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Inhibition of Pollen Tube Elongation by Microinjected Anti-Rop1Ps Antibodies Suggests a Crucial Role for Rho-Type GTPases in the Control of Tip Growth

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Microinjection of anti-Rop1Ps antibodies was used to assess the function of a tip-localized Rho-type GTPase, Rop, in controlling pollen tube growth. Injected antibodies induced sustained growth arrest within 1 to 2 min after injection but did not affect cytoplasmic streaming. Coinjection with Rop rescued antibody-induced growth inhibition, indicating that injected antibodies specifically block the activity of Rop GTPases. Antibody-induced inhibition was significantly enhanced in the presence of a lower threshold of extracellular [Ca^{2+}] or a subinhibitory dosage of caffeine. In contrast, injection of the C3 toxin, which inactivates a different Rho-type GTPase, arrested tube elongation 10 to 20 min after injection. C3-induced growth arrest was accompanied by the cessation of cytoplasmic streaming. These data suggest that Rho-type GTPases play a pivotal role in the control of pollen tube elongation. We propose that Rop may regulate a Ca^{2+}-dependent pathway involved in vesicle docking/fusion, whereas a C3-sensitive Rho GTPase may mediate cytoplasmic streaming.

INTRODUCTION

Pollen tubes undergo a long and tortuous journey through several tissues to deliver male gametes to the ovule during sexual reproduction in plants. Diffusible chemical signals, extracellular matrices, and electrical and mechanical cues have been implicated in directing pollen tubes toward the ovule (Mascarenhas, 1993; Hepler et al., 1994; Lord, 1994; Nasrallah et al., 1994; Cheung et al., 1995; Hülskamp et al., 1995; Wu et al., 1995; Wilhelmi and Preuss, 1996). However, the molecular mechanism underlying directional pollen tube growth remains to be explored.

Pollen tube elongation is dependent on polarized delivery of Golgi vesicles by actomyosin-driven cytoplasmic streaming and localized fusion of these vesicles to the apex—a process called tip growth. Tip growth is common in eukaryotes, for example, root hair extension in plants, hyphal growth in fungi, and neuronal outgrowth in animals. In vitro–cultured pollen tubes provide a simple system for analyzing molecular events that lead to localized exocytosis during tip growth. In vitro, pollen tube growth is generally unidirectional; however, growth orientation can be manipulated by using Ca^{2+} or glucose gradients or an electrical field (Mascarenhas, 1993; Malhó et al., 1994; Malhó and Trewevas, 1995, 1996). Recent studies have shown that a transmitting tissue–localized extracellular glycoprotein TTS attracts pollen tubes as well as promotes their growth on culture media (Cheung et al., 1995; Wu et al., 1995). This important finding implies a mechanism whereby the rate of pollen tube growth is coupled with its orientation, analogous to the regulation of axon guidance in animal cells (Cheung et al., 1995; Wu et al., 1995).

Ca^{2+} signaling appears to play a critical role in the regulation of both the rate and orientation of pollen tube growth. Early work showed that pollen germination and tube growth require a window of extracellular [Ca^{2+}] (reviewed in Hepler et al., 1994). The need for Ca^{2+} can be accounted for at least in part by a tip-focused cytosolic Ca^{2+} gradient and a tip-localized extracellular Ca^{2+} influx (Pierson et al., 1994, 1996; Malhó et al., 1995; Malhó and Trewavas, 1996). Perturbation of the apical Ca^{2+} influx and gradient led to the inhibition of pollen tube elongation as well as the alteration of growth orientation (Pierson et al., 1994, 1996; Malhó et al., 1995; Malhó and Trewavas, 1996). It is not clear how Ca^{2+} signaling modulates localized growth in pollen tubes, although it is proposed to mediate polarized exocytosis (Battey and Blackbourn, 1993; Hepler et al., 1994; Bush, 1995). Annexins, a family of Ca^{2+}-dependent phospholipid binding proteins, are involved in the regulation of exocytosis in animal cells (Raynal and Pollard, 1994) and might also be Ca^{2+} effectors that control pollen tube exocytosis (Blackbourn et al., 1992; Blackbourn and Battey, 1993; Hepler et al., 1994).

Recent studies have shown that Rop1, a member of the Rho family of small GTP binding proteins, is preferentially expressed in pollen and pollen tubes in pea and Arabidopsis (Lin et al., 1996; H. Li and Z. Yang, unpublished results).

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Rop1 belongs to the Rop subfamily of Rho GTPases that appears to be unique to plants (H. Li, G. Wu, D. Ware, D. Zhou, C.L. Cramer, K.R. Davis, and Z. Yang, unpublished results). Rho family GTPases (members of the Ras superfamily) are key signaling switches that control a variety of cellular processes in yeast and mammals, including actin cytoskeletal organization, membrane trafficking and organization (e.g., exocytosis and endocytosis), cell cycle progression, mitogen-activated protein kinase cascades, formation of focal adhesion, and activation of glucan synthase and NADPH oxidase (Hall, 1994; Chant and Stowers, 1995; Vojtek and Cooper, 1995; Lamaze et al., 1996; Larochelle et al., 1996; Murphy et al., 1996; Nagata and Hall, 1996; Ridley, 1996). In the budding yeast, two Rho-type GTPases (CDC42 and Rho1) known to control polarized growth are localized to the site of bud emergence and growth (Ziman et al., 1993; Yamochi et al., 1994). Interestingly, our studies indicate that Rop1 and/or a related GTPase is localized to the apical cortex of pollen tubes (Lin et al., 1996). In this region, vesicle fusion and tip-focused Ca^{2+} gradients occur (Hepler et al., 1994). This localization pattern is consistent with a role for Rop in signaling localized exocytosis.

In this study, we demonstrate that injected anti-Rop1Ps antibodies cause rapid growth arrest of pea pollen tubes and that antibody-induced growth inhibition is potentiated by lower extracellular [Ca^{2+}] and by treatments with a subinhibitory dosage of caffeine. In contrast, a bacterial exoenzyme, C3, which inhibits a distinct subfamily of Rho GTPases, induces a more gradual cessation of growth that is associated with the inhibition of cytoplasmic streaming. These results suggest that at least two distinct Rho GTPases are required for pollen tube growth. Rop seems to participate in a Ca^{2+}-dependent pathway that leads to polarized exocytosis, whereas a C3-sensitive Rho GTPase might be involved in actin-mediated cytoplasmic streaming.

RESULTS

Anti-Rop1Ps Antibodies Specifically React with Rop in Pollen Tubes

To analyze the function of Rop proteins in pollen tubes by using injected anti-Rop1Ps antibodies, it was important to determine whether the antibodies react with native Rop proteins in a specific manner. In a previous study, protein gel blot analyses showed that affinity-purified polyclonal rabbit antibodies against Rop1Ps specifically detected a protein with the molecular mass of Rop1Ps from pea pollen protein extracts (Lin et al., 1996). To determine whether native Rop proteins also react with these antibodies, we extracted pollen tube proteins under nondenaturing conditions. Native proteins were allowed to react with affinity-purified anti-Rop1Ps rabbit antibodies immobilized onto nitrocellulose membranes. Reactive pollen tube proteins were eluted, separated by SDS-PAGE, and detected by silver staining or by using the anti-Rop1Ps antibodies and a chemiluminescent detection system. As shown in Figure 1A, two bands were detected by using silver staining, an ~57-kD band corresponding to the molecular mass of a rabbit IgG heavy chain and an ~22-kD band corresponding to the molecular mass of Rop proteins and an IgG light chain. Protein gel immunoblotting detected an additional minor band of ~45 kD (Figure 1B). As shown in Figure 1C, the 57-kD band, but not the 22- and 45-kD bands, was also detected in the absence of the primary antibodies, thereby confirming that the 57-kD protein is the IgG heavy chain (anti–rabbit second antibodies were produced against the rabbit IgG heavy chain).

We conclude that the 22-kD band contains a native Rop protein monomer, whereas the 45-kD band is a dimer of the Rop protein because the same band was also present in purified Rop1Ps protein expressed in *Escherichia coli* (Yang and Watson, 1993). Obviously, an aliquot of immobilized antibodies was coeluted with the antibody-associated Rop proteins. Nonetheless, these results indicate that immobilized anti-Rop1Ps antibodies specifically reacted with native Rop1 and/or a closely related Rop isoform(s) from pea pollen tubes. Thus, the purified native Rop proteins served as an important control for our microinjection experiments described below.

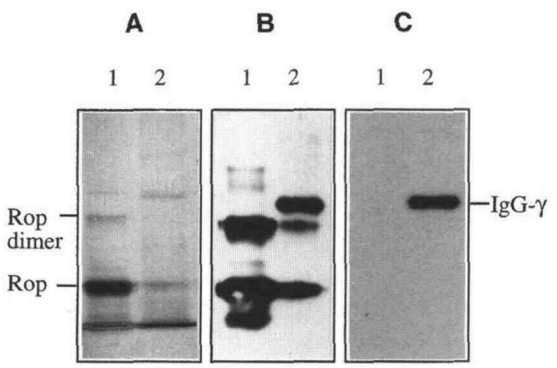

Figure 1. Gel Blot Analyses of Native Rop Proteins Isolated from Pollen Tubes.

Native Rop proteins were isolated using the immunopurification technique described in the text. Two micrograms of eluted proteins was separated by electrophoresis on an SDS–polyacrylamide gel, blotted onto nitrocellulose membranes, and detected using anti-Rop1Ps antibodies and a chemiluminescence kit. Lanes 1 contain 5 μg of Rop1Ps protein expressed in *E. coli* (Yang and Watson, 1993); lanes 2 contain 2 μg of immunopurified native Rop protein preparations. The 22- and 45-kD bands on lanes 1 are the Rop monomer and dimer, respectively, as indicated at left; the 57-kD band on lanes 2 is the rabbit IgG light chain (IgG-γ), as indicated at right.

(A) Silver-stained protein gel.

(B) Rop protein detection using anti-Rop1Ps antibodies and goat anti–rabbit secondary antibodies.

(C) Detection of anti-Rop1Ps antibodies using goat anti–rabbit secondary antibodies.

The anti-Rop1Ps polyclonal antibodies react with members of the Rop subfamily that are closely related to Rop1Ps (Lin et al., 1996). It is not clear how many Rop members are expressed in pea pollen tubes; therefore, it is possible that the antibodies react with other members in addition to Rop1Ps in pea pollen tubes. In this study, we refer to Rop1Ps and related proteins that specifically react with the anti-Rop1Ps polyclonal antibodies as Rop.

Anti-Rop1Ps Antibodies Induce Rapid Growth Arrest in Pea Pollen Tubes

The expression pattern for Rop1 genes in pollen and the localization of Rop proteins to the tip of pollen tubes are consistent with a role for Rop in the regulation of tip growth (Lin et al., 1996). To assess directly the function of Rop GTPases in pollen tubes, we microinjected anti-Rop1Ps antibodies into pea pollen tubes cultured on agarose medium. Microinjection of proteins, peptides, or other agents into living plant cells has become a useful approach to study plant signal transduction (Neuhaus et al., 1993; Bowler et al., 1994; Malhó et al., 1994; Pierson et al., 1994, 1996; Staiger et al., 1994; Wolniak and Larsen, 1995; Malhó and Trewavas, 1996). However, injection of antibodies into higher plant cells apparently has not been reported, although this technique has been used frequently for functional analyses of signaling proteins in animal cells.

Injection of high molecular mass proteins, such as antibodies, into walled and high-turgor plant cells is technically difficult and may cause significant mechanical damage to the cytoplasm. In this study, we developed a protocol to introduce antibodies into pea pollen tubes by using pressure injection. To minimize mechanical damage, each tube was gently and gradually loaded with multiple small aliquots of antibodies (see Methods for details). To demonstrate that injected antibodies induced specific effects, several different controls were used—anti-Rop1Ps antibodies coinjected with the aforementioned purified native Rop proteins or with purified, *E. coli*–expressed Rop1Ps fusion proteins and a preimmune serum prepared using a procedure identical to that for the isolation of affinity-purified antibodies from anti-Rop1Ps antisera (Lin et al., 1996).

Elongation rates of individual pea pollen tubes on agarose medium vary from <100 to >200 μm/hr (Figure 2). Similar variable growth rates among individual pollen tubes from other species have also been observed (Pierson et al., 1994). Pressure injection of Tris-HCl buffer or preimmune serum reduced the rate of growth to some extent within the first 10 min after injection, evidently because of pollen responses to mechanical stimuli or damage. However, normal growth rapidly recovered. In addition, these control injections did not cause growth cessation, and no obvious changes in cytoplasmic streaming were observed (see Table 1). In contrast, the majority of pollen tubes injected with anti-Rop1Ps antibodies ceased growth within 1 to 2 min after injection (see Table 1 and Figure 3). Although the pattern of cytoplasmic streaming may be altered (i.e., streaming invades the apical region), the rate of streaming is not significantly affected by injected antibodies. Twenty to 40 min after injection, antibody-loaded tubes typically started gradual recovery of growth, presumably because of de novo accumulation of Rop proteins or turnover of injected antibodies. Arrested tubes sometimes undergo tip swelling before growth resumption. A small number of cells injected with the antibodies did not cease to grow, but their growth rate was considerably lower compared with that of cells injected with preimmune preparations. Like untreated or control-injected pollen tubes, elongation rates for individual pollen tubes injected with antibodies were variable. Nonetheless, the mean elongation rate for antibody-injected tubes was dramatically reduced compared with that of tubes injected with buffer or preimmune preparations (see Table 1).

When antibodies were premixed with native Rop proteins purified from growing pollen tubes or Rop1Ps fusion proteins purified from *E. coli*, the antibody-induced inhibition of tube elongation was essentially abolished (Figures 3 and 4 and Table 1). These results clearly indicate that injected anti-Rop1Ps antibodies specifically blocked the activity of Rop1 and/or related Rop proteins in pollen tubes. This conclusion is further supported by the distinct behavior of tip growth and cytoplasmic streaming induced by the injected C3 toxin (an inhibitor specific for the Rho subfamily of GTPases), as described below.

Distinct Growth Behavior Is Induced by the C3 Toxin, a Rho-Specific ADP–Ribosyltransferase

According to amino acid sequence similarity, yeast and animal Rho-type GTPases are categorized into at least three subfamilies, CDC42, Rac, and Rho (Chardin, 1993). The C3 exoenzyme from *Clostridium botulinum* has been shown to preferentially ADP–ribosylate and thus to inactivate the Rho subfamily (Aktories et al., 1989; Sekine et al., 1989; Sugai et al., 1992). Thus, the C3 toxin is useful for distinguishing the biological function for the Rho subfamily from other subfamilies (Chardin et al., 1989; Ridley and Hall, 1992; Sugai et al., 1992; Takaishi et al., 1993).

To assess whether the C3 toxin has similar effects on pollen tube growth as anti-Rop1Ps antibodies, growth behavior and cytological changes in pollen tubes injected with the toxin were determined. As shown in Figures 5 and 6 and Table 1, the injected C3 toxin clearly induced pollen tube growth inhibition, as did the anti-Rop1Ps antibodies. However, C3 induced two distinct aspects of changes in pollen tube growth behavior. First, little growth inhibition was observed in the first few minutes after injection and was followed by gradual inhibition within 5 to 10 min and complete growth arrest between 10 and 20 min (Figure 6). Second, growth arrest was accompanied by the cessation of cytoplasmic streaming. The streaming lane became disrupted,

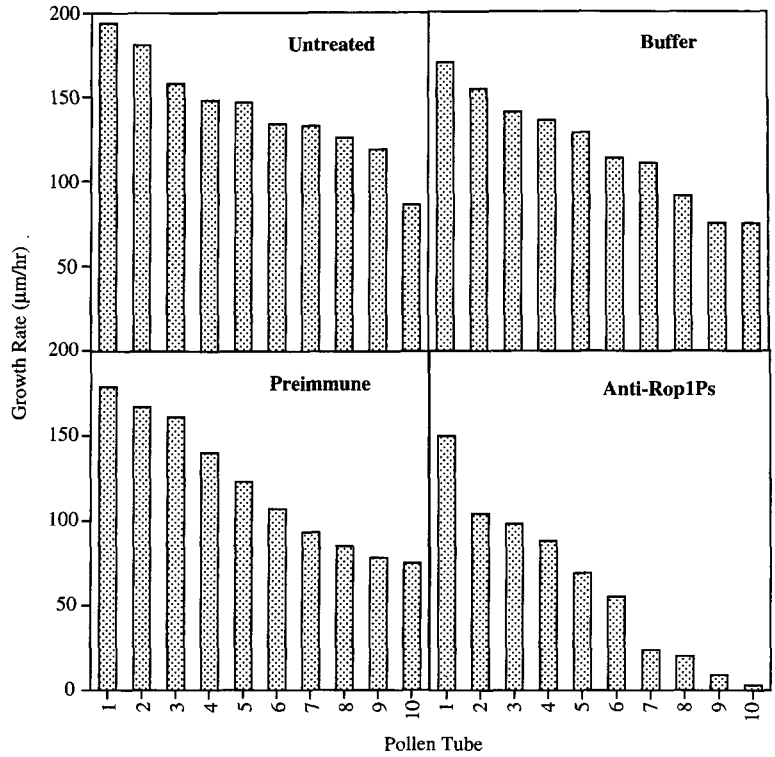

Figure 2. Inhibition of Pollen Tube Elongation by Microinjected Anti-Rop1Ps Antibodies.

Pollen tubes were cultured on an agarose medium containing 10 mM Ca^{2+} and injected with Tris-HCl buffer, preimmune preparation, or anti-Rop1Ps antibodies using the procedure described in Methods. Elongation rates were measured ~1 hr after injection. Untreated pollen tubes were randomly chosen from noninjected pollen tubes, and elongation was measured between 10 and 70 min after germination. The x-axis represents the number of individual treated pollen tubes, ordered according to their elongation rates.

and organelles exhibited Brownian movement. This cytoplasmic change is identical to the changes induced by treatments of pollen tubes with cytochalasins, drugs that disrupt actin filaments and lead to the cessation of cytoplasmic streaming (Heslop-Harrison et al., 1991). These results suggest that a C3-sensitive Rho GTPase, most likely belonging to the Rho subfamily, may be involved in the control of pollen tube elongation by way of a mechanism that is different from a Rop-dependent mechanism.

Low Extracellular [Ca^{2+}] Potentiates Antibody-Induced Effects

The similarity of anti-Rop1Ps–induced effects to those caused by the dissipation of tip-focused Ca^{2+} gradients and tip-localized Ca^{2+} influx (Malhó et al., 1994, 1995; Pierson et al., 1994, 1996; Malhó and Trewavas, 1996) led us to speculate that Rop GTPases might be involved in a Ca^{2+}-dependent mechanism that modulates tip growth. To test this idea, we assessed whether changes in extracellular [Ca^{2+}] potentiate antibody-induced growth inhibition. Pea pollen tubes exhibit similar growth rates when [Ca^{2+}] in the medium varies from 2 to 10 mM, although pollen tubes are slightly wavy at lower [Ca^{2+}] (e.g., 2 mM Ca^{2+}). Interestingly, extracellular [Ca^{2+}] had a significant effect on growth inhibition induced by anti-Rop1 antibodies. As shown in Figure 7 and Table 1, the number of antibody-loaded tubes that exhibited sustained growth arrest increased significantly (from 60 to 90%), and mean elongation rates were reduced considerably (from 65 to 33 μm/hr) when pollen tubes were cultured in the presence of 2 mM Ca^{2+} compared with 10 mM Ca^{2+}. The elongation of pollen tubes injected with preimmune preparations was comparable under these different Ca^{2+} conditions. These results indicate that lower extracellular [Ca^{2+}] potentiates growth inhibition induced by anti-Rop1Ps antibodies.

Subinhibitory Dosage of Caffeine Enhanced Anti-Rop1Ps Antibody–Induced Effects

Caffeine has been shown to affect many Ca^{2+}-dependent cellular processes in plants (Becerra and López-Sáez, 1978;

Roberts and Haigler, 1992; Pierson et al., 1996). In particular, caffeine at a concentration >1.5 mM dissipates the intracellular tip-focused Ca^{2+} gradient and extracellular Ca^{2+} influx and inhibits the elongation of lily pollen tubes (Pierson et al., 1996). We showed that >1.0 mM caffeine has similar inhibitory effects on pea pollen tube growth (data not shown) but that 0.5 mM caffeine has no effects on tube growth (Figure 8). We reasoned that the subinhibitory dosage of caffeine may reduce the extracellular Ca^{2+} influx and intracellular Ca^{2+} concentration to the extent that it would enhance the effect of anti-Rop1Ps antibodies. Thus, microinjections were performed with pollen tubes cultured in a medium containing 0.5 mM caffeine.

As shown in Figure 8, 0.5 mM caffeine dramatically enhanced antibody-induced growth inhibition, whereas it did not affect the elongation of pollen tubes injected with the preimmune control. The caffeine effect was analogous to that of low extracellular [Ca^{2+}], providing additional support for the idea that Rop interacts with Ca^{2+} signaling in the control of pollen tube growth. Interestingly, caffeine treatment did not potentiate the effect of the C3 toxin (Figure 5 and Table 1). These results further support the hypothesis that the Rop/Ca^{2+}-dependent mechanism for the regulation of pollen tube growth is distinct from the one mediated by a C3-sensitive Rho GTPase.

DISCUSSION

The Rho family of small GTPases has emerged as a most important class of molecular switches in signal transduction pathways that modulate diverse cellular functions in eukaryotes (Nobes and Hall, 1994; Chant and Stowers, 1995; Nagata and Hall, 1996; Ridley, 1996). Higher plants contain a unique subfamily of Rho GTPases, Rop (Yang and Watson,

Table 1. Behavior of Microinjected Pollen Tubes[a]

Treatments[b]	No. of Tubes Treated	Elongation Rate Mean (μm/hr)	No. of Tubes Affected (% Affected)	
			Elongation Cessation[c]	Streaming Cessation[d]
Untreated				
10 mM Ca^{2+}	16	144	0 (0)	0 (0)
2 mM Ca^{2+}	14	152	0 (0)	0 (0)
10 mM Ca^{2+}/0.5 mM caffeine	20	147	0 (0)	0 (0)
Buffers[e]				
Tris-HCl	10	119	1 (10)	0 (0)
Hepes	13	118	1 (7.7)	0 (0)
Preimmune				
10 mM Ca^{2+}	34	117	4 (12)	0 (0)
2 mM Ca^{2+}	19	109	4 (21)	0 (0)
10 mM Ca^{2+}/0.5 mM caffeine	18	115	3 (17)	0 (0)
Anti-Rop1Ps				
10 mM Ca^{2+}	75	65	45 (60)	0 (0)
2 mM Ca^{2+}	29	33	26 (90)	1 (3.5)
10 mM Ca^{2+}/0.5 mM caffeine	25	17	22 (88)	0 (0)
Purified Rop1Ps fusion[e]	13	128	0 (0)	0 (0)
Anti-Rop1Ps plus Rop1Ps fusion[e]	21	127	1 (4.8)	0 (0)
Purified native Rop[e]	10	111	1 (10)	0 (0)
Anti-Rop1Ps plus native Rop[e]	28	102	4 (14)	0 (0)
C3 toxin				
10 mM Ca^{2+}	17	55	12 (71)	12 (71)
10 mM Ca^{2+}/0.5 mM caffeine	20	75	12 (60)	6 (60)

[a] A summary of data collected from all experiments performed with pollen tubes derived from different batches of plants, regardless of growth conditions and plant ages, is provided.

[b] Pollen tubes cultured on media containing various concentrations of Ca^{2+} and caffeine were injected with the indicated agents. Untreated tubes refer to noninjected tubes grown on agarose media under the indicated conditions.

[c] Sustained cessations of tube elongation (10 min or longer growth arrest) were scored.

[d] Complete inhibition of "reverse fountain"–type streaming was scored.

[e] Pollen tubes for these injections were grown on a medium containing 10 mM Ca^{2+}.

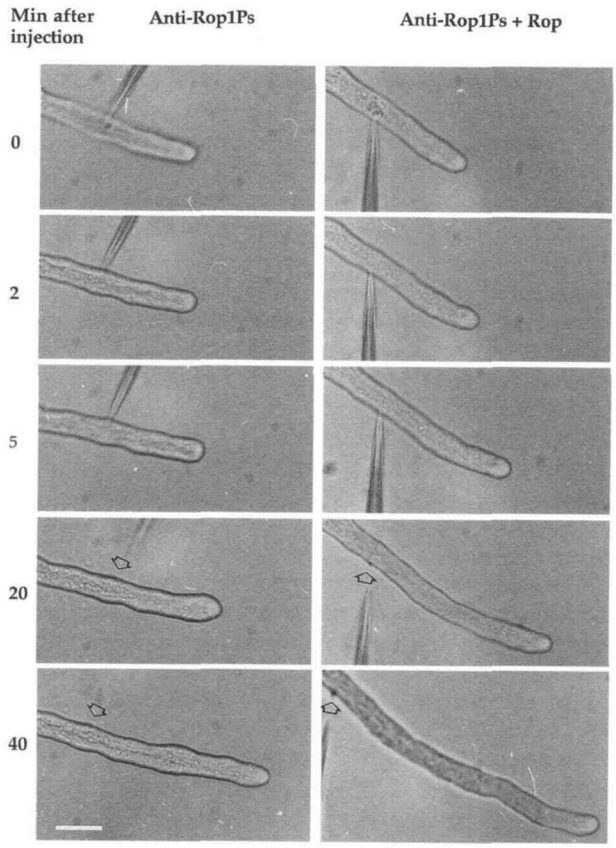

Figure 3. Time Course Analyses of Pea Pollen Tube Behavior after Microinjection with Anti-Rop1Ps Antibodies.

Pollen tubes were cultured on an agarose medium containing 10 mM Ca^{2+} and injected with anti-Rop1Ps antibodies (left) or the antibodies premixed with native Rop protein preparations (right), as described in the legend to Figure 2. Tubes were photographed at various times after injection, as indicated at left. Injection positions on pollen tubes are indicated by the pipette tip or by arrows after the removal of the tip from the tubes. Bar = 20 μm.

1993; Delmer et al., 1995; H. Li, G. Wu, D. Ware, D. Zhou, C.L. Cramer, K.R. Davis, and Z. Yang, unpublished results). However, the cellular function of Rop GTPases in plants remains unknown. Our current data, together with the polar localization of Rop GTPases to the tip of pollen tubes and preferential expression of Rop1 in mature pollen and growing pollen tubes (Lin et al., 1996), suggest that a Rop GTPase-dependent molecular pathway is involved in the control of polarized growth in pollen tubes.

We propose that Rop GTPases modulate localized exocytosis of Golgi vesicles that leads to polarized growth. Among the most prominent cellular functions mediated by the Rho family of GTPases are the organization of actin cytoskeleton, the establishment of cell polarity, exocytosis, endocytosis, focal adhesion, and mitogen-activated protein kinase cascade signaling (Ridley, 1996). In budding yeast, bud site–localized CDC42 and Rho1 are thought to mediate the polarization of the cortical actin cytoskeleton, which is essential for both the establishment of bud sites and subsequent polarized growth (Ziman et al., 1991, 1993; Yamochi et al., 1994). Interestingly, Rop GTPases are localized to the cortex of pollen tube apex (Lin et al., 1996). This location appears to correspond to that of a fine meshwork of F-actin that has been observed in unfixed pollen tubes or tubes fixed by conventional techniques (Pierson and Cresti, 1992; Lin et al., 1996). Given the existence of this actin meshwork, we speculated that Rop GTPases might participate in controlling the organization of this potential tip actin network, which might then direct localized vesicle docking and fusion (Lin et al., 1996). However, use of live cells and cells fixed by rapid freezing and freeze substitution (a technique that provides much more faithful preservation of ultrastructures) did not reveal such a fine actin network at the tip (Miller et al., 1996). Thus, the potential involvement of Rop GTPases in the regulation of a possible tip actin network cannot be validated until the existence of this network in pea pollen tubes is determined.

Polarized delivery of secretory vesicles to the apex of pollen tubes requires a "reverse fountain"–type cytoplasmic streaming, which is most likely controlled by axial actin cables (Heslop-Harrison et al., 1991; Pierson and Cresti, 1992; Pierson and Li, 1992). One possible function for Rop GTPases in pollen tube growth could be regulation of the formation and the dynamics of actin cables. However, injected anti-Rop1Ps antibodies did not significantly inhibit cytoplasmic streaming, suggesting that Rop GTPases are unlikely to mediate the organization or the activity of axial actin cables.

Interestingly, we showed that the C3 toxin induced the cessation of cytoplasmic streaming coupled with growth inhibition. C3, a bacterial exoenzyme that preferentially inactivates the Rho subfamily of GTPases, has been used to demonstrate the function of the Rho subfamily of GTPases, which is distinct from that of the Rac and CDC42 subfamilies (Aktories et al., 1989; Ridley and Hall, 1992; Ridley et al., 1992). Because the Rop subfamily is close to the Rac subfamily (65% amino acid sequence identity) and relatively distant from the Rho subfamily (50% identity), we anticipate that C3 is inactive toward Rop GTPases. Thus, the effect of C3 on cytoplasmic streaming supports the hypothesis that a Rho subfamily GTPase but not a Rop GTPase controls the organization of axial actin cables involved in the delivery of vesicles to the tip of pollen tubes. This hypothesis is also in accord with our observations that it takes much longer (10 to 20 min after injection) for C3 to completely inhibit tube growth, whereas anti-Rop1Ps antibody injection induces rapid growth arrest (1 to 2 min after injection). In addition, caffeine treatments had no effect on C3-induced growth inhibition. The Rho subfamily of GTPases has yet to be identified in plants.

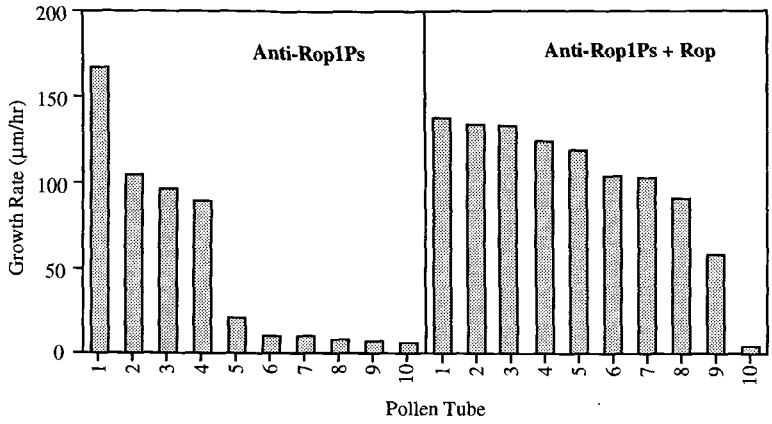

Figure 4. Rescue of Anti-Rop1Ps Antibody–Induced Inhibition by Native Rop Proteins.

Anti-Rop1Ps antibodies were premixed with purified native Rop proteins, as described in Methods, and the mixture was injected into cultured pea pollen tubes, as described in the legend to Figure 2.

Our results are consistent with a crucial role for Rop GTPases in the pathway that leads to targeted vesicle docking and/or fusion. This hypothesis is supported by the following observations. First, injected anti-Rop1Ps antibodies induced immediate cessation of tube growth (within 2 min after injection) (see Figure 3). The rapid arrest of growth is unique to the antibody-induced effect and is not induced by injection with buffer, preimmune preparations, Rop-neutralized antibodies, or the aforementioned C3 toxin. The timing of the anti-Rop1Ps–induced growth arrest is remarkably similar to the 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid–induced dissipation of tip-localized Ca^{2+} gradients and extracellular Ca^{2+} influx, which is accompanied by growth arrest (Pierson et al., 1994). Ca^{2+} is thought to be involved in the regulation of vesicle fusion (Hepler et al., 1994). Thus, the antibody-induced rapid growth arrest is most likely due to the inhibition of vesicle docking/fusion but not to the actin-dependent delivery of vesicles. Second, Rop is localized to the cortex of apical regions, where vesicles fuse with the plasma membrane and the tip-focused Ca^{2+} gradient is found. In the budding yeast, CDC42 is also localized to the growing plasma membrane and adjacent Golgi vesicles in the process of docking and fusion (Ziman et al., 1993). This led to the suggestion that CDC42 may also be involved in the regulation of vesicle fusion (Ziman et al., 1993). In addition, several Rho-type GTPases have been shown to function in signaling pathways that regulate exocytosis in mammalian cells (Price et al., 1995; Mariot et al., 1996). Finally, our

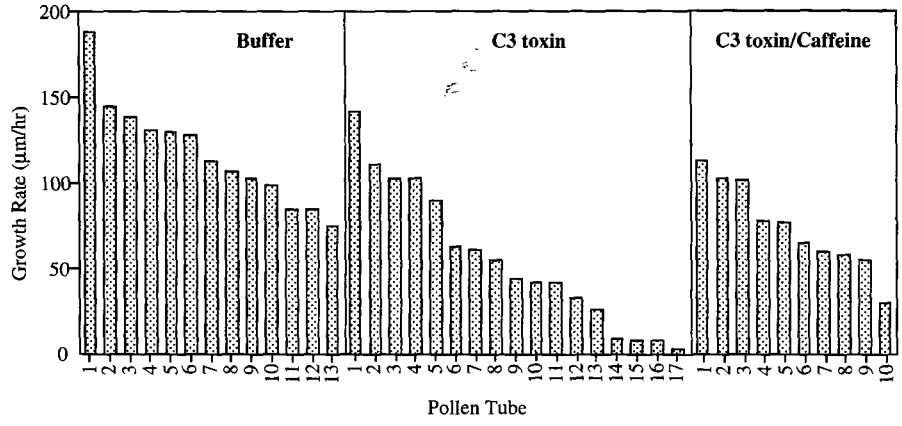

Figure 5. Effects of Microinjected C3 Toxin on Pea Pollen Tube Elongation.

Pollen tubes were cultured on a medium containing 10 mM Ca^{2+} and injected with the C3 toxin or Hepes buffer, as described in the text. Injections were performed with pollen tubes that were cultured in the absence or presence of 0.5 mM caffeine. Microinjection and elongation rate measurement are described in the legend to Figure 2.

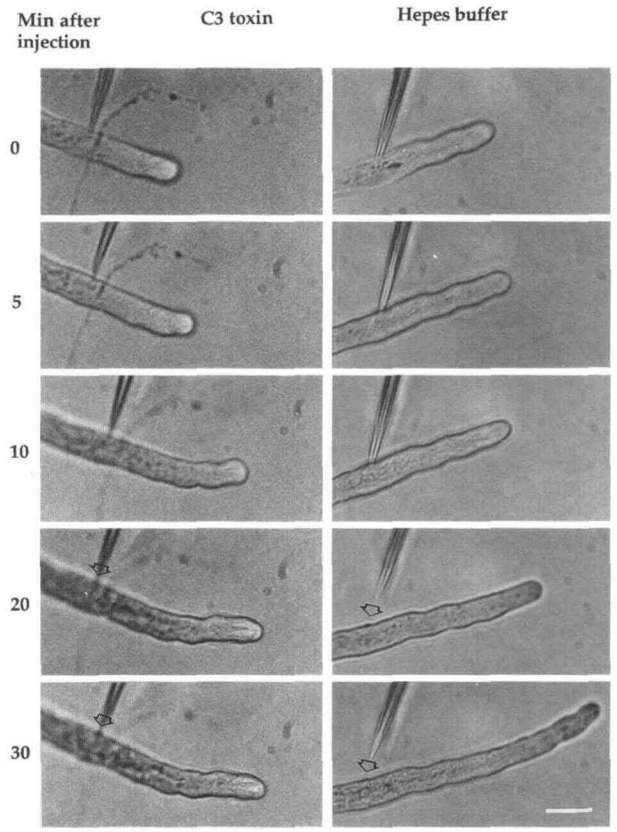

Figure 6. Time Course Analyses of Pea Pollen Tube Behavior after Microinjection with the C3 Toxin.

Pollen tubes were cultured on a medium containing 10 mM Ca^{2+} and injected with the C3 toxin (left) or Hepes buffer (right), as described in the legend to Figure 5. Injected tubes were photographed as described in the legend to Figure 3. The positions of injection on the tubes are indicated by the pipette tip or arrows after the removal of the tip from the tubes. Bar = 20 μm.

injection data support an interaction of Rop with Ca^{2+} signaling that is assumed to play a key role in polarized exocytosis in pollen tubes (Battey and Blackbourn, 1993; Hepler et al., 1994).

We showed that treatment with a subinhibitory dosage of caffeine as well as lowering of extracellular [Ca^{2+}] potentiate the inhibition of pollen tube elongation caused by injected anti-Rop1Ps antibodies. Evidence suggests the existence of tip-localized Ca^{2+} channel activity, which may be responsible for the establishment and maintenance of tip-focused cytosolic Ca^{2+} gradients in cultured pollen tubes from several species (Pierson et al., 1994, 1996; Malhó et al., 1995; Malhó and Trewavas, 1996). Pea pollen tube elongation is most rapid in the presence of 2 to 10 mM extracellular Ca^{2+}, suggesting that optimal tube elongation probably occurs within a window of intracellular [Ca^{2+}]. Thus, it appears that the potentiation of the antibody-induced effect by a lower threshold of extracellular [Ca^{2+}] is actually due to low intracellular [Ca^{2+}].

This idea is further supported by the effect of subinhibitory caffeine treatments on antibody-induced inhibition. We showed that 0.5 mM caffeine has no effect on the elongation of noninjected pollen tubes or tubes injected with preimmune control. In contrast, this caffeine treatment considerably potentiated the effect caused by injected antibodies. The caffeine effect is most likely associated with caffeine-induced changes in cytosolic [Ca^{2+}]. In animal cells, caffeine alters intracellular Ca^{2+} homeostasis by activating the release of the intracellular Ca^{2+} store (Rousseau and Meissner, 1989). In plant cells, the effect of caffeine has been tightly linked to Ca^{2+}- and secretion-dependent processes, such as cytokinesis, xylem differentiation, and tip growth (Becerra and López-Sáez, 1978; Roberts and Haigler, 1992; Pierson et al., 1996). However, caffeine action appears to be very different in plant cells. Evidence suggests that caffeine does not activate the release of the intracellular Ca^{2+} store (Keifer et al., 1992). In contrast, 1.5 mM caffeine completely dissipates tip-focused intracellular Ca^{2+} gradient and extracellular Ca^{2+} influx (Pierson et al., 1996). Thus, the most reasonable explanation for our observations is that caffeine treatment leads to reduced intracellular [Ca^{2+}], which in turn potentiates the effect of injected antibodies. Interaction of the Rho family of GTPases with Ca^{2+} signaling has also been reported in animal and fungal cells (Miyamoto et al., 1987; Bender and Pringle, 1989; Hirata et al., 1992; Grönroos et al., 1996; Peppelenbosch et al., 1996).

We postulate that Rop GTPases may mediate a Ca^{2+}-dependent pathway that leads to polarized exocytosis. It is possible that Rop GTPases regulate the activity of Ca^{2+}-dependent annexins. Phosphorylation of animal annexins that mediate exocytosis has been shown to regulate their Ca^{2+} binding affinity and membrane localization (Sarafian et al., 1991; Chasserot-Golaz et al., 1996). Plant annexins contain potential phosphorylation sites (Battey et al., 1996), raising the possibility that plant annexins may also be regulated by phosphorylation events mediated by a Rop GTPase–activated protein kinase. Several observations are consistent with this notion. First, most identified effector proteins for fungal and mammalian Rho GTPases are protein kinases (Nagata and Hall, 1996). Importantly, Rop GTPases contain the conserved effector domain that is known to interact with effector protein kinases (Yang and Watson, 1993). Second, we have shown that a tonoplast annexin is colocalized with Rop proteins on developing vacuolar membranes in pea tapetal cells and that tonoplast accumulation of Rop precedes that of annexin in these cells (Y. Lin, S. Randall, D. Seals, and Z. Yang, unpublished results). Third, maize annexins have been localized to the apical region of pollen tubes, the region where Rop is localized (Blackbourn et al., 1992; Battey and Blackbourn, 1993).

Although the above-described model for Rop–Ca^{2+} interaction is most attractive, alternative models cannot be ex-

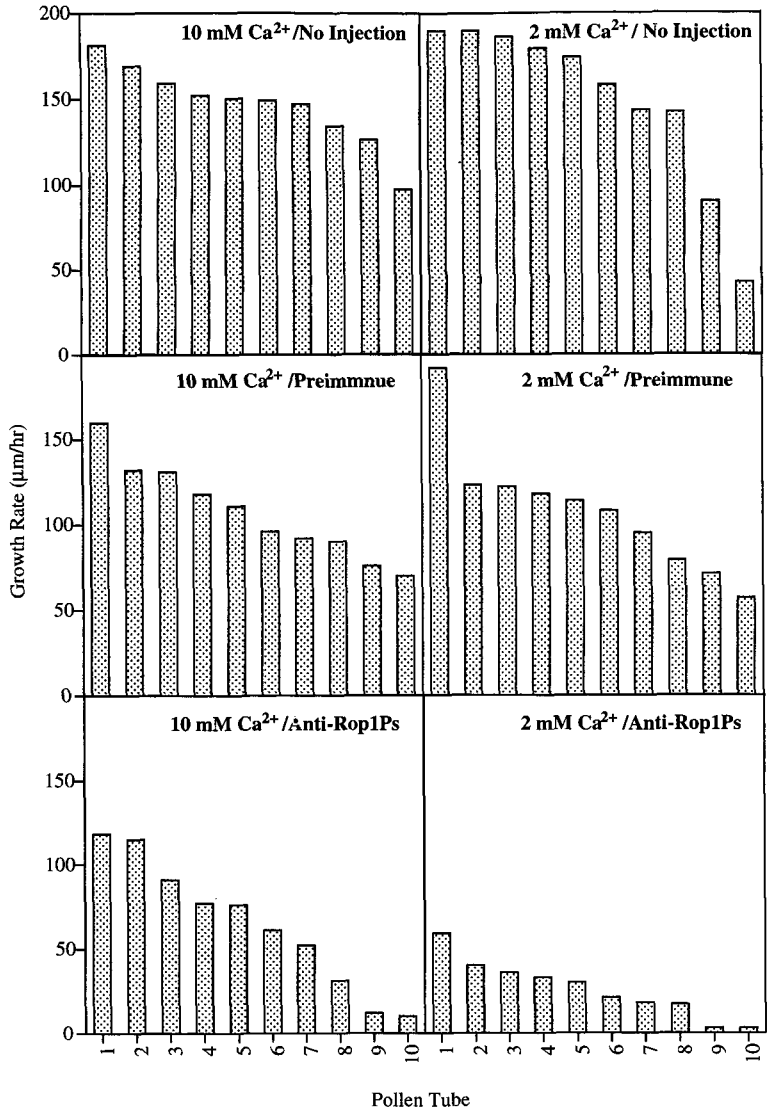

Figure 7. Enhancement of Antibody-Induced Growth Inhibition by a Lower Threshold of Extracellular [Ca^{2+}].

Pollen tubes were cultured on media containing 10 or 2 mM Ca^{2+} and microinjected with preimmune preparations or anti-Rop1Ps antibodies, as described in the legend to Figure 2.

cluded. For instance, Rop GTPases might interact with a pathway linked to a Ca^{2+}/calmodulin–dependent protein kinase, which was also implicated in the regulation of pollen tube exocytosis (Hepler et al., 1994). It is also possible that Rop might mediate ligand-dependent Ca^{2+} influx, as shown in animal cells (Peppelenbosch et al., 1996). In addition, Rop might mediate the synthesis of cell walls whose integrity is mediated by extracellular Ca^{2+}. The introduction of constitutively active or dominant inhibitory forms of Rop GTPases should facilitate verifying the potential functional interaction of Rop with Ca^{2+} signaling and delineating the mechanism underlying this interaction.

METHODS

Anti-Rop1Ps Antibody Purification

The production of polyclonal anti-Rop1Ps antisera was described previously (Lin et al., 1996). The antibodies were affinity purified according to the method of Lin et al. (1996), except that they were eluted using 50 mM glycine-HCl, pH 2.5. For microinjection, the pH of the antibody solution was adjusted to 7.0 with 1.0 M Tris-HCl, pH 8.0. Protein concentrations in purified preparations were determined using a DotMETRIC protein microassay kit (Geno Technology, Inc., St. Louis, MO).

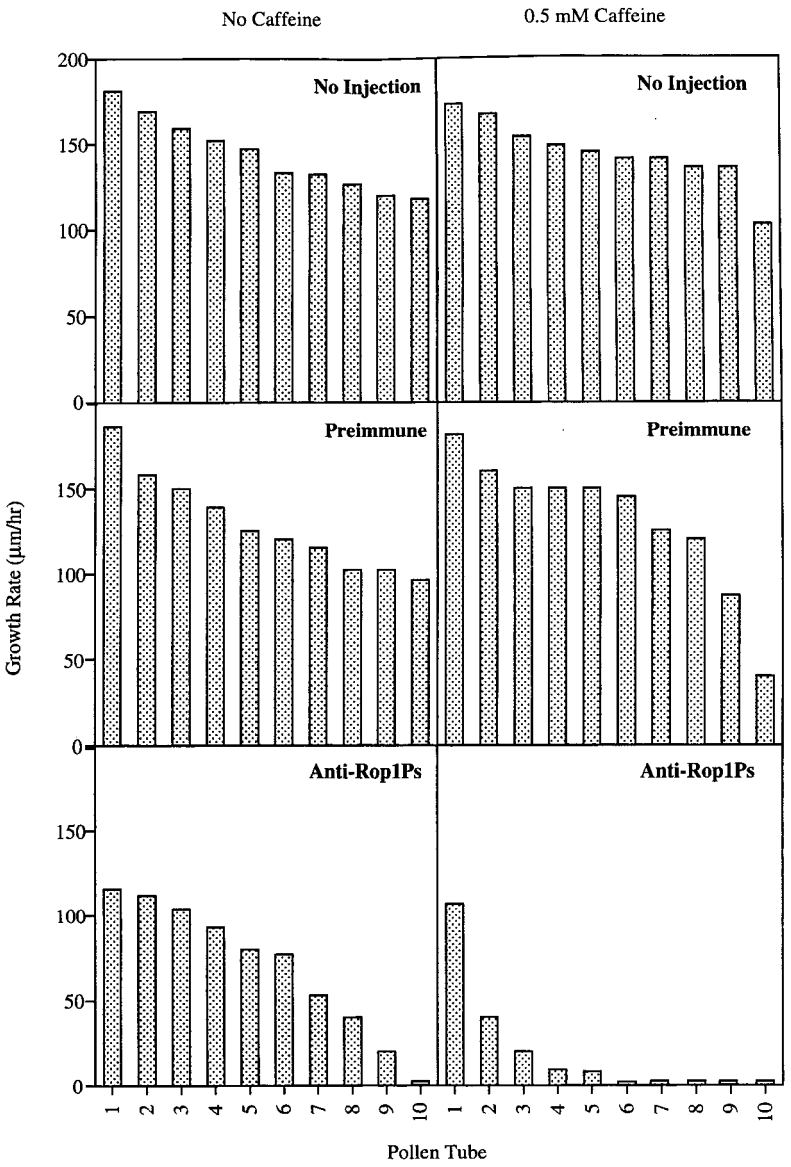

Figure 8. Enhancement of Antibody-Induced Growth Inhibition by Caffeine Treatment.

Pollen tubes were cultured on media containing 10 mM Ca^{2+} in the presence or absence of 0.5 mM caffeine. Elongation rates were measured for noninjected tubes or tubes injected with a preimmune control or anti-Rop1Ps antibodies, as described in the legend to Figure 2.

Purification and Protein Gel Blot Analyses of *Escherichia coli*–Expressed Rop1Ps Fusion Proteins and Native Rop Proteins from Pea Pollen Tubes

Fifty milligrams of pollen grains was harvested from pea plants grown in a growth chamber at 22°C with a light regime of 16 hr of light and 8 hr of dark and germinated in a liquid medium (25% sucrose, 0.01% boric acid, and 10 mM $CaCl_2$). Germinated tubes were sedimented by centrifugation, and the culture medium was removed. Pollen tubes were resuspended in 2 mL of Tris-HCl buffer, pH 7.4, containing 1 μg/mL proteinase inhibitors (aprotinin, pepstatin A, chymostatin, and leupeptin; Sigma) and homogenized using a glass microhomogenizer. Cell debris was removed by centrifugation in an Eppendorf microcentrifuge for 5 min. Extracts were used for the purification of native Rop proteins. Affinity-purified anti-Rop1Ps antibodies were absorbed to nitrocellulose membrane strips, and membranes were incubated in PBS buffer containing 3% nonfat dry milk at room temperature for 1 hr. The membrane strips were then incubated in pollen tube protein extracts at 4°C overnight and washed three times with PBS containing 0.3% Tween 20 and then twice with

PBS. Proteins that remained associated with the antibodies were eluted in 60 μL of 50 mM glycine-HCl, pH 2.5. Eluted proteins were adjusted to pH 7.0 using 1 M Tris-HCl, pH 8.0, and concentrated to 10 μL using a SpeedVac (Savant Instruments, Inc., Farmingdale, NY). Protein concentrations were determined using a DotMETRIC protein microassay kit as described above. The concentration of proteins was estimated to be 1.6 μg/mL.

To determine whether purified proteins specifically react with anti-Rop1Ps antibodies, 2 μg of proteins was separated by electrophoresis on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were detected by using the affinity-purified anti-Rop1Ps antibodies and a chemiluminescence kit (Chemiluminescence Western Blotting kit; Boehringer Mannheim) as described by Lin et al. (1996). To determine whether the purified protein preparations contained any primary antibodies, a duplicate membrane was detected by using the chemiluminescence kit in the absence of primary antibodies. As shown in Figure 1, the protein preparations contained a significant amount of anti-Rop1Ps antibodies. Based on silver staining and immunoblotting, the preparations were estimated to contain ~0.6 mg/mL Rop proteins and ~1.0 mg/mL anti-Rop1 antibodies.

To produce Rop1Ps fusion protein in *E. coli*, the Rop1Ps-coding sequence was cloned in frame with the glutathione-*S*-transferase gene into EcoRI and SstI sites in pGEX-KG (Guan and Dixon, 1991). The Rop1Ps portion of the fusion protein was cleaved with thrombin and was affinity purified using glutathione-conjugated agarose as described (Guan and Dixon, 1991). The resulting purified protein consisted of the Rop1Ps polypeptide and 10 additional amino acid residues encoded by the linker sequence in pGEX-KG. The identity of this fusion protein was confirmed by gel blot analyses using anti-Rop1Ps as described above. Purified proteins were dialyzed against 50 mM Tris-HCl buffer, pH 8.0.

Microinjection Procedures

Pea pollen grains described above were dusted on an agarose medium (25% sucrose, 0.01% boric acid, 10 mM $CaCl_2$, and 1% low-melting agarose [NuSieve GTG; FMC Inc., Rockland, ME]) in a 9-cm Petri dish and immediately covered with a thin layer of the agarose medium. Plates were flooded with a liquid medium of the same components, with the exception of agarose. Approximately 40 min after germination, pollen tubes ~100 μm in length were chosen for injection. Microinjection was performed on an Axiovert 100 inverted microscope (Carl Zeiss, Inc., Jena, Germany). Micropipettes for injection were made from borosilicate glass capillaries (GD-1; Narishige Scientific Instruments, Tokyo, Japan), using a micropipette puller (model BP-7; Narishige Scientific Instruments). Micropipette tips were backfilled to the shank with injection agents by using a 1-mL plastic syringe whose tip was made into a capillary by pulling the heated tip into a thin thread that fit within the inner filament of the micropipettes. The remaining part of micropipettes was filled with mineral oil (Sigma).

The pipette tip was inserted ~40 μm from the tip of pollen tubes by using a micromanipulator and a manual pressure injector (models MMO-203 and IM-5B; Narishige Scientific Instruments). The pipette tip reached no more than 3 μm into the cytoplasm of pollen tubes, and agents were gently loaded into the cytoplasm. Gentle, gradual loading was necessary to avoid severe mechanical damage to the cytoplasm, which usually led to immediate cell death or rupture of pollen tubes. This was accomplished by loading a small aliquot of injected agents at a time (one-sixtieth turn of the injector knob); five loadings were generally performed for each pollen tube. Five loadings were estimated to deliver ~1 nL of solution into the pollen tube cytoplasm. The volume of injected solution was estimated according to the method of Wolniak and Larsen (1995). This estimate may not be accurate because of high turgor pressure within the cytoplasm. Nonetheless, roughly equal volumes of agents were injected into each pollen tube, as determined by the visualization of injected lucifer yellow fluorescent dye.

Five minutes after injection, micropipette tips were slowly removed. Pollen tubes that suffered severe mechanical damage or leakage of the cytoplasm were discarded. The behavior of the injected pollen tubes was recorded using a black-and-white CCD camera (model KP-M1U; Hitachi Ltd., Tokyo, Japan) and a time lapse VCR (model 6730; Panasonic, Mississauga, Ontario, Canada) or a 35-mm camera. Video-recorded images were displayed on a Sony Triniton monitor (Sony, Inc., San Jose, CA). The lengths of pollen tubes were measured at the time of injection and ~1 hr after injection, and the rate of pollen tube elongation was calculated.

For the injection of affinity-purified anti-Rop1Ps antibodies, a concentration of 1 mg/mL was used. To determine whether the antibody-induced effect was specifically caused by blocking Rop GTPase activity, one part of purified native Rop protein preparations (containing 0.6 mg/mL Rop proteins and 1.0 mg/mL anti-Rop1Ps antibodies) was mixed with three parts of anti-Rop1Ps antibodies (1 mg/mL), and the mixture was used for microinjections. We anticipated that each Rop molecule would contain multiple epitopes that could bind anti-Rop1Ps antibodies and that less than a 1:1 molar ratio of Rop proteins to antibodies would be sufficient for blocking most of the antigen binding sites of the antibodies in the mixture. This was consistent with the observation that the purified Rop protein preparations alone did not induce greater growth inhibition than did the preimmune control (see Table 1).

To further confirm the ability of Rop proteins to block anti-Rop1Ps antigenic sites, the antibodies were coinjected with Rop1Ps fusion proteins purified from *E. coli*. Ten micrograms of purified fusion proteins was freeze-dried and resuspended in 10 μL of 1 mg/mL anti-Rop1Ps antibodies. The mixture containing 1 mg/mL Rop1Ps fusion proteins and 1 mg/mL anti-Rop1Ps antibodies was used for injections.

For additional control injections, preimmune sera were subjected to a purification procedure identical to that used for the purification of anti-Rop1Ps antibodies described above. The amounts of preimmune sera and buffer used for protein elution were equivalent to those for anti-Rop1Ps purification. Volumes of injected preimmune preparations were also equivalent to those of purified antibodies.

Most microinjection experiments were performed with pea pollen tubes cultured in a medium containing 10 mM Ca^{2+}. To determine the effect of extracellular Ca^{2+} on anti-Rop1Ps–induced behavior, pollen tubes were cultured on plates containing different Ca^{2+} concentrations in the flooding medium. To examine the effect of caffeine treatments, 0.5 mM caffeine was included in the liquid medium that was used to flood the agarose medium.

The bacterial C3 exoenzyme was purchased from CalBiochem (La Jolla, CA). C3 was resuspended in 5 mM Hepes buffer, pH 6.5, and injected at a concentration of 1 mg/mL, as described for the anti-Rop1Ps antibody.

We noticed that the rate of cultured pea pollen tubes may vary, depending on growth conditions and ages of plants. To minimize growth variability, each set of data was collected from microinjection experiments performed with pollen tubes derived from the same batch of pea plants that were at the same developmental stages. Figures 2, 3,

5, 7, and 8 represent data from the microinjection experiment that was performed using the same batch of plants within a 1-week period. Table 1 summarizes data collected from all experiments performed, regardless of the ages of the plants and growth conditions.

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