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# Small GTPases and Spatiotemporal Regulation of Pollen Tube Growth

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**Abstract** During in vivo growth, pollen tubes make a long journey toward the ovule, responding to long- and short-distance guidance cues and elongating through different female tissues. Thus, pollen tube growth and guidance require complicated inter- and intracellular signaling, integration of multiple signals, and spatiotemporal coordination of the downstream responses necessary for targeted exocytosis. ROP, a plant-unique family of Rho small G proteins, is known to function as a versatile molecular switch in a variety of processes such as cell morphogenesis, stress and defense responses, hormonal responses, and directional growth of pollen tubes and root hairs. Current evidence suggests that ROP GTPase controls pollen tube growth temporally and spatially, coordinating multiple downstream signaling pathways. This chapter will review up-to-date findings about ROP GTPase signaling in pollen tubes and will discuss how ROP regulates pollen tube growth.

## 1 Introduction

Rho-family small GTPases are pivotal signaling switches conserved in eukaryotic organisms. By cycling between inactive GDP-bound and active GTP-bound forms, Rho GTPases participate in signaling to cytoskeletal organization and dynamics and membrane trafficking, and the list of cellular processes regulated by these GTPases keeps increasing (Burridge and Wennerberg 2004; Gu et al. 2004). Examples of processes controlled by Rho GTPase signaling include cell polarity development, polar cell growth, cell morphogenesis, cell migration, and cell division.

An important feature of Rho family GTPases is their capacity to act at key nodes of a signaling network, because of their ability to integrate multiple upstream signals, to coordinate multiple downstream pathways, and to participate in feedback regulation. This feature lies in the large number of functional partners that can physically associate with the small GTPases. Four classes of Rho-interacting proteins are known to act as upstream regulators. Docking/scaffolding proteins target Rho GTPases to specific membranes or membrane domains. Guanine nucleotide exchange factors (GEFs)

activate membrane-associated GTPases by replacing GDP with GTP. GTPase-activating proteins (GAPs) promote GTP hydrolysis leading to the inactivation of GTPases, whereas guanine nucleotide-dissociation inhibitors (GDIs) inhibit the activation of GTPases by suppressing nucleotide exchange and sequestering GTPases in the cytosol. Any of these regulators is capable of perceiving an upstream signaling, giving Rho GTPases the ability to integrate multiple signals. A single Rho GTPase is capable of activating multiple functionally distinct effector proteins, achieving the coordination of multiple downstream pathways.

Another key feature of Rho GTPases is their functional diversity due to their ability to orchestrate a wide range of intracellular signaling networks. In humans, at least 20 genes encode Rho family proteins, which are roughly classified into five functionally distinct subfamilies: Rho, Rac, Cdc42, Rnd, and RhoBTB (Burridge and Wennerberg 2004). Similarly, their interacting partners are usually encoded by gene families with functionally distinct members. For example, two structurally unrelated classes of Rho GEFs have been described in humans. Classic Dbl homology–Pleckstrin homology (DH–PH) domains containing Rho GEF proteins form the largest family of RhoGEF in humans, composed of 69 homologs (Erickson and Cerione 2004; Rossman et al. 2005). The divergent functional motifs flanking the DH–PH domain indicate that these GEFs are capable of linking Rho to diverse extracellular stimuli and intracellular pathways. In addition, another family of GEFs (DOCK180-related RhoGEFs) has emerged (Brugnera et al. 2002; Meller et al. 2005). Regardless of the structural dissimilarity, DOCK180-related RhoGEFs activate Rho G proteins probably via a similar mechanism of GDP–GTP exchange as shown in SopE of *Salmonella typhimurium* (Erickson and Cerione 2004). The presence of numerous Rho GEF molecules emphasizes the capability of Rho proteins as versatile molecular switches.

Interestingly, plants possess a single subfamily of Rho-like GTPases, named ROP (Rho-like GTPase from plants) (Yang and Watson 1993; Winge et al. 2000; Zheng and Yang 2000; Vernoud et al. 2003). The *Arabidopsis* genome encodes 11 different ROP genes, which can be classified into four subgroups based on amino acid sequence homology (Zheng and Yang 2000). Because no apparent orthologs of mammalian Rho, Rac, or Cdc42 have been reported, ROP appears to mediate diverse cellular responses as the sole family of Rho GTPase in plants. Studies using gain of function and loss of function approaches have revealed that ROPs have many physiological roles such as control of pollen tubes and root hair growth, cell shaping, stress response, defense to pathogen attack, and hormonal signaling (Gu et al. 2004). The functional diversity and specificity of Rop GTPases in plants can be accounted for by different isoforms of ROPs as well as by the diversity of their interacting proteins (Table 1).

**Table 1** Expression of ROPs and upstream regulators and effectors in mature *Arabidopsis* pollen. The expression of ROPs, upstream regulators GEF, GDI, and GAP, and effector RIC, are indicated. Information from several independent studies is summarized (sources shown in References). "Present" indicates that the transcript of a gene is present in mature pollens. "Abundant" and "selective" stand for high expression and preferential expression in mature pollens compared to other vegetative tissues, respectively. If the presence of transcript, either abundant or not, was reported by at least one study, the gene is considered to be expressed in mature pollens. "Not determined" indicates that the gene's expression has never been tested. Three of 11 RICs have not been given AGI numbers (RIC8, 9, and 11)

| Gene name | | AGI no. | Expression in pollen | Refs. |
|---|---|---|---|---|
| ROP | ROP1 | At3g51300 | Abundant | [1, 4–6] |
| | ROP2 | At1g20090 | Absent | [1, 4–6] |
| | ROP3 | At2g17800 | Abundant | [1, 4–6] |
| | ROP4 | At1g75840 | Absent | [1, 4–6] |
| | ROP5 | At4g35950 | Abundant | [1, 2, 4–6] |
| | ROP6 | At4g35020 | Absent | [1, 2, 4–6] |
| | ROP7 | At5g45790 | Absent | [4–6] |
| | ROP8 | At2g44690 | Present | [3–6] |
| | ROP9 | At4g28950 | Present | [3–6] |
| | ROP10 | At3g48040 | Present | [3–6] |
| | ROP11 | At5g62880 | Present | [3–5, 7] |
| GEF | RopGEF1 | At4g38430 | Present | [5, 6, 8] |
| | RopGEF2 | At1g01700 | Absent | [5, 6, 8] |
| | RopGEF3 | At4g00460 | Absent | [5, 6, 8] |
| | RopGEF4 | At2g45890 | Absent | [6, 8] |
| | RopGEF5 | At5g05940 | Absent | [5, 8] |
| | RopGEF6 | At3g55860 | Absent | [8] |
| | RopGEF7 | At5g02010 | Absent | [5, 6, 8] |
| | RopGEF8 | At3g24620 | Abundant and selective | [5, 6, 8] |
| | RopGEF9 | At4g13240 | Abundant and selective | [5, 6, 8] |
| | RopGEF10 | At5g19580 | Abundant | [5, 6, 8] |
| | RopGEF11 | At1g52240 | Abundant and selective | [5, 6, 8] |
| | RopGEF12 | At1g79880 | Abundant | [6, 8] |
| | RopGEF13 | At3g16130 | Abundant and selective | [6, 8] |
| | RopGEF14 | At1g31650 | Present | [5, 6, 8] |
| | SPK1 | At4g16340 | Absent | [5, 6] |
| GDI | RhoGDI1 | At3g07880 | Abundant | [5–7] |
| | RhoGDI2 | At1g12070 | Abundant | [5–7] |
| | RhoGDI3 | At1g62450 | Abundant and selective | [5–7] |

**Table 1** (continued)

| Gene name | | AGI no. | Expression in pollen | Refs. |
|---|---|---|---|---|
| GAP | RopGAP1 | At5g22400 | Present | [5–7] |
| | RopGAP2 | At4g03100 | Absent | [5–7] |
| | RopGAP3 | At2g46710 | Present | [5–7] |
| | RopGAP4 | At3g11490 | Absent | [5, 6] |
| | RopGAP5 | At1g08340 | Absent | [5–7] |
| | RopGAP6 | At2g27440 | Absent | [5–7] |
| | RopGAPx | At5g61530 | Abundant | [5, 6] |
| RIC | RIC1 | At2g33460 | Abundant | [5, 6, 9] |
| | RIC2 | At1g27380 | Present | [5, 9] |
| | RIC3 | At1g04450 | Abundant | [9] |
| | RIC4 | At5g16490 | Present | [5, 6, 9] |
| | RIC5 | At3g23380 | Abundant | [9] |
| | RIC6 | At2g20430 | Abundant | [5, 6, 9] |
| | RIC7 | At4g28560 | Abundant | [6, 9] |
| | RIC8 | | Not determined | |
| | RIC9 | | Present | [9] |
| | RIC10 | At4g04900 | Present | [6, 9] |
| | RIC11 | | Not determined | |

1. Li et al. (1998); 2. Kost et al. (1999); 3. Gu et al. (2003); 4. Cheung et al. (2003); 5. Pina et al. (2005); 6. Honys and Twell (2004); 7. Wu G, Hwang JU, Yang Z, unpublished; 8. Gu Y, Li S, Lord EM, Yang Z, unpublished; 9. Wu et al. (2001)

## 2 ROP GTPases and Pollen Tube Growth

Gene expression analysis using RT-PCR, promoter-GUS assay, and microarrays has revealed that up to seven ROP genes are expressed in mature *Arabidopsis* pollen (Table 1). Three members of group IV (*ROP1*, *ROP3*, and *ROP5*) are highly expressed in mature pollen grains (Li et al. 1998; Kost et al. 1999; Honey and Twell 2004; Pina et al. 2005). ROP8 of group I and all three members of group II (ROP9, ROP10, and ROP11) are also expressed in mature pollen (Gu et al. 2003; Cheung et al. 2003). ROP1, ROP3, and ROP5 share a high level of amino acid sequence identity (> 86%), and thus they are expected to be functionally redundant in the control of pollen tube tip growth (Li et al. 1999; Gu et al. 2004). Indeed, ROP1 and ROP5 displayed similar cellular localization and caused similar growth depolarization when overexpressed in pollen tubes (Li et al. 1999; Kost et al. 1999; Wu et al. 2001). In the following sections of this chapter, we will refer to the members of

subgroup IV collectively as "ROP1" for simplicity. However, there is still the possibility that they play subtle distinct in vivo functions during pollen tube development. A study from maize pollen supports this possibility. In maize pollen, only a subset of ROPs (*ROP2*, *8*, and *9*) are highly expressed (Christensen et al. 2003). ROP2 and ROP9 share higher identity in amino acid sequence (> 97%), even in untranslated regions, and yet knocking out one of the two nearly identical ROP genes produced minor but significant transmission defects via male gametophytes without any obvious cellular defect (Arthur et al. 2003).

The importance of ROPs in pollen tube growth was first implicated by a study showing that pea *Rop1Ps*, the first known plant Rho GTPase (Yang and Watson 1993), is preferentially expressed in pollen and that Rop1Ps is localized to the apical region of the plasma membrane in pollen tubes (Lin et al. 1996). In yeast and animal cell systems, Rho such as Rac and Cdc42 play an essential role in cell polarity establishment and maintenance, primarily through their role in the regulation of the actin cytoskeleton (Burridge and Wennerberg 2004). Subsequent studies accumulated evidence supporting the hypothesis that ROP regulates both temporal and spatial aspects of pollen tube growth. Inhibition of ROP activity by microinjection of anti-Rop1Ps antibody into pea pollen tube arrested tip growth without affecting cytoplasmic streaming (Lin and Yang 1997). Overexpression of dominant negative forms (T20N, D121A) of *Arabidopsis* ROP1 inhibited the tip growth of *Arabidopsis* or tobacco pollen tubes (Li et al. 1999; Fu et al. 2001). Dominant negative forms of ROP1 are expected to block endogenous ROP1 signaling by sequestering upstream activators (GEFs). Antisense *ROP1* expression also brought about pollen tube growth inhibition in *Arabidopsis* (Li et al. 1999). The growth retardation caused by ROP1 inactivation indicates that the activity of ROP1 and/or its closely related ROPs is essential for tip growth. Overexpression of DN-rop5 mutant also blocked pollen tube growth in tobacco (Kost et al. 1999). As mentioned above, it is likely that ROP1, ROP3, and ROP5 have a redundant cellular function in their control of pollen tube growth. However, the relative contribution of each of these ROPs in pollen tube growth regulation needs to be evaluated using individual and multiple knockout mutants.

When GFP-ROP1 was expressed in *Arabidopsis* pollen, the pollen with low green fluorescent protein (GFP) fluorescence (low level of ROP1 expression and small increase of ROP1 activation) displayed better germination and tube growth (Gu et al. 2003). This observation further supports the role of ROP1 in the control of pollen tube growth. Interestingly, pollen tubes with strong GFP fluorescence (higher level of ROP1 expression and excess ROP1 activation) produced bulbous tubes, resulting from the depolarization of pollen tube growth (Li et al. 1999; Gu et al. 2003). Expression of constitutively active (CA) forms of ROP1 (G15V or Q64L) caused much more severe depolarization of pollen tube growth (Li et al. 1999; Gu et al. 2003). The isotropic growth

(bulbous pollen tube formation), rather than polar growth, induced by excess ROP1 activation suggests the importance for the control of ROP1 activity of the spatial regulation of tip growth in pollen tubes. This observation also implies a role for ROP1 in the control of pollen tube growth polarity, and thus the directional control of pollen tube growth (see the chapter by Malhó, this volume).

Orthologs of ROP1 appear to be present in monocot species, supporting a common ROP-dependent mechanism for the control of pollen tube growth in plants. Rice and maize possess *ROP* genes closely related to *Arabidopsis ROP1* (*OsRacB* and *OsRacD*, and *ZmROP2*, 4, and 9) (Christensen et al. 2003). Recent studies support the view that these ROPs may be the functional orthologs of ROP1 in the control of pollen tube growth in monocots, by showing that maize *ROP2* and *ROP9* are highly expressed in pollen and that loss-of-function mutants of maize *ROP2* displayed transmission defects in the male gametophyte (Arthur et al. 2003).

Unlike ROP1, the function of other ROPs expressed in pollen, i.e., ROP8 to ROP11, remains mysterious but appears to be distinct from that of ROP1. Overexpression of ROP8, 9, 10, or 11 did not induce severe depolarization of pollen tube growth as ROP1 and ROP5 did (Gu et al. 2005; Hwang et al. 2005). The cellular localization tested with GFP-fused forms of these ROPs was not confined to the tip apex like GFP-ROP1 (Cheung et al. 2003; Vernoud et al. unpublished data). In addition, the expression of these ROPs did not alter ROP1 signaling significantly in the pollen tube apex (Gu et al. 2005; Hwang et al. 2005). The recruitment of a ROP1 downstream effector, RIC4 (ROP-interactive CRIB motif-containing protein 4), to the apical plasma membrane was not affected significantly by overexpression of these ROPs, whereas ROP1 overexpression did have an effect (Gu et al. 2005; Hwang et al. 2005). Thus, ROP8–11 may play an in vivo role yet to be identified but clearly distinct from that of ROP1.

## 3 Tip-Localized Active ROP1 Couples the Spatial and Temporal Control of Pollen Tube Elongation

The preferential localization of ROP1 to the apex and the effect of ROP1 activation on both tip growth and growth polarity suggest that localized ROP1 activation in the tip is not only critical for growth control, but may also define growth polarity or the site for tip growth. A recent investigation of in vivo ROP1 activity using a live GFP-based ROP1 activity marker supports this hypothesis. An active ROP1 reporter was developed based on RIC4 (Hwang et al. 2005). RIC4 is a downstream effector molecule of ROP1 (Wu et al. 2001; Gu et al. 2005). It preferentially binds to the GTP-bound active ROP1 both in vitro and in vivo (Gu et al. 2005). A mutant of RIC4, in which C-terminal 21-

amino acids behind the CRIB motif were deleted (RIC4ΔC), retained the full capacity of RIC4 to specifically bind to the active form of ROP1 but did not alter pollen tube growth when transiently expressed in tobacco pollen tubes (Hwang et al. 2005). Visualization of GFP-RIC4ΔC reveals that active ROP1 forms a tip-high gradient in the extreme of the pollen tube plasma membrane (PM), termed the apical cap of active ROP1, which apparently corresponds

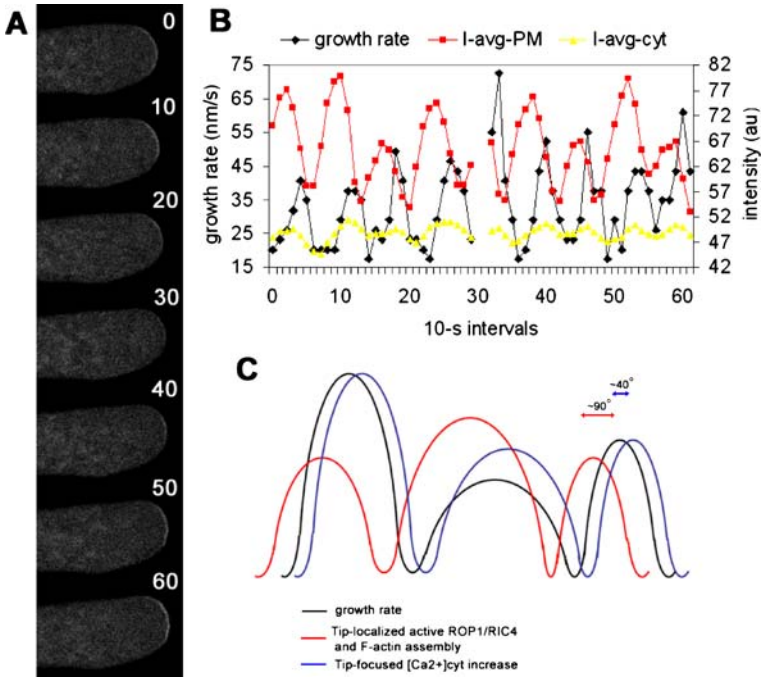


**Fig. 1** The oscillatory tip-localized ROP1 activity coordinates two downstream pathways, $[Ca^{2+}]_c$ dynamics and F-actin assembly. **a** A typical round of tip-localized ROP1 oscillation of ~ 60–70 s period. In vivo ROP1 activity was visualized by GFP-RIC4ΔC and the average intensity of GFP-RIC4ΔC localized to the tip apical PM oscillates with the tip growth rate. Numbers at the *top right* indicate the elapsed time (seconds) from the beginning (0 s). **b** Quantitative data showing that a tip-localized ROP1 activity increase leads the burst of tip growth, supporting the control of tip growth by active ROP1. The graph was reproduced from Hwang et al. (2005) with permission of MBC (This needs to be obtained). The average activity of ROP1 in the tip apical PM was quantified by measuring the average intensity of GFP-RIC4ΔC localized to the tip PM (I-avg-PM). I-avg-PM oscillates 10–20 s ahead of tip growth rate, whereas the amount of GFP-RIC4ΔC in the tip (I-avg-c) remains relatively constant with only minor fluctuations. **c** A cartoon showing the phase relations between tip growth, tip-localized ROP1 (RIC4) activity, F-actin assembly, and $[Ca^{2+}]_c$ increase. Tip-localized ROP1 activity oscillation is on average ~ 90° ahead of tip growth. Two downstream targets of active ROP1 appear to be activated differentially: F-actin assembly is stimulated early, which is in similar phase with ROP1 activation, while the $[Ca^{2+}]_c$ increase is stimulated late (~ 40° behind tip growth)

to the active growth domain (Fig. 1a). The tip-localized ROP1 activity is very dynamic in normal growing pollen tubes and was found to oscillate with the same frequency as the growth rate (Hwang et al. 2005; Fig. 1). Tip-localized ROP1 activity starts increasing about 10–20 s ahead of the tip growth rate increase in a 70-s period of oscillation (i.e., 90° ahead of growth). This phase relationship is different from oscillations of ion fluxes such as $Ca^{2+}$, $Cl^{-}$, $K^{+}$, and $H^{+}$, which appear to be associated with the growth or post-growth events (Holdaway-Clarke et al. 1997; Messerli et al. 1999, 2000). The temporal relation between ROP1 activation and growth thus supports a crucial role of active ROP1 in pollen tubes (Fig. 1c).

The formation of the apical cap of active ROP1 at the presumed active growth site indicates that ROP1 activation may be spatially regulated to de-

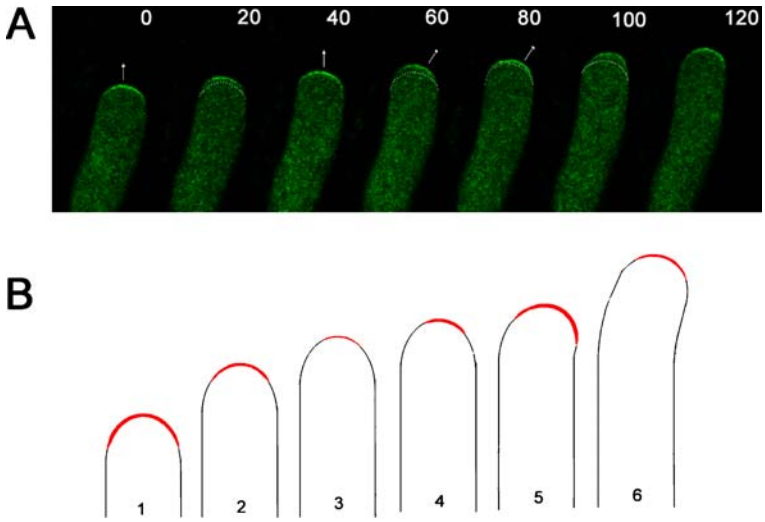


**Fig. 2** The tip-localized ROP1 activation defines the growth polarity. **a** Representative pollen tube images with tip-localized ROP1 activity. The pollen tube grew in a rapid oscillatory mode of 40–60-s period. Numbers at the *top right* indicate the elapsed time (seconds) from the beginning (0 s). The repositioning of the active ROP1 apical cap is indicated by *arrows*, which point in the future growth direction. The position of the tip apex at the previous time point is outlined with *white dots*. **b** A cartoon showing the spatiotemporal regulation of tip growth by tip-localized ROP1. The *red lines* indicate the amount and distribution of active ROP1 in the tip apical PM. One round of oscillation is divided into six phases (1–6): (1) tip-localized active ROP1 at the peak; (2) growth burst with tip active ROP1 amount being decreased; (3) interpulse state of ROP1 oscillation with growth rate being decreased; ROP1 activity starts increasing again from the minimum level; (4) ROP1 activity increases, pointing in new growth direction; the growth rate is in the minimum; (5) the new growth polarity is stabilized with increased ROP1 activity; ROP1 activity is in the maximum with growth rate being increased slowly; and (6) tip growth bursts toward new growth direction defined by active ROP1

fine the region of the plasma membrane for growth. This notion is supported by a tight correlation between the distribution of GFP-RIC4ΔC in the apical region of the plasma membrane and spatial changes in pollen tube growth. In pollen tubes overexpressing ROP1 or RIC4, an increase in the size of the GFP-RIC4/RIC4ΔC-containing apical cap was associated with an increase in tube width (Wu et al. 2001; Gu et al. 2005; G Wu et al. unpublished data). Conversely, a reduction of tube width was correlated with a decrease in the size of the apical cap in tubes expressing ROP negative regulators (G Wu et al. unpublished data). In reorienting pollen tubes, the GFP-RIC4ΔC apical cap was relocated to the future growth site before observable growth direction occurred (Hwang et al. 2005). Tobacco pollen tubes treated with 0.5 nM latrunculin B (LatB) displayed transient growth retardation and then resumed the oscillatory growth in a new direction (Hwang et al. 2005). The apical cap of GFP-RIC4ΔC relocation toward a future growth direction was detected clearly before the growth surge (Fig. 2). Consistent with the effects of ROP1 inactivation and overactivation on pollen tube growth, these observations strongly support the hypothesis that the spatiotemporal dynamics of tip-localized ROP activation couples the spatial and temporal regulation of tip growth (Fig. 2b). The apical cap of ROP1 activity can thus be considered as a dynamic growth organizer in time and space, which predicts the timing of the next growth surge and the position or direction of new growth. Given that Rho GTPases have been established as an integrator of different upstream signals, it is reasonable to speculate that ROP1 can integrate various signals from female tissues that regulate and guide pollen tube growth as the pollen tube is targeted to the ovule for fertilization (see the chapter by Johnson and Lord, this volume). Therefore, it is not surprising that the study of pollen tube signaling has been focused on two most interesting questions: (1) how the spatiotemporal dynamics of ROP1 activity is regulated and (2) how the localized ROP1 activity signals to localized growth.

## 4 Tip-Localized ROP1 Controls Pollen Tube Growth by Coordinating Multiple Pathways

Pollen tubes need to coordinate several cellular activities necessary for localized growth, including the production and targeting of secretory vesicles, fusion of these vesicles to the target site, and remodeling of cell walls (see the chapters by Malhó and by Geitmann and Steer, this volume). We postulate that ROP1 signaling can coordinate various pathways that, in turn, regulate these cellular activities. Recent studies provide strong evidence that support such a hypothesis (Kost 1999; Li et al. 1999; Wu et al. 2001; Gu et al. 2005). Perhaps the most insightful study is the identifica-

tion of putative ROP target proteins, named RICs (ROP-interactive CRIB-motif-containing proteins). The *Arabidopsis* genome encodes 11 RICs, which share the ROP-binding CRIB motif but are highly diverse in their primary structures.

## 4.1 RIC3-mediated Formation of the Tip Focused $[Ca^{2+}]_c$ Gradient

Growing pollen tubes form a steep gradient of cytosolic free calcium ($[Ca^{2+}]_c$) in the apex (see the chapter by Hepler et al., this volume). Blocking of the $[Ca^{2+}]_c$ gradient formation abolished tip growth (Pierson et al. 1994; Malhó et al. 1995), and localized $[Ca^{2+}]_c$ increase induced by releasing $Ca^{2+}$ ionophores or photolysis of caged $Ca^{2+}$ caused growth reorientation to the direction of $[Ca^{2+}]_c$ increase (Malhó and Trewavas 1996). Thus, the tip-focused $Ca^{2+}$ gradient is not only required for tip growth but also appears to be involved in the control of polar growth. How is the tip-focused $[Ca^{2+}]_c$ gradient established?

Several lines of evidence support the notion that ROP1 mediates the establishment of the tip $[Ca^{2+}]_c$ gradient. Inhibition of ROP signaling by microinjection of ROP-specific antiserum completely abolished the tip-focused $[Ca^{2+}]_c$ gradient, causing immediate growth inhibition (Li et al. 1999). *Arabidopsis* pollen tubes overexpressing *ROP1* or antisense *ROP1* gene displayed altered sensitivity of pollen tube growth to external $Ca^{2+}$ concentrations (Li et al. 1999; Gu et al. 2005). In root hairs, it has been shown that ROP overexpression induced growth inhibition and delocalized the tip-focused $[Ca^{2+}]_c$ gradient (Molendijk et al. 2001). In *Agapanthus umbellatus* pollen tubes, Camacho and Malhó (2003) also reported a tight interaction between ROP function and the $[Ca^{2+}]_c$ gradient in the control of apical secretion and membrane recycling. Using caged analogs of GTP and antisense oligonucleotides to reduce ROP expression, Camacho and Malhó (2003) obtained data that led them to suggest that $Ca^{2+}$ and ROPs act differentially but in a concerted form in the sequential regulation of pollen tube secretion and membrane retrieval. The most recent experiment that links ROP1 to $Ca^{2+}$ is the functional study of RIC3 (Gu et al. 2005), a ROP1 target in pollen tubes.

RIC3 is one of the 11 *Arabidopsis* RICs. RIC3 interacted specifically with the active form of ROP1 in both in vitro pull down and in vivo fluorescence resonance energy transfer (FRET) assays (Gu et al. 2005). It induced the depolarization of tip growth as ROP1 did, and its action is dependent upon ROP1 activation (Wu et al. 2001; Gu et al. 2005). RIC3 overexpression increased cytosolic $Ca^{2+}$ levels and induced an extended $[Ca^{2+}]_c$ gradient. RIC3 appears to regulate the influx of extracellular $Ca^{2+}$ because the putative $Ca^{2+}$ channel blocker, $La^{3+}$, suppressed the RIC3 overexpression phenotype (Gu et al. 2005). Furthermore, RIC3 overexpression or loss-of-function mutant pollen tubes displayed altered sensitivity to extracellular (ext) $Ca^{2+}$. RIC3-overexpressing

*Arabidopsis* pollen tubes grew well in the environment of low $[Ca^{2+}]_{ext}$, whereas wild-type pollen tube growth was generally suppressed under such conditions. In contrast, RIC3 loss-of-function mutant pollen tubes needed a higher $[Ca^{2+}]_{ext}$ to achieve growth (Gu et al. 2005). Since RIC3 is a novel protein, the mechanism by which RIC3 promotes the influx of extracellular $Ca^{2+}$ remains unknown.

### 4.2 RIC4-Dependent Accumulation of Tip-Localized F-actin

The actin cytoskeleton in pollen tubes can be said to be composed of two major subpopulations: the extensive longitudinal actin cables, which appear to be responsible for the reverse fountain pattern of cytoplasmic streaming, and the dynamic F-actin structures in the tip (see the chapter by Yokota and Shimmen, this volume). There is controversy over the detailed description of the dynamic F-actin structure in the tip, depending on the kind of F-actin markers and cell preservation methods applied (Fu et al. 2001; chapters by Hepler et al. and by Yokota and Shimmen, this volume), but it is generally believed that the dynamic F-actin structure in the tip is more directly related to the control of secretory vesicle targeting and fusion. The selective disruption of tip-localized dynamic F-actin with low concentration of actin-depolymerizing drugs (e.g., cytochalasin D and latrunculin A/B) inhibited pollen tube growth without disrupting major cytoplasmic streaming (Gibbon et al. 1999; Vidali et al. 2001). It has been shown that the dynamic tip F-actin assembly is affected by active ROP1. Overexpressed ROP1 promoted the formation of short actin filaments in the tip apex, whereas inhibition of ROP1 by expressing DN-rop1 or RopGAP1 and GDI1 led to the extension of longitudinal actin cables to the tube tip (Fu et al. 2001). Similar results were reported with a tobacco ROP1 homolog (NtRac1). NtRac1 overexpression promoted the formation of a dense meshwork of actin filaments in pollen tube tips (Chen et al. 2003).

In mammalian cells, Rho stimulates F-actin formation primarily by activating actin nucleating factors or via Rho kinases, which in turn activates actin stabilizing factors or inactivates actin depolymerizing factors (ADFs). However, it is not well understood how ROP regulates F-actin dynamics in plant systems. Recently, RIC4 was proposed to mediate ROP1-induced F-actin assembly (Gu et al. 2005). RIC4 specifically interacts with the active form of ROP1 and is localized to the tip apical plasma membrane in an active ROP1-dependent way (Gu et al. 2005). RIC4 overexpression enhanced the formation of tip-localized F-actin as ROP1 did, but did not increase $[Ca^{2+}]_c$ in pollen tubes (Fu et al. 2001; Gu et al. 2005). RIC4-induced depolarized tip growth could be suppressed by increasing actin depolymerization with a treatment of LatB or coexpression of profilin. *Arabidopsis* pollen tubes overexpressing RIC4 showed the enhanced accumu-

lation of tip F-actin and displayed increased resistance to LatB-induced growth inhibition, whereas loss-of-function mutants were hypersensitive to LatB (Gu et al. 2005). In animal cells, the Arp2/3 complex is pivotal for actin polymerization, primarily associated with Cdc42/Rac. *Arabidopsis* possesses homologs of subunits of the Arp2/3 complex, but genetic disruption of Arp2/3 complex subunits barely affected pollen tube growth (Li et al. 2003). This suggests that ROP-mediated F-actin assembly in pollen tubes differs from Cdc42/Rac-mediated actin assembly in animal and yeast cells. The mechanism by which RIC4 regulates actin organization remains to be determined.

### 4.3 ROP and Phosphoinositide Signaling

Phosphoinositides (PIs) are important signaling molecules, either serving as substrates for the production of the inositol-1,4,5-trisphosphate (IP3) second messenger or directly binding to a signaling protein. Emerging evidence supports a role for PIs in pollen tube growth (Monteiro et al. 2005; chapter by Žárský et al., this volume). Interestingly, it was shown that recombinant ROP5 was associated with a phosphatidylinositol phosphate (PIP) kinase activity from tobacco pollen tubes (Kost et al. 1999). Using a GFP-tagged PH domain, which specifically binds PIP2, the product of PIPK, it was shown that PIP2 was localized to the apical region of the pollen tube plasma membrane (Kost et al. 1999). Blocking PIP2 by a specific PH domain also inhibited pollen tube growth. These results suggest that ROP1 and PIP2 signaling may converge for control of pollen tube growth. Although it is unclear how PIPK and its product PIP2 are implicated in pollen tube growth, both of F-actin assembly and $[Ca^{2+}]_c$ accumulation are potential targets (see the chapter by Žárský et al., this volume).

### 4.4 ROP Regulation of Actin Depolymerization Factors (ADFs)

In addition to Arp2/3 complex-mediated nucleation, animal Rho is known to regulate several actin binding proteins (ABPs) such as ADF/cofilin, filamin, and formin (Maekawa et al. 1999; Vadlamudi et al. 2002). The involvement of ADFs in ROP1-mediated control of F-actin dynamics was reported in tobacco pollen (Chen et al. 2003). Tobacco pollen-specific ADF (NtADF1) suppressed the NtRac1-induced depolarization of tip growth and F-actin assembly. The nonphosphorylatable mutant ADFs with an Ala substitution at the Ser-6 position were much stronger, whereas a phospho-mimicking mutant with Ser-to-Asp change was not effective in suppressing NtRac1 phenotype development (Chen et al. 2003). Interestingly, NtRac1 overexpression increased the phosphorylation of NtADF1 and presumably its inactivation, suggesting

that ROP regulates the phosphorylation status of ADF and thereby controls the dynamics of F-actin in the pollen tube (Chen et al. 2003). In maize, it was reported that ADF is phosphorylated by a $Ca^{2+}$-dependent protein kinase activity (Allwood et al. 2001). Whether ROP regulates the phosphorylation status of ADF and whether it is $Ca^{2+}$ dependent are questions now requiring an answer.

### 4.5 Other Possible Downstream Pathways: Formins, Exocyst, and RabA4

In animal cells, the actin nucleating process by formins is the rate-limiting step for de novo actin filament synthesis, and some formins are known to be activated by interaction with Rho via a G protein-binding domain (Evangelista et al. 2003). It is reported that a group I formin (AFH1) of 20 *Arabidopsis* formin family members promoted pollen tube growth at a low level of expression and induced depolarization of tip growth at a high level of expression (Cheung and Wu 2004; Michelot et al. 2005). This suggests that actin nucleation via AFH1 is involved in pollen tube growth. However, it is unclear whether formins are involved in ROP-mediated F-actin assembly, because plant formins lack the G protein-binding domain.

For polar cell growth, directional vesicle delivery, targeted fusion, and secretion are critical. Polar exocytosis most likely requires F-actin reorganization and $Ca^{2+}$ increases. In addition to F-actin formation and $Ca^{2+}$ increase, Rho is known to regulate the exocytosis via other mechanisms. One potent candidate of ROP effector for targeted exocytosis is exocyst. Exocyst is a conserved eight-subunit protein complex, which is localized to sites of polarized exocytosis and required for vesicle targeting and docking to specific plasma membrane domains (Roumanie et al. 2005; chapter by Žárský et al., this volume). In yeast cells, one of eight subunits, Sec3, is known to interact directly with GTP-bound Rho1 and functional Rho1 is required both to establish and to maintain polarized Sec3 localization (Guo et al. 2001). Sec3 is not the only target of Rho to control exocyst. Another subunit, Exo70, interacts with Rho3 independently from Sec3 (Roumaine et al. 2005). The *Arabidopsis* genome contains homologs of all eight subunits of exocyst complex (Elias et al. 2003; Cole et al. 2005). Particularly, pollen of Sec8 knockout mutants showed transmission defects, and some of Sec3 and Exo70 coding genes are highly expressed in pollen (Cole et al. 2005). It will thus be interesting to investigate a putative link between exocyst and ROP1.

Another potential linkage is the family of RAB small G proteins. *Arabidopsis* RabA4 proteins and tobacco Rab11 (homologs of animal Rab11) are reported to play a critical role in polarized vesicle delivery and exocytosis in polar growing cells (de Graaf et al. 2005; Preuss et al. 2004). Both localize at the pollen tube tip (de Graaf et al. 2005; YJ Lee et al. unpublished data) and mutations on NtRab11 caused defects in tip growth and tip F-actin structure

in tobacco pollen tubes (de Graaf et al. 2005). In *Arabidopsis* trichoblasts, the recruitment of ROP to the growth initiation site was dependent on Rab function, because brefeldin A inhibited the polar localization of ROPs (Molendijik et al. 2001). Thus, crosstalk between ROP and RAB small G proteins for coordinating targeted secretion is quite likely.

## 5
## Check and Balance Between RIC3 and RIC4 Pathways: Roles in Actin Dynamics

As discussed above, active ROP1 stimulates two important downstream pathways, the assembly of tip-localized F-actin and the tip-focused $[Ca^{2+}]_c$ gradient, via two distinct mediators, RIC3 and RIC4 (Gu et al. 2005) (see Fig. 3). The assembly of tip-localized F-actin could provide forces for targeted delivery and accumulation of secretory vesicles to the future growth site; too much and less dynamic F-actin could be the physical barrier to vesicle fusion (Giner et al. 2005). Cytosolic $Ca^{2+}$ is also required for vesicle fusion, but high levels of cytosolic $Ca^{2+}$ disrupt F-actin (see the chapter by Yokota and Shimmen, this volume). Efficient tip growth thus requires a critical balance between $Ca^{2+}$ and dynamic F-actin. Not surprisingly, RIC3-dependent $Ca^{2+}$ and RIC4-dependent F-actin pathways counteract each other (Gu et al. 2005). RIC4-overexpressing pollen tubes showed enhanced F-actin assembly but a less pronounced tip-focused $Ca^{2+}$ gradient. In contrast, RIC3-overexpressing tubes developed higher tip $[Ca^{2+}]_c$ but completely lost the tip fine F-actin structure. RIC4 coexpression suppressed RIC3-induced depolarized growth in an F-actin dependent manner, and LatB treatment blocked the suppression of RIC3 signaling by RIC4 coexpression. RIC3 coexpression suppressed RIC4-induced depolarized growth in a $Ca^{2+}$-dependent manner, and blocking of $Ca^{2+}$ influx by $La^{3+}$ inhibited the counteracting effect of RIC3 on RIC4 signaling (Gu et al. 2005). Clearly, control mechanisms are needed to check the activation of these two pathways and make them balanced for optimum growth. It was found that when RIC3 and RIC4 were expressed at 1 : 2 molecular ratio in tobacco pollen tubes, the apical morphology was comparable to the control tubes and a normal tip F-actin structure was restored (Gu et al. 2005). Interestingly, *ric3* and *ric4* double loss-of-function *Arabidopsis* pollen tubes grew with normal apical morphology (although shorter than wild-type tubes), whereas single loss-of-function mutants displayed retarded growth (Gu et al. 2005). In the RNAi *ric3* and *ric4* double mutant, cells could still express diminute amounts of RIC3 and RIC4, thus justifying normal morphology but shorter growth.

How do the RIC3- and RIC4-dependent pathways counteract each other? An increase of cytosolic $[Ca^{2+}]$ activates several actin binding proteins (ABPs) (see chapter by Yokota and Shimmen, this volume, for detailed dis-

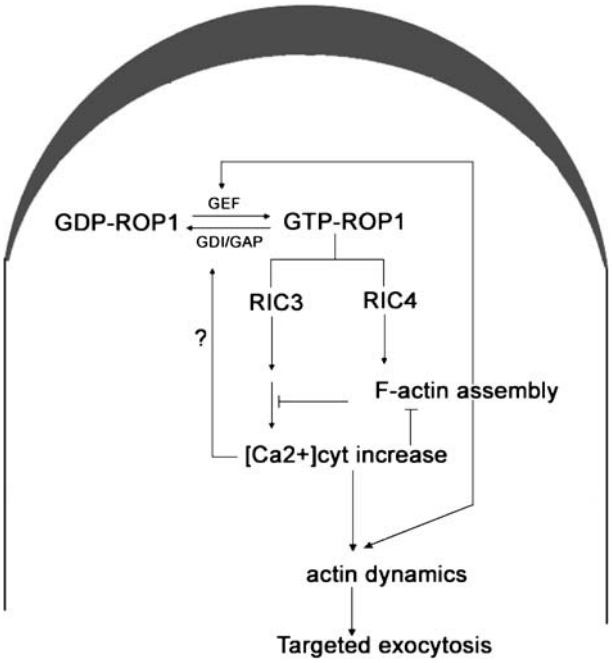


**Fig. 3** A model for a ROP signaling network regulating pollen tube growth. Active ROP1 stimulates F-actin assembly and $[Ca^{2+}]_c$ increase via RIC4- and RIC3-dependent pathways. F-actin assembly may control the targeted accumulation of secretory vesicles, and late $[Ca^{2+}]_c$ increase induces actin depolymerization, promoting vesicle fusion. By regulating targeted vesicle delivery and fusion, tip-localized active ROP1 couples the temporal and spatial aspects of tip growth. F-actin assembly feed-forwardly promotes the accumulation of active ROP1 to the tip, forming a positive feedback loop of signal amplification. A $[Ca^{2+}]_c$ increase terminates the active ROP1 amplification probably by counteracting the F-actin pathway. It remains to be answered whether tip high $Ca^{2+}$ directly activates the downregulation of ROP1 activity via, or independently of, F-actin

cussion). For example, in tobacco pollen tubes, profilin and RIC3 completely restored the RIC4 overexpression phenotype (Gu et al. 2005).

$Ca^{2+}$ channels at the pollen tube plasma membrane are also putative targets for F-actin to suppress a $[Ca^{2+}]_c$ increase. RIC3-induced $Ca^{2+}$ increase is dependent on the influx of extracellular $Ca^{2+}$ (Gu et al. 2005), and recent studies of $Ca^{2+}$ channel activities in pollen protoplasts provide some hints to explain why increased F-actin assembly by RIC4 suppressed RIC3-dependent $Ca^{2+}$ accumulation in the tip. Wang et al. (2004) have shown that the voltage-operated inward $Ca^{2+}$ channel activity found in the plasma membrane of pollen protoplasts was stimulated by actin depolymerization. Increased tip-localized F-actin assembly by RIC4 may affect the activities of these $Ca^{2+}$ influx channels in the tip plasma membrane, thereby inhibiting the increase of $[Ca^{2+}]_c$.

## 6
## ROP1 Temporally Coordinates $Ca^{2+}$ and F-actin

How are RIC3- and RIC4-dependent pathways balanced to promote rapid tip growth? A temporal lag between RIC4 activation of the actin assembly and the RIC3 activation of $Ca^{2+}$ accumulation may underscore the mechanism that coordinates these two important signaling pathways. In normal growing pollen tubes, tip-localized ROP1 activity (RIC4 activity), tip-localized F-actin accumulation, and tip-focused $[Ca^{2+}]_c$ seem to oscillate with the same frequency as tip growth oscillation (Messerli et al. 2000; Fu et al. 2001; Hwang et al. 2005). The correlation test based on frequency (period length) indicates that RIC4-dependent F-actin accumulation is stimulated earlier than RIC3-dependent $[Ca^{2+}]_c$ increase (Fig. 2c). The lag of the RIC3 activation of $[Ca^{2+}]_c$ increase could result from a more lengthy multistep signaling for this pathway, although the details have yet to be worked out.

The tip-localized ROP1 activity/RIC4 localization to the tip peaks approximately 90° ahead of growth rate (Hwang et al. 2005). The RIC4-dependent assembly of tip-localized F-actin oscillates in the same phase with or slightly lagging the tip-localized ROP1 activity, but leading tip growth (Fu et al. 2001; Hwang et al. 2005). In contrast, the RIC3-modulated tip-focused $[Ca^{2+}]_c$ gradient was found to oscillate slightly behind growth. Temporally differential activation of the RIC3-dependent $Ca^{2+}$ pathway and RIC4-dependent F-actin pathway by active ROP1 and the counteraction between the two pathways may enable the rapid oscillatory tip growth and tip-localized ROP1 activation (Gu et al. 2005; Hwang et al. 2005). Increase in F-actin assembly immediately follows the increase in the amount of active ROP1 and may subsequently modulate vesicle transport. At the same time, cortical F-actin assembly may lead to inhibition of $Ca^{2+}$ influx, thus preventing premature $[Ca^{2+}]_c$ increase from interfering with polarity stabilization via F-actin. Subsequently, the delayed RIC3-dependent $[Ca^{2+}]_c$ increase could promote F-actin depolymerization via $Ca^{2+}$-sensitive ABPs, causing a growth burst toward the determined growth polarity.

### 6.1
### How is ROP1 Activity Regulated?

As discussed above, in vitro cultured pollen tubes display dynamic spatiotemporal changes in ROP1 activity. In vitro pollen tube growth does not require exogenous growth stimuli, so the dynamics of ROP1 activity in these tubes must be achieved by a self-organizing mechanism. In migrating animal cells and budding yeast cells, random initiation of local activation of Rho GTPases is stabilized by positive feedback amplification (Weiner et al. 2002; Wedlich-Soldner et al. 2003). In yeast, Cdc42-mediated positive feedback activation involves the regulation of Cdc24 (Cdc42 GEF) by both actin (a target of Cdc42

signaling)-dependent and -independent mechanisms (Wedlich-Soldner et al. 2003; Wedlich-Soldner and Li 2003). In animal cells, $PIP_3$ and Rac accumulate to the leading edge of migrating cells and are amplified via a positive feedback loop that requires PI3K and Rac GTPase (Weiner et al. 2002). Similar mechanisms may be implicated in the regulation of the apical cap of active ROP1 in growing pollen tubes (Hwang et al. 2005). Polymerization of F-actin in the apex appears to participate in the positive feedback regulation of ROP1 activity. Increased F-actin assembly by RIC4 overexpression induced sustained increase in tip-localized ROP1 activity, whereas LatB treatment did the opposite (Hwang et al. 2005). To maintain the tip-localized ROP activity, negative regulation is also required to confine ROP activity to the apex. Otherwise, ROP1 would be excessively activated, resulting in the delocalization of active ROP1 and depolarization of tip growth. Furthermore, in normal tubes undergoing growth oscillation, a negative feedback mechanism must be required to downregulate ROP1 activity once it reaches a peak. A detailed molecular basis for the feedback mechanisms is thus required.

During pollination, tip-localized ROP activity is most likely regulated by guidance signals so that pollen tubes can properly navigate toward the ovule. It is not understood how ROP activation is regulated by spatial cues. Female reproductive tissues are known to release guidance cues (see the chapters by Johnson and Lord and by Guermonprez et al., this volume). Receptors in the pollen tube plasma membrane are speculated to perceive the spatial cues and convey the signal into the cytoplasm, which may finally trigger ROP activation. Plants possess two types of unconventional GEFs, RhoGEFs and SPIKE1, but lack the classic Dbl homology domain containing GEF. RopGEFs have been recently identified to have GEF activity toward ROPs, and have a plant-unique guanine nucleotide exchange domain (Berken et al. 2005; G Ying et al. unpublished data). However, it is not understood if and how RopGEF is involved in ROP activation by intrinsic or extrinsic stimuli.

Recent studies suggest the possibility that pollen receptor-like protein kinases (RLKs) may link guidance cues to ROP signaling by recruiting RopGEF. RopGEF is also known as a kinase partner protein (KPP) in tomato (Kaothien et al. 2005). KPP is a pollen-specific protein and interacts with the cytoplasmic domain of receptor-like kinases (LePRK1 and LePRK2). KPP was phosphorylated in tomato pollen, and induced depolarization of pollen tube growth and abnormal F-actin structure like ROP overexpression did (Kaothien et al. 2005). It is also known that ROP may form a complex with RLKs like CLAVATA1 (Trotochaud et al. 1999). These results support an exciting hypothesis that ROP activity is regulated by RopGEF, which is regulated by its interaction with or phosphorylation by membrane receptor kinases. In mature *Arabidopsis* pollens, up to eight members of RopGEF are expressed (Table 1). When transiently overexpressed in tobacco pollen tubes, these RopGEFs induced depolarization of tip growth (Y Gu et al. unpublished data). In rice, a heterotrimeric G protein was shown to function upstream of the small GT-

Pase OsRac1 in the early steps of signaling (Suharsono et al. 2002), and such proteins have been implicated in the regulation of pollen tube growth (Ma et al. 1999). Future studies should address whether RopGEF is regulated by receptor-like kinases and/or heterotrimeric G protein-coupled receptors.

## 7 Perspectives

Active ROP1 is localized to the tip apical plasma membrane and oscillates with tip growth, stimulating downstream pathways in a temporally coordinated way, e.g., an early RIC4-dependent F-actin pathway and a delayed RIC3-dependent $Ca^{2+}$ pathway. Tip-localized active ROP1 forms a tip-high gradient (apical cap), which corresponds to the area where secretory vesicles accumulate. Active ROP1 defines the active growth domain probably by spatially regulating the vesicle delivery and fusion. Thus, tip-localized dynamic ROP1 activity couples the temporal and spatial aspects of the tip growth. Since the first plant Rho was identified in pea pollen, considerable progress has been made in understanding the functional roles that ROP plays in pollen tube growth. Major future challenges will be to unravel how the spatial cues regulate ROPs in the pollen tube tip, which may involve signal perception via specific receptors, transduction to ROP activation, and/or inactivation via GEF, GDI, or GAP. The mechanisms by which active ROP1 stimulates the downstream pathways such as F-actin assembly and $[Ca^{2+}]_c$ increase also need to be characterized. In addition to RIC3 and RIC4, future studies may reveal the new ROP1 effectors that may be needed for ROP1 to coordinate other downstream pathways such as RabA4 and exocyst components. Investigation of a potential interaction of the ROP1 signaling network with other mechanisms in the control of pollen tube growth should provide a full picture of the molecular mechanisms underlying pollen tube growth and guidance.

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