could be localized to the site of growth as well as activated by a localized cue. Our results suggest that the recruitment of Rop1At to and its removal or recycling from the growth site also play a critical role in the control of tip growth. In chemically fixed wild-type cells, only a small fraction of total Rop protein is localized to the plasma membrane and is concentrated to the apical region of the membrane (Lin et al., 1996). In living cells, tip membrane localization is barely detectable using GFP-tagged Rop1At. *GFP–Rop1At* overexpression leads to a greater abundance of Rop1At proteins accumulating across to a wider area of the plasma membrane at the apex of pollen tubes, which can be readily detected by GFP tagging. Thus, depolarized growth induced by *Rop1At* overexpression involves the ectopic localization of Rop1At proteins in the plasma membrane. Furthermore, the localization of Rop1At to the plasma membrane appears to be critical for its function, because a mutation in the geranylgeranylation site renders Rop1At inactive. These results suggest that proper polar localization of Rop1At to the apical region of the plasma membrane is required for tip growth, although the membrane localization of Rop1At is more concentrated at the tip but does not seem to be restricted to the site of growth (Figure 3). This localization implies that the specification of growth sites also involves the activation of Rop1At by a localized cue. Thus, we propose that both Rop1At activation by a localized cue and localized protein distribution contribute to the specification of pollen tube growth sites.

An interesting question is whether the site of growth is first established during pollen germination through the activation of Rop1At by a localized cue or through the localization of Rop1At to a predetermined growth site. Constitutively active *rop1at* mutants under the control of either the strong *LAT52* or the weak *Rop1At* promoter induces the isotropic growth of the pollen grain protoplast when germinated, whereas the highest level of wild-type *Rop1At* overexpression does not cause such phenotypes. These results are consistent with a uniform distribution of Rop1At in the plasma membrane of the vegetative cell of pollen grains before germination. Thus, it is more likely that a spatial cue localized at the germinal pore activates Rop1At, leading to the establishment of polar growth sites. These sites then could be maintained or propagated by the localization of Rop1At to the growth site through a positive feedback loop of Rop1At signaling, as suggested by the depolarized growth induced by the ectopic accumulation of Rop1At to the plasma membrane at the apex of pollen tubes. We propose that such a Rop1At signaling–localization feedback loop is a crucial molecular mechanism governing tip growth.

A Rop1At-Dependent Pathway Couples the Spatial Control of Tip Growth with Temporal Control

We have shown that Rop1At also controls the rate of pollen tube elongation. Dominant negative *rop1at* mutants cause the inhibition of pollen tube growth but no significant changes in tube morphology. The slight increase in diameter of dominant negative *rop1at* tubes could be due either to the accumulation of Golgi vesicles in the apex that forces them to fuse slowly to a greater area at the tip or to greater amounts of wild-type Rop proteins localized at the tip. The dominant negative *rop1at* mutants specifically affect the Rop1At-dependent pathway, as confirmed by the same effects caused by antisense *rop1at* expression in Arabidopsis (this study) and anti-Rop1Ps antibodies in pea (Lin and Yang, 1997). Because the antibodies were shown to completely arrest pea pollen tube growth, complete inactivation of Rop GTPases in Arabidopsis pollen was expected to prevent pollen germination. A future study should be directed toward eliminating the expression of all *Rop* genes expressed in pollen.

A role for Rop1At in the specification of growth sites as well as in the control of growth indicates that a Rop1At-dependent pathway couples spatial control with temporal control of tip growth in pollen tubes. In pollen tubes, temporal control may involve both the frequency of growth oscillation and the rate of growth. However, it remains to be determined whether both aspects of temporal control are regulated by the Rop1At-dependent pathway. Although our current data do not reveal a precise mechanism for the Rop1At-mediated coupling of spatial and temporal control of tip growth, this coupling can be explained by the continuous operation of the Rop1At signaling–localization positive

feedback loop after the establishment of an initial tip growth site at the germinal pore. According to this model, Rop1At signaling would regulate the polar localization of Rop1At (spatial control) on the one hand and mediate the rate of exocytosis (temporal control) on the other hand. The coupling of the spatial and temporal control of tip growth by this pathway could be realized through a Rop1At signaling–dependent regulation of the site and the rate of Ca^{2+} influx.

The Rop1At-Dependent Pathway Directly Regulates Tip-Localized Ca^{2+} Influx

The tip-focused Ca^{2+} gradient plays an essential role in tip growth, probably by mediating polar exocytosis, and tip-localized Ca^{2+} influx is required for the generation of the Ca^{2+} gradient (Miller et al., 1992; Pierson et al., 1994; Malhó and Trewavas, 1996; Hepler, 1997; Messerli and Robinson, 1997; Roy et al., 1999). Our results demonstrate that Rop GTPase signaling acts upstream of tip-localized Ca^{2+} signaling. Injected anti-Rop1Ps antibodies cause the dissipation of tip-focused Ca^{2+} gradients, which appears to precede or coincide with the inhibition of tip growth in injected pea pollen tubes. Although these injection experiments did not allow conclusive demonstration of whether Rop GTPase signaling regulates the formation of the tip-focused Ca^{2+} directly or indirectly through the regulation of growth, evidence suggests that the effect of Rop GTPases on Ca^{2+} is direct. First, all treatments that dissipate the Ca^{2+} gradient have been shown to block tip growth (Pierson et al., 1994). On the contrary, treatments with Yariv reagents inhibit growth but do not dissipate Ca^{2+} gradients in lily pollen tubes (Roy et al., 1999). These results raise doubts about the requirement of growth for the formation of the Ca^{2+} gradients.

More importantly, our experiments with transgenic plants involving antisense and dominant negative *rop1at* mutants indicate that the effect of Rop1At signaling on Ca^{2+} is direct. At 0.5 mM extracellular Ca^{2+}, dominant negative rop1at proteins strongly inhibit Arabidopsis pollen tube growth, but expression of antisense *rop1at* weakly inhibits growth. The dominant negative *rop1at*-induced inhibition is partially rescued by increasing extracellular Ca^{2+} levels (2 to 10 mM), whereas the weak growth inhibition by antisense *rop1at* is completely reversed by 2 mM extracellular Ca^{2+}. High levels of extracellular Ca^{2+} (10 to 20 mM) are inhibitory to wild-type pollen tubes, probably as a result of negative feedback regulation of Ca^{2+} influx (see below). In contrast, antisense *rop1at* expression releases this growth inhibition. These results clearly indicate that Rop1At signaling and Ca^{2+} operate on the same pathway and that the regulation of Ca^{2+} by Rop1At is not growth dependent. Together with the anti-Rop1Ps antibody-induced dissipation of intracellular Ca^{2+} gradients, our transgenic experiments suggest that the Rop1At-dependent pathway directly regulates tip-localized Ca^{2+} influx.

Our results also provide a strong support for the existence of negative feedback regulation of Ca^{2+} influx in the presence of high extracellular Ca^{2+} levels. We found that the tip-high intracellular Ca^{2+} gradient is much less steep when pea pollen tubes are incubated at 10 mM extracellular Ca^{2+} than at 2 mM Ca^{2+}. This is consistent with an increased optimal concentration of extracellular Ca^{2+} in pollen tubes expressing the antisense *rop1at* gene. The negative feedback regulation of Ca^{2+} influx also is found in animal cells (Lee et al., 1999). The negative feedback regulation may allow the cell to coordinate exocytosis with cellular metabolism, such as the synthesis of cell wall materials required for pollen tube extension. This negative regulation also might be a mechanism underlying the oscillation of the tip-focused Ca^{2+} gradient correlated with growth rates (Pierson et al., 1996; Messerli and Robinson, 1997).

Although these studies clearly connect Rop1At signaling with tip-localized Ca^{2+} signaling, the most definitive demonstration of the Rop1At-dependent direct regulation of the tip-localized Ca^{2+} influx and the tip-focused Ca^{2+} gradient await the identification of a tip-localized Ca^{2+} channel and intermediate components connecting Rop1At and the channel. Tip-localized Ca^{2+}-dependent protein kinase activity could be a key factor involved in this connection (Moutinho et al., 1998). It is possible that a component downstream of Rop1At could directly activate plasma membrane Ca^{2+} channels. Alternatively, this component could first regulate the generation of inositol triphosphate, which in turn could regulate store-operated Ca^{2+} influxes known to exist in animal cells (Scharenberg and Kinet, 1998). Interestingly, evidence suggests that inositol triphosphate also may play a role in the regulation of the tip-localized Ca^{2+} and directional tube growth (Malhó, 1998a). This possible pathway is also consistent with the results of Kost et al. (1999), which implicate phosphatidylinositol kinase as a potential downstream component of Rop in tobacco pollen tubes.

It also remains possible that Rop1At regulates the organization of a specific subpopulation of cortical actin filaments (or network) in the apical region, which could either regulate the activation of Ca^{2+} channels at the apex or define the apical region of the tube, at which active Ca^{2+} channels are localized. Kost et al. (1999) suggested that At-Rac2/Rop5At might be involved in the regulation of the formation of the cortical actin cables involved in vesicle transport, but such cables are unlikely to be involved in the specification of growth sites and the regulation of Ca^{2+} influx. Nonetheless, the identification of additional components in the Rop1At-dependent pathway will be critical for understanding how Rop signaling regulates the formation of the tip-focused Ca^{2+} gradient.

The role of the tip-localized Ca^{2+} gradient in the regulation of tip growth appears to be universal (Silver et al., 1990; Schiefelbein et al., 1992; Garrill et al., 1993; Schumaker, 1996; Bibikova et al., 1997; Kuhn et al., 1998). Furthermore, the function of Rho GTPases in the control of polarized cell

growth is conserved (Luo et al., 1994; Drubin and Nelson, 1996). Hence, the Rop-dependent pathway could provide a paradigm for tip growth signaling in eukaryotic organisms. It would be interesting to determine whether Rop GTPases provide a universal molecular switch for the control of cell polarity and cell morphogenesis in plants.

METHODS

Plant Materials

Plants (*Arabidopsis thaliana* ecotype Columbia wild type) were grown at 22°C in growth rooms with a light regimen of 12 hr of dark and 12 hr of light. Pea plants (*Pisum sativum* cv Extra Early Alaska) were grown at 25°C in growth chambers with 16 hr of light and 8 hr of dark.

Construction of *rop1at* Mutants, Antisense Expression and Overexpression, and Fusion with the Green Fluorescent Protein

To create dominant mutations of *Rop1At*, we amplified the *Rop1At* coding sequence (Li et al., 1998) by using the polymerase chain reaction (PCR) and subcloned the amplified fragments into the XbaI and HindIII sites of pSELECT (Promega). Site-directed mutagenesis was conducted by using the Altered Sites in vitro mutagenesis system (Promega). Four oligonucleotides containing the desired changes (underlined) were used: G15V, 5′-GTTGGTGATGTAGCTGTCCC-3′; T20N, 5′-GTCGGAAAAAATTGTTTGTTG-3′; Q64L, 5′-CACTGCAGGTCTAGAGGATTAC-3′; and D121A, 5′-GAACAAAGCTCGCTCTTCGAGATG-3′. The sites of mutations were confirmed by sequencing. The mutant genes then were cloned into XbaI and SacI sites of pBI101 vector behind the *LAT52* promoter. For overexpression of the *Rop1At* wild-type gene, the coding sequence amplified by PCR was subcloned behind the *LAT52* promoter in the same vector.

To create the G15V/C194S double mutant, we used the antisense primer 5′-CCGAGCTCATCATAGAATGGAGCTTGCCTTCTG-3′ containing a T-to-A change at the cysteine residue 194 of Rop1At. This change results in the replacement of the cysteine residue with serine in the four–amino acid CSIL geranylgeranylation motif of Rop1At. This primer was used to amplify the *rop1at* G15V mutant gene. The double mutant G15V/C194S *rop1at* gene was subcloned into pBI101 in the same way as other mutant *rop1at* genes.

To create an antisense *rop1at* construct, we subcloned the full-length *Rop1At* cDNA sequence in the antisense orientation behind the *LAT52* promoter. The antisense fusion gene also was cloned into pBI101 at SalI and EcoRI sites.

To construct the green fluorescent protein *GFP–Rop1At* chimeric gene, a BglII site was introduced to replace the stop codon of the *mGFP4* gene (Haseloff et al., 1997) by PCR amplification. The PCR product was cloned into pBluescript SK+ (Stratagene, La Jolla, CA). Wild-type *Rop1At* coding sequence was amplified by PCR by using 5′ and 3′ primers containing BglII and SacI sites, respectively. The *Rop1At* sequence was ligated with the *mGFP4* gene to generate the *GFP–Rop1At* fusion gene. The fusion gene was subcloned behind the *Rop1At* promoter in pBI101. As a control, the *mGFP4* gene was cloned behind the same promoter in pBI101.

Arabidopsis Transformation

The above constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and transformed into Arabidopsis ecotype Columbia wild type by using the vacuum infiltration method (Bechtold et al., 1993). Transgenic plants were selected on Murashige and Skoog medium (Gibco BRL) containing kanamycin. Transgenic seedlings were transferred to soil and grown at 22°C in a growth room with 12-hr-light and 12-hr-dark cycles.

In Vitro Pollen Germination and Growth Measurement

Flowers collected from Arabidopsis plants 1 to 2 weeks after bolting were used for the examination of pollen tube phenotypes. Pollen was germinated on an agar medium containing 18% sucrose, 0.01% boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 0.5% agar, pH 7. Open flowers were dehydrated at room temperature for at least 2 hr. Pollen grains were transferred to agar medium by dipping the flowers on agar. Pollen was germinated at room temperature for 6 hr, examined under a light microscope, and photographed with a 35-mm camera. To examine the effect of different extracellular Ca^{2+} concentrations on pollen tube growth, we germinated pollen in the same agar medium containing various concentrations of Ca^{2+} (with an equal molar ratio of $CaCl_2$ and $Ca[NO_3]_2$).

Mature pollen from homozygous transgenic and Columbia wild-type plants was placed on agar media containing different concentrations of Ca^{2+} to allow germination at room temperature for 6 hr. For each transgenic plant, at least 100 pollen tubes were chosen randomly for length measurements. Measurements were performed under a light microscope using a ×20 objective and a ruler in the eyepiece. Average length and standard deviations among 100 pollen tubes were calculated. For antisense plants, analyses of two independent transgenic lines showed similar growth responses to extracellular Ca^{2+}. Data from one transgenic line were used.

Ca^{2+} Ratio Imaging and Microinjection

Pea pollen tubes were germinated on a pollen germination medium containing 2 mM $CaCl_2$, 0.01% boric acid, 1% low melting agarose, and 25% sucrose for 40 min. Pollen tubes were injected with 5 mg/mL of fura-2 dextran (with a molecular weight of 10 kD) and dissolved in Hepes buffer (5 mM, pH 7.0) or fura-2 dextran plus affinity-purified anti-Rop1Ps antibodies (a pipette tip concentration of 1 mg/mL), as described previously (Lin and Yang, 1997). Beginning 5 sec after injection, fluorescence images at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were captured every 10 sec on a Photon Technology International (Monmouth Junction, NJ) Ca^{2+} imaging system. Ratio images were acquired in 340:380 nm, using ImageMaster software (Photon Technology International).

Protein Gel Blot Analyses

Open flowers of the same stage were collected from transgenic or wild-type plants. Proteins were extracted from the flowers in protein gel loading buffer. One hundred micrograms of total protein was separated on 12% SDS–polyacryamide gels and transferred to nitrocel-

lulose membranes. Rop proteins were detected using affinity-purified anti-Rop1Ps antibodies as described previously (Lin et al., 1996).

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Control of Pollen Tube Tip Growth by a Rop GTPase–Dependent Pathway That Leads to Tip-Localized Calcium Influx

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