Localization of a Rho GTPase Implies a Role in Tip Growth and Movement of the Generative Cell in Pollen Tubes

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The Rho family GTPases function as key molecular switches, controlling a variety of actin-dependent cellular processes, such as the establishment of cell polarity, cell morphogenesis, and movement in diverse eukaryotic organisms. A novel subfamily of Rho GTPases, Rop, has been identified in plants. Protein gel blot and RNA gel blot hybridization analyses indicated that one of these plant Rho GTPases, Ropal, is expressed predominantly in the male gametophyte (pollen and pollen tubes). Cell fractionation analysis of pollen tubes showed that Rop is partitioned into soluble and particulate fractions. The particulate Rop could be solubilized with detergents but not with salts, indicating that it is tightly bound to membranes. The membrane association appears to result from membrane anchoring via a geranylgeranyl group because an in vitro isoprenylation assay demonstrated that Rop1Ps is geranylgeranylated. Subcellular localization, using indirect immunofluorescence and confocal microscopy, showed that Rop at the apex forms a gradient with decreasing concentration from tip to base and appears to be associated with the plasma membrane. These results suggest that the apical Rop GTPase may be involved in the signaling mechanism that controls the actin-dependent tip growth of pollen tubes. Localization of the Rop GTPase to the periphery of the generative cell is analogous to that of myosin, suggesting that the Rop GTPase plays an important role in the modulation of an actomyosin motor system involved in the movement of the generative cell.

INTRODUCTION

The Rho family of small GTPases (GTP binding proteins) plays a central role in the modulation of many cellular processes related to the actin cytoskeleton (reviewed in Chant, 1994; Hall, 1994; Chant and Stowers, 1995). Many Rho and related proteins have been identified in various eukaryotes and categorized into three major subfamilies: CDC42, Rac, and Rho. Their categorization is based on their cellular functions and sequence homology (Chant, 1994; Hall, 1994; Chant and Stowers, 1995). In motile mammalian cells, each subfamily has a distinct function, yet they constitute a hierarchical cascade of molecular switches for the control of actin-mediated cell morphogenesis, movement, and adhesion (Ridley and Hall, 1992; Ridley et al., 1992; Hall, 1994; Nobes and Hall, 1995). Within this cascade, CDC42 controls Rac, which in turn controls Rho.

Similar Rho GTPase cascades also appear to be involved in cell morphogenesis in other diverse systems. In the yeast Saccharomyces cerevisiae, CDC42 controls the establishment of cell polarity associated with the asymmetrical distribution of cortical actin filaments at the site of bud emergence (Adams et al., 1990; Johnson and Pringle, 1990). CDC42 also appears to regulate the activity of a series of Rho-related proteins (Rho01, Rho02, Rho03, and Rho04) that are required for subsequent bud growth (Matsui and Toh-e, 1992; Yamochi et al., 1994). Consistent with their functions, both CDC42 and Rho01 are localized to the site of bud emergence and growth (Ziman et al., 1993; Yamochi et al., 1994). In Schizosaccharomyces pombe, CDC42 controls actin-mediated asymmetrical growth (Miller and Johnson, 1994). CDC42 is also involved in the modulation of neuronal cell differentiation and cell coupling in the immune system (Luo et al., 1994; Stowers et al., 1995). Animal CDC42 is able to complement yeast cdc42 temperature-sensitive mutations, indicating remarkable functional conservation (Shinjo et al., 1990; Chen et al., 1993).

The establishment and maintenance of cell polarity are fundamental cellular attributes to differentiation and development in plants. Polarized growth is exhibited in many plant cell types, such as zygotes, specialized epidermal cells (e.g., root hairs, trichomes, and guard cells), and pollen tubes. Although the actin cytoskeleton has been implicated in the polarized growth of plant cells, molecular mechanisms controlling the organization of F-actin and growth polarity remain largely obscure. As a first step toward understanding the possible role of Rho in controlling these cellular behaviors in plants, we have cloned...
and characterized genes encoding several Rho-type GTPases from pea and Arabidopsis (Yang and Watson, 1993; H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished data). These plant Rho proteins belong to a novel subfamily of Rho GTPases, Rop, which is distinct from those found in fungi and animals. In Arabidopsis, the expression of Rop1At, the Arabidopsis counterpart of pea Rop1Ps (Yang and Watson, 1993), is correlated with pollination and pollen tube growth (H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished data). This provides the first clue that Rop1 may be involved in the control of pollen and pollen tube function.

Pollen and pollen tubes are an attractive model system for investigating the mechanism controlling cellular polarity. First, pollen grains must establish a growth polarity before the emergence of the tube because only one tube emerges from one of the three or four apertures on the grain during germination. Second, growth occurs only at the apex by fusion of vesicles to the apical plasma membrane. This tip growth requires targeted delivery of vesicles containing membranes and wall-matrix materials to the site of growth. Third, the direction of pollen tube extension is highly regulated both in vitro and in vivo. In vitro tube growth can be guided by electrical potential and chemical gradients (Mascarenhas, 1993). In vivo pollen tube growth is guided through the transmitting tract of styles and toward the embryo sac (Hulskamp et al., 1995). Because the extension of pollen tubes is totally dependent on tip growth, pollen tube guidance must involve directed fusion of vesicles to the site of growth. In addition to polarized growth, cellular polarity in pollen tubes is also reflected by the distinct functional zonation along the length of the pollen tube (Cresti and Tiezzi, 1992). Behind an apical growing zone and an organelle-rich subapical zone, there is a wide nuclear zone that contains vegetative nuclei and a generative cell. As the pollen tube extends, the generative cell, accompanied by the vegetative nucleus, moves down the tube as a male germ unit (Palevitz and Tiezzi, 1992).

There is evidence that an actomyosin system is involved in the movement of vesicles to the tip and the generative cell (Heslop-Harrison and Heslop-Harrison, 1989; Palevitz and Tiezzi, 1992; Pierson and Cresti, 1992; Pierson and Li, 1992). Actin filaments form extensive axial bundles that appear to guide the delivery of vesicles to the apical zone. Actin filaments also are associated with both the vegetative nucleus and the generative cell. Actin also has been proposed to determine the polarity of pollen germinal aperture and tip growth (Tiwari and Polito, 1988). A fine actin network has been observed both close to the pore from which the tube will emerge and at the tip of the pollen tube (Tiwari and Polito, 1988; Pierson and Cresti, 1992; Pierson and Li, 1992). Cytochalasin treatment leads to inhibition of germination, random vesicle distribution, and inhibition of tip growth (reviewed in Pierson and Cresti, 1992). Together, these studies suggest that the actin cytoskeleton is dynamic during pollen activation and tube growth and plays an important role in the function of the male gametophyte. However, the mechanism controlling the dynamic organization of the actin cytoskeleton in pollen tubes is unknown.

In this study, we report that a unique member of the conserved Rho GTPase family is expressed predominantly in pea pollen and pollen tubes. Furthermore, our results indicate that the pea Rho GTPase is localized preferentially to what appear to be specific regions of the plasma membrane, that is, the apex of pollen tubes and the periphery of the generative cell. These results support the hypothesis that one or more Rho GTPases modulate both the tip growth of pollen tubes and the movement of the generative cell.

RESULTS

Preferential Accumulation of the Rop Protein and mRNA in the Male Gametophyte

We have shown that an Arabidopsis homolog of Rop1Ps, Rop1At, is expressed specifically in fully developed flowers and that its mRNA accumulates to a maximum level during pollination and fertilization, suggesting that Rop1 plays a fundamental role in pollen and pollen tube function (H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished results). As a first step in testing this possibility, the accumulation of Rop1Ps protein in different parts of pea flowers

![Figure 1. Protein Gel Blot Analysis of Rop Proteins in Different Parts of Pea Flowers.](image)

Total proteins were extracted from different parts of flowers as indicated. Equivalent amounts of proteins were separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, reacted with the affinity-purified anti-Rop1Ps antibody, and detected with a chemiluminescence kit as described in Methods.
was estimated by protein gel blot analysis, using a polyclonal antibody raised against Rop1Ps. As shown in Figure 1, a specific band of ~21 kD, corresponding to the estimated molecular mass of Rop1Ps, was detected. The intensity of this band was strongest in anthers and pollinated stigma and styles. A weak signal was detected in filaments and petals only after extended exposure. However, no Rop protein was found in unpollinated stigma and styles. It should be noted that the anti-Rop1Ps polyclonal antibody is likely to react with other members of the Rop subfamily in pea, because all plant Rop members that have been characterized to date share >80% sequence identity (H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished data). Hence, these experiments did not address specifically whether any of the proteins detected by the antibody raised against Rop1Ps are specifically encoded by Rop1Ps.

To study further the relationship of the Rop protein found in different parts of the flower to Rop1Ps gene expression, Rop1Ps transcript levels from different vegetative and reproductive pea organs were determined by RNA gel blot hybridization analysis. As shown in Figure 2, low levels of Rop1Ps mRNA were also detected in floral buds, vegetative buds, and root tips, but the Rop1Ps transcript was most abundant in pollen among various parts of pea plants, indicating that Rop proteins detected in the male gametophyte are indeed encoded primarily if not specifically by Rop1Ps. This is consistent with the expression pattern of Rop1At during floral development in Arabidopsis (H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished data).

Subcellular Localization of Rop Proteins in Pollen Tubes

To provide evidence for the cellular function of Rop proteins in pollen tubes, their subcellular localization was investigated using indirect immunofluorescence. In vitro–cultured pollen tubes were stained using the affinity-purified Rop1Ps antibody and fluorescein isothiocyanate–conjugated second antibody. Fluorescent images were visualized using confocal or epifluorescence microscopy. Figures 3A to 3D show confocal images of a series of laser sections of a labeled pollen tube. Punctate dots of fluorescence were observed uniformly throughout the vegetative cytoplasm of the pollen tube. However, highly intense fluorescence was observed in the cortex of the apical region of pollen tubes. The cortical fluorescence has a gradient with decreasing intensity from the tip to the base. This pattern of fluorescence was found consistently in pollen tubes with different growth stages (data not shown). In pollen tubes that had just emerged, the cortical fluorescence was restricted to the tip. As the tube elongated, the area of cortical stain increased, but the most intense fluorescence always was restricted to the apical 10 to 20 μm. A much weaker cortical signal was found uniformly throughout the basal region in elongated pollen tubes (Figure 3D). This cortical fluorescence in the basal region also was composed of punctate dots. These patterns of fluorescence are unlikely to be artifacts, because the second antibody alone did not stain pollen tubes (data not shown) and the affinity-purified Rho1Ps primary antibody reacted specifically with the Rop protein, as determined by protein gel blot analysis (see above).

As shown in Figure 4A, punctate fluorescence from the anti-Rop1Ps antibody also was concentrated on the periphery of the generative cell. The pattern of fluorescence was in the shape typical of the generative cell, that is, spindle shaped with a tail. The generative cell was identified by the bright staining of nuclear DNA with propidium iodide, whereas vegetative nuclei were stained weakly (Figure 4B).

Attempts to colocalize the actin cytoskeleton and the Rop protein were not successful, because the protocol that was optimal for the Rop protein severely disrupted the structure of F-actin. However, by staining unfixed pollen tubes with rhodamine–phalloidin, extensive actin filaments were visualized throughout the tube. Figures 5A to 5D are a series of laser scans of rhodamine–phalloidin fluorescence revealed by confocal microscopy. The stained F-actin includes the cortical and cytoplasmic actin cables and the less organized apparent actin network at the tip. The apical fluorescence appeared to be most intense in the cortex. Although it remains to be determined whether this cortical actin is colocalized with the cortical Rop proteins at the apex, the presence of both Rop and actin in the apical cortex suggests that there might a correlation between the two.
Rop Proteins Are Distributed in Particulate and Soluble Fractions of Pollen Tubes

The localization of Rop proteins on the periphery of the generative cells and in the cortex of the apical region of pollen tubes suggests that Rop is associated with the plasma membrane in those regions. This is consistent with the requirement of membrane localization for the biological activity of Rho GTPases (Hancock and Marshall, 1993). To confirm the membrane association of Rop proteins, their subcellular distribution was determined by subcellular fractionation. Pollen tubes, which were frozen in liquid nitrogen, were ground and centrifuged at low speed (1000g) to remove cell debris. The 1000g supernatant was then centrifuged at 10,000g, and the distribution of the Rop protein was determined by protein gel blotting. Figure 6A shows that Rop protein was found in both particulate and supernatant fractions. An aliquot of the 10,000g supernatant was also centrifuged at 100,000g to obtain microsomal and soluble fractions. As shown in Figure 6A, Rop proteins once again were found in both the supernatant and particulate fractions.

As shown in Figure 6B, the Rop protein in both particulate fractions could be solubilized with 2% Triton X-100 but not with 0.5 M NaCl, indicating that this protein is bound tightly to membranes. Figure 6 also shows that although most Rop protein is soluble, a significant amount of this protein was associated with membranes. The fact that the Rop protein is found in both 10,000g and 100,000g particulate fractions suggests that the membrane Rop protein might be associated primarily with the plasma membrane (Hodges and Leonard, 1974; Sze, 1980; Ziman et al., 1993). Thus, these fractionation data are in agreement with the immunolocalization of Rop proteins in pollen tubes as described above.

In Vitro Isoprenylation of Rop1Ps

In fungal and animal cells, the membrane association of Rho GTPases results from their isoprenylation by the addition of a geranylgeranyl group at the C terminus (Adamson et al., 1992; Ando et al., 1992; Hancock and Marshall, 1993). Rop1Ps contains a C-terminal signature sequence for protein
Localization of a Rho GTPase in Pollen Tubes

geranylgeranyltransferase I, suggesting that it is also geranylgeranylated (Yang and Watson, 1993). To determine whether Rop1Ps is indeed geranylgeranylated, an in vitro assay was performed using cell-free extracts from the plant Atriplex nummularia as a source of plant protein isoprenyltransferases (Zhu et al., 1993). As shown in Figure 7, when the assay containing protein extracts from an Escherichia coli strain expressing Rop1Ps fusion protein was used (see Figure 7A), a protein of the size of the fusion protein was efficiently labeled with tritiated geranylgeranyl pyrophosphate but barely labeled with tritiated farnesyl pyrophosphate (Figure 7B). The converse labeling pattern was observed with an E. coli strain expressing a DnaJ homolog from the plant A. nummularia (Figure 7C), which was shown to be farnesylated (Zhu et al., 1993). Protein extracts from a strain containing the expression vector alone were not labeled with either isoprenyl substrates. These results indicate that Rop1Ps is geranylgeranylated, and this modification may be responsible for the membrane association of the Rop proteins in pollen and pollen tubes.

DISCUSSION

A central theme has emerged from recent studies of Rho GTPases in various animal and fungal systems: they function as common molecular switches that are turned on by various internal and external signals and control specific actin-related cellular behaviors (Hall, 1994; Chant and Stowers, 1995). Although Rho-like GTPases also have been identified in plants, their functions are not known. This report provides evidence for the polarized localization of Rop1Ps in specific regions of the pollen tube, the apical cortex, and the periphery of the generative cell. This polarized localization suggests that the plant Rho protein plays a role in the control of the actin cytoskeleton involved in the function of these specific regions, that is, the tip growth of pollen tubes and the movement of the generative cell.

The conservation of the Rho family of GTPases is reflected not only in their primary structure and function but also in their regulation. For instance, all Rho GTPases contain a C-terminal CAAL motif (C, cysteine; A, aliphatic amino acids; L, leucine), the site of isoprenylation by protein geranylgeranyltransferase I. Isoprenylation is a prerequisite for membrane association of Rho GTPases, which in turn are required for regulation of the actin cytoskeletal organization (Ziman et al., 1991; Hancock and Marshall, 1993). However, Rho GTPases are distributed in both particulate and soluble fractions in a number of cells; the proportion of membrane-associated Rho varies with cell type and kind of Rho GTPase (Ziman et al., 1991, 1993; Adamson et al., 1992; Yamochi et al., 1994), suggesting that the membrane localization is regulated. Although the mechanism for this regulation is not understood fully, it is known that solubilization from membranes involves a ubiquitous protein called RhoGDI (reviewed in Takaishi et al., 1993; Hall, 1994). It appears that active and inactive forms of Rho GTPases cycle between the membrane and the cytosol. Upon geranylgeranylation and GTP binding, Rho is targeted to membranes and becomes activated. RhoGDI forms a complex with the geranylgeranylated GDP-bound form, leading to the dissociation of inactive Rho from membranes.

Subcellular fractionation and indirect immunofluorescence microscopy indicated that the Rop proteins also are distributed in both membrane and soluble fractions in pollen tubes. The fractionation study showed that a significant amount of the Rop GTPases is membrane associated (Figure 6); this is analogous to the distribution of Rho1 in yeast, CDC42 in monocytes, and RhoA and RhoC in various mammalian cells (Adamson et al.,

Figure 4. Localization of Rop Proteins on the Periphery of the Generative Cell in the Pea Pollen Tube.

Pollen tubes were first stained with the anti-Rop1Ps antibody, as described in the legend to Figure 3. For nuclear DNA visualization, propidium iodide was added to stained pollen tubes 10 min before mounting. Stained pollen tubes were visualized using an epifluorescence microscope, and the mid-plane image was photographed with a 35-mm camera.

(A) Fluorescence from anti-Rop1Ps antibody staining.

(B) Nuclear DNA staining of the same pollen tube as shown in (A). The more intense staining indicates the nucleus of the generative cell (basal end), and the weaker staining indicates a vegetative nucleus (apical end). Bar = 10 µm.
Figure 5. Actin Visualization in Pea Pollen Tubes.

In vitro-germinated pollen tubes were stained with rhodamine-phalloidin as described in Methods and visualized using a confocal microscope. A series of 1-μm sections was scanned, and alternate sections are shown. (A) to (D) Sequential serial scans from the mid-plane (A) to the surface of the pollen tube (D). Bar in (D) = 10 μm.

1992; Aepfelbacher et al., 1994; Yamochi et al., 1994). This also agrees with the results of immunocytochemical localization, which suggested that the Rop GTPases appear to be localized differentially to certain specific regions of the plasma membrane (Figures 3 and 4). If the conservation of its function and regulatory mechanisms also holds true in plants, the membrane-localized Rop protein represents the active pool of the Rop GTPase, whereas the soluble Rop is inactive. Thus, this would be consistent with the notion that the function of the Rop GTPase is related to the specific localization on the plasma membrane.

Given its localization at the tip, it is most likely that Rop GTPase plays a pivotal role in the control of polarized growth in pollen tubes, that is, tip growth and pollen tube guidance. The polarized localization to the apparent apical plasma membrane of pollen tubes is similar to that of CDC42 and Rho1 at the site of bud emergence and growth, where they regulate the polarized organization of actin cytoskeleton, bud formation, and polarized growth induced by mating factors (Ziman et al., 1993; Chant, 1994; Yamochi et al., 1994). Immunogold electron microscopy has shown that CDC42 is localized to the growing plasma membrane and to the vesicles that are in the process of fusing with the plasma membrane (Ziman et al., 1993). Our studies suggest that Rop and CDC42 are similarly located, although this needs to be confirmed by immunogold electron microscopy.

Because of this analogous localization and the conserved function of the Rho GTPase in regulating the actin cytoskeleton, it is tempting to postulate that the Rop GTPase controls directional pollen tube growth by modulating the organization of the actin cytoskeleton. Two major forms of F-actin, the axial actin cables and the apical actin network, have been observed in various pollen tubes (Figure 5; Tiwari and Polito, 1988; Pierson and Cresti, 1992; Pierson and Li, 1992). The former probably directs the transport of vesicles to the apical region; the latter probably mediates the fusion of vesicles to the specific site of growth leading to tip growth and pollen tube guidance (reviewed in Steer, 1990; Pierson and Cresti, 1992).

Using unfixed pollen tubes, we observed the apical actin network in pea pollen tubes (see Figure 5), which apparently is consistent with several earlier studies using conventional fixation techniques (Tiwari and Polito, 1988; Pierson and Cresti, 1992; Pierson and Li, 1992). However, actin preservation, using either unfixed pollen tubes or conventional fixation techniques,
has been questioned, because few tip actin filaments were observed in lily pollen tubes prepared by the rapid freeze fixation and freeze substitution technique (Lancelle and Hepler, 1992). This technique is thought to result in much improved ultrastructural preservation (Lancelle and Hepler, 1992). Though the presence of the apical actin network remains to be resolved, additional functional studies of the Rop GTPase, especially involving dominant mutations of the GTPase, will provide direct evidence concerning the role of Rop1Ps in the modulation of pollen tube growth and guidance in relation to the organization of actin cytoskeleton. These studies also might assist in clarifying the controversial issue regarding the apical actin network.

The localization of the Rop GTPase on the periphery of the generative cell is consistent with the notion that the movement of the generative cell is controlled by an actomyosin system in the vegetative cytoplasm (Palevitz and Tiezzi, 1992). Several lines of evidence support this model (reviewed in Palevitz and Tiezzi, 1992). First, the generative cell is enmeshed in a microfilament network in the vegetative cytoplasm, whereas the generative cell appears to lack actin filaments. Second, cytochalasin treatment inhibits the movement of the generative cell. Finally, myosin has been localized to the periphery of the generative cell. Interestingly, the Rop GTPase shows the same localization pattern as myosin. Thus, Rop could control the organization of the actin filaments surrounding the generative cell or might be involved in the signaling mechanism that regulates the actomyosin-mediated movement. There is evidence that a novel myosin, Myr5, is a Rho effector (Reinhard et al., 1995). It remains to be determined whether and how the myosin localized to the periphery of the generative cell interacts with the Rop GTPase.

Because the anti-Rop1Ps polyclonal antibody probably cross-reacts with other Rop isoforms, our data do not directly show which Rop isoform is localized to the apical cortex or to the periphery of the generative cells. However, Rop1Ps mRNA accumulation in pollen (Figure 2) is correlated with the abundance of Rop protein detected by protein gel blot analysis (Figure 1), suggesting that Rop1Ps is responsible primarily if not solely for the pollen tube localization. Our studies in Arabidopsis also suggest that a specific Rop isoform may be involved in pollen tube growth (H. Li, D. Ware, D. Zhou, K.R. Davis, C.L. Cramer, and Z. Yang, unpublished results). The expression pattern of Rop1At (an Arabidopsis homolog of Rop1Ps) appears to be similar to that of Rop1Ps. Among four different Arabidopsis Rop isoforms, Rop1At is the only one expressed in open flowers (H. Li and Z. Yang, unpublished results). Furthermore, an indirect immunofluorescence study involving Rop1Ps antibody showed that Rop is also localized preferentially to the apical cortex in Arabidopsis pollen tubes (Y. Lin and Z. Yang, unpublished results). These results are consistent with the notion that plants have evolved a specific Rop, Rop1, which may play a distinct role in sexual reproduction. The pea genome contains at least six Rop isoforms. Additional studies are needed to determine whether other Rop isoforms are also expressed in pea pollen tubes.

Conceivably, different Rop isoforms might be involved in the regulation of other aspects of plant development, such as spatial control of cell division. Among various vegetative tissues from pea, Rop is most abundant in meristematic tissues, as shown by protein gel blot analysis, using Rop1Ps antibody (Y. Lin and Z. Yang, unpublished results). This suggests that the

Figure 6. Distribution of Rop Proteins in Particulate and Soluble Fractions.

(A) Fifty micrograms of pea pollen grains was germinated, ground, and fractionated by centrifugation. The 1000g supernatants were centrifuged at 10,000g. Supernatants obtained with 10,000g centrifugation were again centrifuged at 100,000g. Particulate fractions were resuspended in a buffer equal to the volume of supernatants. Equal volumes of each fraction were separated on SDS–polyacrylamide gels and analyzed by protein gel blotting as described in the legend to Figure 1. (B) The 10,000g pellets as described in (A) were washed with 2% Triton X-100 or 0.5 M NaCl. The remaining pellets were resuspended in an equal volume of washes. Both particulate and solubilized proteins were analyzed by protein gel blotting, as described in Figure 1.
Figure 7. In Vitro Protein Isoprenylation Assays.

Total protein extracts isolated from *E. coli* strains expressing substrate proteins were used for protein isoprenylation assays, as described in Methods. The isoprenyl substrates were either $^3$H-geranylgeranyl pyrophosphate ($^3$H-GGPP) for the geranylgeranylation assay or $^3$H-farnesyl pyrophosphate ($^3$H-FPP) for the farnesylation assay. Mixtures of isoprenylation reactions were separated on a 10% SDS–polyacrylamide gel and subjected to fluorography. pDS, an expression vector without inserts; pSRHO1, plasmid containing the Rop1Ps fusion gene (see Yang and Watson, 1993); pANJ1, plasmid-containing gene encoding the ANJ1 protein (Zhu et al., 1993). The positions of the ANJ1 and Rop1Ps fusion proteins are indicated.

(A) Total *E. coli* proteins separated on a 10% SDS–polyacrylamide gel and stained with Coomassie Brilliant Blue R 250.
(B) Fluorography of the geranylgeranylation reaction mixtures.
(C) Fluorography of the farnesylation reaction mixtures.

Rop GTPase might play a role in controlling cell division. In plants, the position of the division plane is determined by the preprophase band and the phragmoplast, both of which contain F-actin and microtubules (Lloyd, 1991; Wick, 1991). Future studies will reveal whether distinct Rop GTPases modulate specific cellular behaviors such as polarized growth and spatial organization of cell division in plants.

**METHODS**

**Plant Materials**

Root tips and apical buds were harvested from 2-day-old seedlings germinated on moist paper towels at room temperature. Pollen grains and different parts of mature pea plants were harvested from plants (*Pisum sativum* cv Extra Early Alaska) grown in a growth chamber at 22°C with a light regime of 16 hr of light and 8 hr of dark.

**RNA Gel Blot Hybridization**

Total RNA from pea pollen and different tissues was isolated by using the Trizol reagent kit (Molecular Research Inc., Cincinnati, OH). Twenty micrograms of total RNA was separated on an agarose gel, transferred to a Magna NT membrane (Micron Separations Inc., Westboro, MA), and hybridized with a Rop1Ps-specific probe, as described previously (Yang and Watson, 1993).

**Production and Purification of Anti-Rop1Ps Polyclonal Antibody**

The Rop1Ps protein was purified from *Escherichia coli* cells expressing a Rop1Fs fusion protein, as described previously (Yang and Watson, 1993). This purified fusion protein was used to prepare anti-Rop1Fs polyclonal antibody in rabbits (Cocalico Biological Inc., Reamstown, PA). Antiserum obtained after the third booster was subjected to affinity purification, using the purified Rop1Ps fusion protein (Yang and Watson, 1993). Briefly, the Rop1Fs protein was separated on an SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Antiserum was incubated with membrane strips containing Rop1Fs. After three washes in PBS buffer (13.7 mM NaCl, 0.27 mM KCl, 0.15 mM KH$_2$PO$_4$, 0.8 mM Na$_2$HPO$_4$, pH 7.4) containing 0.3% Tween 20, the antibody was eluted with 0.3 mL of 0.1 M glycine-HCl, pH 2.5, by gentle shaking for 1 min. The eluent was mixed with 0.3 mL of glycerol and 0.1 mL of 1 M Tris-HCl, pH 8.0. The membrane was washed with 0.1 mL of distilled water. The eluent and the wash were combined, and the purity of the antibody was tested by protein gel blot analysis, using extracts of an *E. coli* strain expressing Rop1Fs fusion protein and extracts from different organs of pea plants. The purified antibody detected a single band of ~21 kD in these extracts.
Plant Protein Extraction, Electrophoresis, and Protein Gel Blot Analysis

For the isolation of total proteins from different pea tissues, 100 mg of tissues was frozen in liquid nitrogen, ground to fine powder with mortar and pestle, and resuspended in 1 mL of protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.025% bromophenol blue, 1 μg/mL proteinase inhibitors [aprotinin, pepstatin A, chymostatin, and leupeptin]). The sample was boiled for 5 min and then centrifuged at 8000g for 10 min. Approximately 20 μL of supernatant containing equivalent amounts of protein for each sample was loaded on a 10% SDS–polyacrylamide gel and separated by electrophoresis. The protein was transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and reacted with the affinity-purified anti-Rop1Ps polyclonal antibody (×500 dilution). The antibody was detected, using a chemiluminescence kit (Chemiluminescence Western Blotting Kit; Boehringer Mannheim) and an anti-rabbit secondary antibody (iGg) conjugated with horseradish peroxidase (Boehringer Mannheim). Membranes were exposed to x-ray film for 1 min to several minutes. To confirm equivalent protein loading, proteins were stained with Ponceau S concentrate (Sigma) after protein gel blot analysis.

Indirect Immunofluorescence Microscopy

Pea pollen grains were germinated in a liquid medium (25% sucrose, 0.01% boric acid, 1 mM CaCl$_2$) for 1 hr at room temperature. Pollen tubes were incubated in fixative (4% paraformaldehyde, 50 mM Pipes buffer, pH 6.9, 2 mM MgSO$_4$) for 1 hr. After washing with PBS, pollen tubes were treated with 2% cellulase R-10 and 1% macerozyme R-10 (Crescent Chemical Co., Hauppauge, NY) in a buffer containing 15 mM Mes, pH 5.5, 400 mM mannitol, 5 mM CaCl$_2$, and 1 μg/mL proteinase inhibitors (see above) at room temperature for 5 min. Digested pollen tubes were washed with PBS twice for 5 min each and once with PBS containing 0.1% Triton X-100 for 5 min. Pollen tubes were transferred to polylysine-coated microscope slides, allowed to settle for 5 min, and blocked with 3% nonfat dry milk in PBS at room temperature for 1 hr. Slides were incubated with the purified anti-Rop1Ps polyclonal antibody (1:40 dilution with 1% nonfat milk in PBS) at 30°C for 1 hr. After three 10-min washes in PBS containing 0.05% Triton X-100, slides were incubated with a secondary antibody (fluorescein isothiocyanate–conjugated, affinity-purified goat anti-rabbit IgG; Cappel Organon Teknika, Durham, NC) at 30°C for 1 hr. After washes as described above, slides were mounted with 0.1% p-phenylendiamine and 50% glycerol in PBS. As controls, pollen tubes were stained as above except for the omission of the primary antibody. Observations and photography were conducted with an Axioshot photomicroscope equipped with epifluorescence optics and specific filters (Zeiss, Thornwood, NY) or a confocal microscope (model MRC-600; BioRad). For confocal microscopy, 2-μm serial sections were examined.

Visualization of Actin Microfilaments

Staining of F-actin in pollen tubes with rhodamine–phalloidin was performed according to the method of Pierson (1990). In vitro–cultured pea pollen tubes were incubated in a staining solution containing 130 nM rhodamine–phalloidin (Molecular Probes Inc., Eugene, OR), 5% DMSO, 25% sucrose, and 100 mM potassium phosphate buffer, pH 7.2, in the dark at room temperature for 2 hr. After a brief wash in 50 mM Pipes, pH 6.9, stained pollen tubes were mounted on microscope slides, examined, and photographed using confocal microscopy as described above.

Nuclear Staining

To visualize vegetative and generative nuclei in pea pollen tubes, pollen tubes stained with the anti-Rop1Ps antibody as described above were incubated with 1 μg/mL propidium iodide in PBS for 10 min just before mounting on slides for observations.

In Vitro Geranylgeranylation of Rop1Ps

The E. coli–expressed Rop1Ps fusion protein (Yang and Watson, 1993) was used for an in vitro isoprenylation assay by employing cell-free extracts of the plant Atriplex nummularia as a source of plant protein isoprenyltransferases, as described previously (Zhu et al., 1993).

Isolation of Microsomal Membrane and Soluble Proteins

Pollen tubes germinated from 50 mg of pollen grains were frozen in liquid nitrogen, ground in a 1.5-mL Eppendorf tube, and resuspended in 500 μL of extraction buffer (10 mM Mops, pH 7.0, 0.8 M sorbitol, 1 mM EDTA, 1 μg/mL proteinase inhibitors). Extracts were centrifuged at 10,000g at 4°C for 10 min. The pellet containing cell debris was discarded. Supernatants were centrifuged at 100,000g at 4°C for 10 min. The pellet was washed once with the extraction buffer and resuspended in the same buffer (volume equal to supernatant, ~500 μL). The supernatant was again centrifuged at 100,000g at 4°C for 1 hr. Pellets were resuspended and washed once with the extraction buffer and resuspended in the same buffer (volume equal to supernatants). Twenty microliters of each fraction was loaded on an SDS–polyacrylamide gel and analyzed by protein gel blotting as described above. For solubilization, NaCl or Triton X-100 was added to 150 μL of the 10,000g particulate fraction to a final concentration of 0.5 M or 2%, respectively. This suspension was incubated at 4°C for 30 min and centrifuged at 10,000g for 10 min. Pellets were resuspended in an equal volume of extraction buffer. Twenty-five microliters of soluble or particulate fractions was used for protein blot analysis, as described above.

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