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ROP GTPase regulation of pollen tube growth through the dynamics of tip-localized F-actin

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Abstract

Pollen tubes expand by tip growth and extend directionally toward the ovule to deliver sperms during pollination. They provide an excellent model system for the study of cell polarity control and tip growth, because they grow into uniformly shaped cylindrical cells in culture. Mechanisms underlying tip growth are poorly understood in pollen tubes. It has been demonstrated that ROP1, a pollen-specific member of the plant-specific Rop subfamily of Rho GTPases, is a central regulator of pollen tube tip growth. Recent studies in pollen from *Arabidopsis* and other species have revealed a ROP-mediated signalling network that is localized to the apical PM region of pollen tubes. The results provide evidence that the localization of this signalling network establishes the site for tip growth and the localized activation of this signalling network regulates the dynamics of tip F-actin. These results have shown that the ROP1-mediated dynamics of tip F-actin is a key cellular mechanism behind tip growth in pollen tubes. Current understanding of the molecular basis for the regulation of the tip actin dynamics will be discussed.

Key words: F-actin, pollen tube tip growth, regulation, ROP GTPase, signalling.

Introduction

Following germination on the surface of the stigma, pollen tubes are directed through the stigma surface, grow within the transmitting track, emerge from the transmitting tract, and finally are targeted toward the micropyle to deliver sperms to the ovule (Johnson and Preuss, 2002). Understanding the mechanisms by which pollen tubes extend and are directed toward the ovule is a fundamentally important problem in the field of sexual reproduction (Franklin-Tong, 1999*b*, 2002). Pollen tubes have also attracted extensive scrutiny by plant cell biologists, as they provide a simple model system for studying cell growth and cell polarity control (Zheng and Yang, 2000). Pollen tubes extend exclusively at the cell apex via an extreme form of polar growth, known as tip growth, producing uniformly shaped cylindrical cells. Pollen tube elongation can be extremely rapid, for example, reaching 1 cm h^{-1} in maize pollen tubes, and growth is usually oscillatory. Thus, pollen tube growth requires both spatial and temporal co-ordination of many cellular functions, including ion fluxes, organization and dynamics of cytoskeletal elements, vesicular trafficking, exocytosis, endocytosis, and wall synthesis, assembly and remodelling (Franklin-Tong, 1999*a*; Yang, 1998, 2002). Most importantly, a defined plasma membrane region of pollen tube must be established, to which post-Golgi vesicles are targeted to and fuse with a defined domain of the plasma membrane at the cell apex, leading to localized extension of the PM (from vesicle membrane) and cell walls (from contents of vesicles). The PM domain is referred as the 'tip growth domain'. Because *in vitro* pollen tubes always extend by tip growth into uniformly shaped cells (e.g. pollen tubes from the same genetic background exhibit constant radial diameters), it is reasonable to assume that internal signals of some kind turn on tip growth mechanisms and define the radial diameter of the pollen tube by spatially regulating the tip growth domain. Similarly, it is anticipated that during *in vivo* growth, external guidance signals also spatially regulate the tip growth domain to redirect pollen tube growth.

The authors were interested in how the tip growth domain is established in pollen tubes, how internal tip growth signals and external growth guidance signals spatially regulate the tip growth domain, and how the tip growth domain signals to the machinery for exocytosis and vesicle targeting. Some answers to these questions was gained from the studies of Rop GTPase signalling in pollen tubes. It has been shown that Rop is a central regulator of tip growth. Recent evidence suggests that Rop localization and activation are spatially regulated to establish the tip growth domain and active Rop localized to the tip growth domain regulates tip growth through modulating the dynamics of tip F-actin and the formation of tip-focused calcium gradients. This article will discuss current knowledge of spatial regulation of ROP activity and of the mechanism by which Rop regulates pollen tube growth.

ROP GTPases and their roles in pollen tube growth

Rop is a plant-specific subfamily of the Rho family of monomeric GTPases (G proteins) that include Cdc42, Rac, and Rho subfamilies from animals and fungi (Li *et al.*, 1998; Zheng and Yang, 2000; Yang, 2002). Rho GTPases are best known for their conserved functions in signal transduction that leads to the reorganization and dynamics of F-actin (Kuhn *et al.*, 2000). However, Rho GTPase signalling has been shown to regulate a wide variety of cellular processes, including microtubule organization, gene transcription, RNA processing, cell cycle progression, and activation of specific enzymes such as NADPH oxidase and glucan synthase (Bokoch, 2000; Kjoller and Hall, 1999; Kuhn *et al.*, 2000; Li and Yang, 2000; Wittmann and Waterman-Storer, 2001). In animals and yeast, different subfamilies of Rho GTPases have distinct functions (Mackay and Hall, 1998).

Rop is the only subfamily of Rho GTPases found in plants and is encoded by a multigene family in different plant species (Yang, 2002). The *Arabidopsis* genome encodes 11 ROPs, which can be divided into four groups based on their amino acid sequence similarities (Zheng and Yang, 2000). Emerging evidence suggests that members within the same group tend to be functionally redundant, whereas different groups appear to be functionally distinct. Three members of group IV (ROP1, ROP3 and ROP5), all members of group II (ROP9, ROP10, and ROP11), and ROP8 (group I) are expressed in pollen (Li *et al.*, 1998; Y Gu, V Vernoud, unpublished data). *ROP3* and *ROP5* transcript levels in pollen are much less abundant than that of *ROP1*. *ROP3* and *ROP5* share 98% identity and belong to recent large duplications (0–50 million years ago (Vision *et al.*, 2000). *ROP3* and *ROP5* genes are most closely related to *ROP1*, and the duplication that resulted in the *ROP1* (*ROP3/ROP5*) gene pair probably belongs to age class C (90–100 million years ago). The evolutionary

analyses together with expression pattern of these ROPs suggest *ROP1* may have a dominant role in pollen tip growth, whereas *ROP3* and *ROP5* may be functionally redundant to *ROP1*. Indeed, both ROP1 and ROP5 have been shown to regulate pollen tube tip growth (Fu *et al.*, 2001; Kost *et al.*, 1999; Li *et al.*, 1999). The function of ROP8, ROP9, ROP10, and ROP11 in pollen tubes is unknown, although transient overexpression suggests that they are functionally distinct from ROP1 and ROP5 in pollen (V Vernoud, Z Yang, unpublished data).

It has been shown that ROP1 regulates pollen germination and pollen tube growth. Pollen tube elongation was inhibited by microinjected anti-Rop1 antibodies or expression of dominant negative (DN) mutants of ROP1 or At-Rac2/ROP5 (Kost *et al.*, 1999; Li *et al.*, 1999). High levels of *DN-rop1* expression also inhibited pollen germination (H Li, Z Yang, unpublished data), whereas overexpression (OX) of wild-type ROP1 promotes pollen germination (V Vernoud, Z Yang, unpublished data). The effect of ROP1 OX on pollen tube growth varies with OX levels, low levels promote tube elongation, whereas high levels caused depolarized growth (Fig. 1). Compared to ROP1 OX, expression of constitutively active (CA) mutants of ROP1 or ROP5 caused much more severe depolarization of pollen tube growth (Kost *et al.*, 1999; Li *et al.*, 1999). These observations indicate that ROP1 and its functionally redundant relatives are essential for growth as well as participation in the regulation of growth polarity in pollen tubes. As shown in Fig. 2, the simplest and most reasonable explanation for these observations is the existence of a tip-localized ROP1-dependent signalling pathway that activates pollen germination and pollen tube growth. According to this model, the localization of the active ROP1 signalling complex defines a PM domain for growth, i.e. the tip growth domain in normal pollen tubes. In ROP1 OX or CA-rop1 tubes (Fig. 2), the depolarized growth is due to the delocalization of the ROP1 signalling complex.

Localized recruitment and activation of ROP1 to the PM is critical for the establishment of the tip growth domain

An interesting and important question is whether the ROP1 localization and/or its localized activation indeed define the tip growth domain. Earlier studies using both immunolocalization in pea pollen tubes and expression of GFP-tagged ROP1 and ROP5 show that a fraction of ROP1 or ROP5 localizes to the apical region of the tube plasma membrane, although the majority of ROPs are cytosolic (Kost *et al.*, 1999; Li *et al.*, 1999; Lin *et al.*, 1996). Furthermore, ROP1 OX leads to increased apical regions of the PM containing ROP1, which are correlated with the severity of depolarized growth (Kost *et al.*, 1999; Li *et al.*, 1999). These observations suggest that ROP1 localization to the PM is subject to spatial regulation,
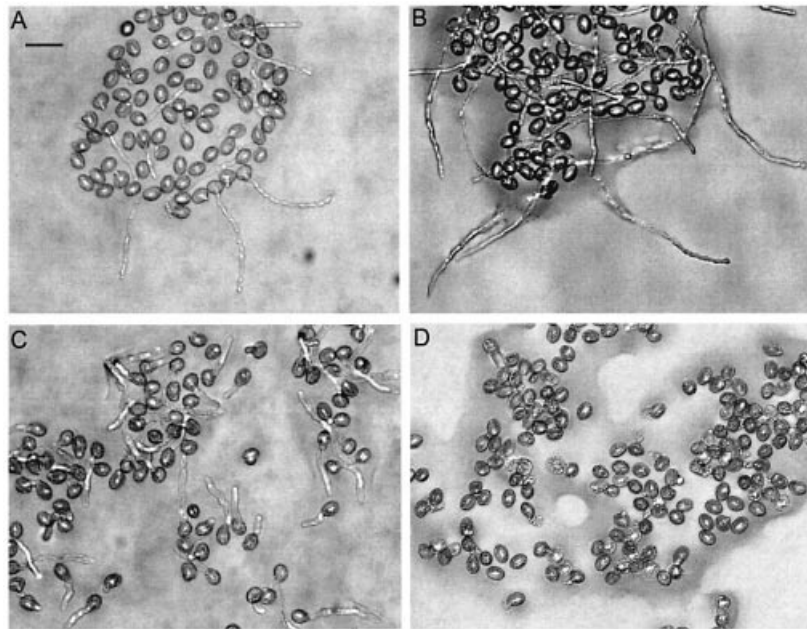
Fig. 1. Effects of ROP1 overexpression on pollen tube elongation and growth polarity. *Arabidopsis thaliana* Col-0 plants were transformed with LAT52::GFP-ROP1 constructs. Transgenic lines that show different levels of GFP expression in pollen were selected. Pollen grains were germinated on agar medium and photographed 6 h after germination. (A) Col-0 wild-type pollen. (B) Pollen from a line expressing a low level of GFP-ROP1. (C) Pollen from a line expressing a moderate level of GFP-ROP1. (D) Pollen from a line expressing a high level of GFP-ROP1. Bar=30 μm.

which plays an important role in the establishment of tip growth domain. The tip localization implies that a tip-localized recruitment factor involved in the control of ROP1 localization exists in pollen tubes. Because the majority of ROP1 is localized in the cytosol, it is also likely that a cytosolic Rop sequestering factor is involved in the regulation of ROP1 localization. In yeast, BEM1 has been shown to act as a Cdc42 recruitment factor, whereas GDIs (guanine nucleotide dissociation inhibitors) act as cytosolic sequestering factors for Rho GTPases in animal cells (Butty *et al.*, 2002; Zarsky *et al.*, 1997). Rop GDIs are also present in plants (Bischoff *et al.*, 2000) and play a role in the regulation of ROP1 localization (Fu *et al.*, 2001), but the ROP1 recruiting factor(s) remains to be identified.

The next question is whether ROP1 activation is required for the establishment of the tip growth domain. To address this question, a marker that reports the localization of GTP-bound active ROP1 is necessary. A ROP1-interacting protein, RIC1, has been identified, which interacts with the GTP-bound CA-rop1, but not with GDP-bound DN-rop1 (Wu *et al.*, 2001). RIC1 contains a CRIB motif (Cdc42/Rac-interactive binding motif) required for RIC1 binding to ROP1. Interestingly, GFP-tagged RIC1 is localized to the apical PM region of tobacco pollen tubes with a tip-high gradient. The localization of GFP-RIC1 to the PM was dependent on its interaction with ROPs, because a mutation in the CRIB motif abolishes its localization to the apical PM region (Wu *et al.*, 2001). Importantly, GFP-RIC1 localization to
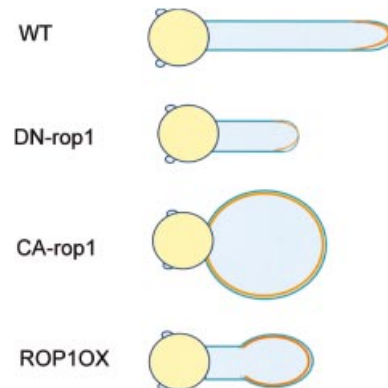
Fig. 2. A schematic model describing the control of pollen tube tip growth by a tip-localized ROP signalling complex. To explain pollen tube phenotypes induced by expression of *DN-rop1*, *CA-rop1* and *WT-ROP1* genes, it is proposed that an active ROP1 signalling complex (shown in orange) is localized to the apical region of the plasma membrane and activates polar exocytosis in wild-type pollen tubes. In *CA-rop1* and *ROP1 OX* tubes, this signalling complex becomes delocalized, leading to depolarized growth, whereas DN-rop1 blocks the activation of this signalling complex resulting in short tubes.

the PM was specifically affected by ROP1 levels. ROP1 OX caused GFP-RIC1 distribution to a wider apical region of the pollen tube PM (Wu *et al.*, 2001; Fig. 3). By contrast, GFP-RIC1 localization was not affected by overexpression of those ROPs that are functionally distinct from ROP1, including ROP9 (Fig. 3), ROP8, ROP10 and ROP11 (V Vernoud, Z Yang, unpublished data). These

GFP   GFP-RIC1   GFP-RIC1   GFP-RIC1
                  + Rop1   +Rop9
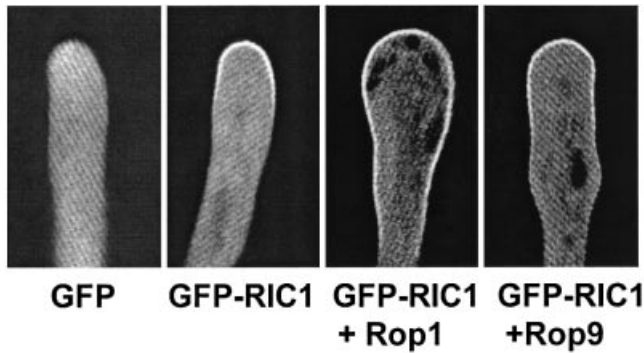
Fig. 3. Effects of ROP overexpression on the localization of GFP-RIC1. LAT52::GFP-RIC1 construct was transiently expressed in tobacco pollen tubes alone or co-expressed with LAT52::ROP1 or LAT52::ROP9. The constructs were introduced into pollen grain by projectile-mediated transformation (Fu *et al.*, 2001) and tubes expressing GFP were examined 5 h after bombardment using laser scanning confocal microscope as described previously (Wu *et al.*, 2001). Shown are medial longitudinal sections of tubes.

results provide evidence that GFP-RIC1 marks the activation of ROP1, although it has yet to be been determined whether or not all PM-localized ROP1 interacts with RIC1. Together with the effect of different levels of ROP1 overexpression on pollen tube growth (Fig. 2) and the requirement for ROP1 (and probably ROP3 and ROP5) in tip growth, these results strongly support the hypothesis that the apical dome of active ROP1 and, subsequently, the localization of the active ROP1 signalling complex establish the tip growth domain (Fig. 2). Future experiments at the EM level should determine whether the localization of active ROP1 exactly delineates the apical region of the PM in which exocytosis occurs.

Given the marking of the tip growth domain by the active ROP signalling complex, external cues that direct pollen tube growth *in vivo* most likely regulate the localization and redistribution of active ROP1. When cultured *in vitro*, pollen tubes tend to extend in one random direction. In this case, the active ROP1-containing tip growth domain must be reproduced rapidly or maintained following the insertion of nascent PM as the result of growth. An intriguing question is how the tip growth domain is reproduced or maintained. Because *CA-rop1* expression caused much more severe depolarized growth compared to similar or higher levels of ROP1 OX, it was proposed that active ROP1 promotes ROP1 recruitment to the apical PM region, forming a ROP activation/recruitment positive feedback loop and that this feedback loop provides a mechanism for the rapid reproduction of tip growth sites (Li *et al.*, 1999). Direct evidence for this hypothesis came from experiments in which changes in GFP-RIC1 and GFP-ROP1 localization was monitored when ROP1 localization and activity were respectively altered by the expression of AtGDI1 and RopGAP1 (G Wu, V Vernoud, Z Yang, unpublished data). RopGAP1 (Rop

GTPase activating protein) promotes GTP hydrolysis in ROP1 and acts as a ROP1 deactivator (Wu *et al.*, 2000). Further studies suggest that the positive feedback loop is activated locally, amplified laterally, and inhibited globally to allow the generation of the dome of active ROP1 with a tip-high gradient (G Wu, V Vernoud, Z Yang, unpublished data). However, it remains possible that additional mechanisms such as endocytosis in the subapical region also play an important role in maintaining the localization of active ROP1 at the tip and/or recycling of the ROP1 signalling complex.

Actin cytoskeletal dynamics and localized ROP1 signalling in pollen tubes

It is generally accepted that the actin cytoskeleton plays a critical role in pollen tube growth as actin-disrupting drugs such as latrunculin B (LatB) and cytochalasins effectively block tip growth (Gibbon *et al.*, 1999). However, which specific forms or populations of F-actin are involved has been a difficult problem to address for at least two reasons. First, available tools for visualizing F-actin, especially highly dynamic F-actin, might not have been optimized. Second, it is difficult to determine how specific a particular drug treatment is against one type of F-actin. The existence of extensive longitudinal actin cables is indisputable, as they are relatively stable and have been visualized using various methods. It has been assumed that they are involved in the reverse fountain pattern of cytoplasmic streaming in pollen tubes, but it is unclear whether they alone are sufficient to determine the streaming pattern. Furthermore, actin cables are unlikely to be the cytoskeletal basis for the accumulation of vesicles in an inverted cone pattern in the extreme apex, since actin cables do not reach to the apex in growing tubes. Microtubules do not play a critical role in tip growth in pollen tubes and are unlikely to be involved in vesicle accumulation at the tip (Astrom *et al.*, 1995). These observations raise an interesting question: Is there a specific form of F-actin distinct from actin cables that provide driving forces for vesicle accumulation at the tip?

Based on earlier studies using staining with fluorescent phalloidin or anti-actin antibodies, either in fixed or live cells, it was proposed that the clear zone in the apex is free or depleted of F-actin (Pierson *et al.*, 1994). More recent studies using improved fixation method by Staiger's group (Staiger *et al.*, 1994) or using expression of GFP-mTalin by Chua's group provided evidence for the presence of an actin collar or ring near the tip of pollen tubes (Kost *et al.*, 1999). This form of F-actin was also observed in fixed root hairs (Miller *et al.*, 1999).

Using a GFP-mTalin construct similar to that from Chua's group, it was found that this actin collar was only present in a small portion of tubes at a given time when tubes were examined (Fu *et al.*, 2001). Time-course

analysis suggests that the actin collar appears to alternate with a highly dynamic form of F-actin localized to the extreme apex (Fig. 4). This dynamic tip actin appears as short actin bundles (Fu *et al*., 2001) in tobacco pollen tubes, but it may be expressed in different forms in different pollen tubes (e.g. as diffuse actin clouds in *Arabidopsis* pollen tubes). In fact, similar diffuse dynamic F-actin is present at the tip of root hairs and growing sites of cells that do not undergo tip growth (Fu *et al*., 2002; Jones *et al*., 2002; Baluska *et al*., 2000). The dynamic F-actin is much more sensitive to LatB than actin cables (Gibbon *et al*., 1999). Interestingly, the dynamics of tip F-actin seems to account for the temporal changes in the accumulation of tip F-actin. It has been shown that the amount of tip F-actin oscillates in the opposite phase with pollen tube elongation rates and that the peak of tip F-actin precedes that of growth (Fu *et al*., 2001). These observations, together with the results showing that low concentrations (0.5–5 nM) of LatB inhibited pollen tube elongation, but did not disrupt actin cables and cytoplasmic streaming, suggest that the dynamic F-actin may play a crucial role in pollen tube tip growth (Pierson *et al*., 1994; Vidali *et al*., 2001).

Because Rho GTPases are conserved signalling switches that regulate actin organization in yeast and animals, the involvement of tip-localized ROP1 in the regulation of tip actin was investigated (Fu *et al*., 2001). The results indicate that ROP1 activation is required for the assembly of tip actin, whereas tip-localized ROP1 signalling regulates the dynamics of this tip actin. Inactivation of ROP1 by overexpression of RopGAP1 or DN-rop1 reduced or eliminated tip F-actin. However, ROP1 overexpression caused the formation of a network of actin filaments at the tip and abnormal transverse actin bands just behind the extreme apex, apparently due to the stabilization of the dynamic tip F-actin. These abnormal forms of F-actin were suppressed by RopGAP1 or AtGDI1 overexpression, which also suppress depolarized ROP1 localization and activation (see above). Interestingly, LatB treatments also suppressed abnormal F-actin and depolarized growth induced by ROP1 OX and recovered normal dynamics of tip F-actin. These results demonstrate that localized ROP1 signalling at the tip is essential for the dynamics of tip F-actin, which, in turn, is crucial for polarized tip growth.

These studies on ROP1 signalling to the organization of F-actin in pollen tubes also demonstrated that actin cables and dynamic tip actin are functionally distinct and are regulated by distinct mechanisms. Treatments with latrunculin B at the concentration that suppressed ROP1 OX phenotype and recovered actin dynamics did not affect actin cables. Similarly, the formation of actin patches and actin cables in yeast are controlled by different mechanisms. Cortical patch assembly is mediated by the Cdc42 Rho GTPase and involves a Wasp/Scar homologue (Bee1/
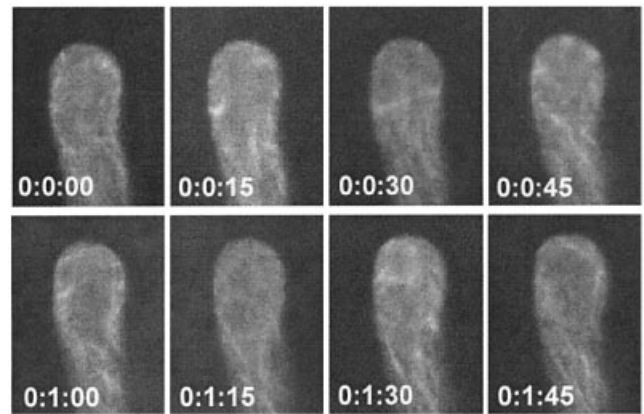
Fig. 4. Dynamic tip F-actin is revealed using GFP-mTalin. LAT52:: GFP-mTalin construct introduced into tobacco pollen tubes and fluorescent tubes were imaged using confocal microscope described in Fig. 3. The medial longitudinal section of pollen tubes were scanned every 15 s. Shown is a time series of these sections for a typical tobacco pollen tube expressing GFP-mTalin. The GFP-mTalin construct was described previously (Fu *et al*., 2001).

Las17), a WIP homologue (Vrp1) and two type I myosins (Myo3 and Myo5), which together activate the Arp2/3 complex. Organization of actin cables is also Rho-GTPase dependent, but Bni1 and Bnr1 are essential for the assembly of polarized actin cables independent of Arp2/3 complex, but dependent of profilins (Evangelista *et al*., 2002). The specific Rop regulation of dynamic F-actin localized to the site of polar growth, but not actin cables, appears to be a general phenomenon in cell morphogenesis in plants, because ROP2 has been shown to be required for the formation of cortical diffuse F-actin in different cell types (Fu *et al*., 2001; Jones *et al*., 2002).

It is unknown why the Rop-dependent dynamics of tip F-actin is important for polar growth in pollen tubes, although the function of the dynamic tip F-actin might be analogous to that of Cdc42-mediated motile actin patches in yeast, which also establishes the site of growth during budding. Cdc42-mediated actin patches have been shown to be required for endocytosis and may also regulate exocytosis (Johnson, 1999). The mode of action for ROP1-mediated dynamics of tip actin may also share parallelism with that of motile actin patches in yeast. The dynamics of tip actin may generate forces required for the concentration of vesicles at the apex in an inverted-cone pattern, whereas the actin collar could regulate endocytosis, which has been shown to occur just behind the extreme apex.

Given the evidence for Rop activation of the assembly of tip F-actin, an interesting question is how localized Rop signalling regulates the dynamics of tip F-actin in pollen tubes. Several classes of actin-binding proteins (ABPs), including ADFs/cofilins, profilins, gesolins/villins, and actin-interacting proteins (AIPs), are known to be involved in the control of actin dynamics by sequestering G-actin, promoting actin severing, or capping barbed ends of

F-actin. Most of these ABPs have been found in plants (Staiger *et al*., 2000; Kovar *et al*., 2001; McCurdy *et al*., 2001) and thus Rop regulation of the dynamics of tip F-actin may involve one or several of these ABPs. As discussed below, it is possible that localized Rop signalling may also activate one or more of these ABPs to regulate F-actin dynamics at the tip of pollen tubes.

Localized Rop signalling may regulate the dynamics of tip F-actin through tip-focused cytosolic Ca^{2+} gradients

Pollen tubes exhibit a tip-focused intracellular Ca^{2+} gradient that can be very steep. For example, cytosolic Ca^{2+} levels in lily pollen tubes can reach as high as 3–5 µM at the extreme apex and fall off sharply to basal levels (100–200 nM) within 20 µm (Pierson *et al*., 1994). It has been shown that Ca^{2+} gradients oscillate in phase with but a few seconds behind growth (Holdaway-Clarke *et al*., 1997; Pierson *et al*., 1994). Importantly, experiments involving the injection of the calcium buffer BAPTA and the localized release of caged calcium show that calcium gradients are essential for tip growth and play an important role in directing tip growth (Malhó and Trewavas, 1996; Pierson *et al*., 1994). Evidence suggests that similar Ca^{2+} gradients are involved in root hair growth (Wymer *et al*., 1997). Based on well-known roles of calcium in regulating exocytosis, it has been proposed that tip-focused Ca^{2+} gradients are also required for exocytosis in pollen tubes, but no direct evidence is available (Roy *et al*., 1999). Unfortunately, few studies have attempted to determine how calcium is involved in the regulation of tip growth. Furthermore, the mechanisms that regulate the formation of tip-focused Ca^{2+} gradients are poorly understood, although tip-focused extracellular Ca^{2+} influxes are thought to be crucial for the formation of cytosolic Ca^{2+} gradients.

Because Rop and calcium are involved in both temporal and spatial control of tip growth in pollen tubes, it is reasonable to speculate a potential functional interaction between Rop and calcium at the tip. An interaction between Rop and Ca^{2+} was first implicated by the observation that low concentrations of extracellular Ca^{2+} and caffeine treatments potentiate tube-growth inhibition by injected anti-Rop1 Ps antibodies (Lin and Yang, 1997). Another study provided evidence that Rop regulates the formation of tip-focused Ca^{2+} gradients (Li *et al*., 1999). First, tip-focused Ca^{2+} gradients were eliminated within 1–2 min after microinjection of an anti-Rop1Ps antibody into pea pollen tubes; this timing coincides with that of antibody-induced growth arrest (Li *et al*., 1999). Second, high levels of [Ca^{2+}]$_{ex}$ (from 10–20 mM) suppressed the growth inhibition caused by *DN-rop1* expression in transgenic *Arabidopsis* pollen tubes, whereas the same concentration of extracellular calcium inhibited wide-type

pollen tube growth. As discussed above, active ROPs are localized in the apical dome of the PM as tip-high gradients, which appears to correspond to the localization of intracellular Ca^{2+} gradients. Thus, these observations are consistent with the hypothesis that tip-localized activated ROP1 regulates the formation of intracellular Ca^{2+} gradients. In root hairs, Rop proteins were found to be polarly localized to the future site of root hair formation in epidermal cells and at the tip of growing root hairs (Jones *et al*., 2002; Molendijk *et al*., 2001). The expression of a constitutively active form of ROP6 delocalized Ca^{2+} gradients. The isotropic growth phenotype of root hairs was also characterized by the presence of multiple Ca^{2+} foci randomly distributed (Jones *et al*., 2002; Molendijk *et al*., 2001), probably as a result of mislocalization of active Rops to the whole root hair PM.

The results described above provide convincing evidence that tip-localized active ROP1 activates both the assembly of tip F-actin and the formation of tip-focused [Ca^{2+}]$_{cyt}$ gradients. Although a temporal relationship between the Rop-dependent tip actin and calcium has not been directly determined, this relationship can be inferred based on the well-established tight correlation between growth and calcium, i.e. tip F-actin and tip-focused calcium gradients oscillate in the opposite phase (Fu *et al*., 2001). If ROP1 regulates both cellular targets, how could these two cellular targets oscillate in the opposite phase? This can be explained if ROP1 signalling coordinates the temporal control of these two cellular targets. In one model, ROP1 signalling, Ca^{2+} oscillation and tip actin dynamics act in a linear pathway, with Ca^{2+} acting upstream of actin or vice versa. Alternatively, ROP1 independently activates Ca^{2+} oscillation and actin dynamics via two different pathways. The observation that accumulation of tip actin alone is not sufficient to induce growth supports the second model (Fu *et al*., 2001). In either model, it is proposed that tip-focused calcium gradients promote actin disassembly to generate the dynamics of tip actin. This explains why tip actin levels reach a minimum when calcium reaches a peak. Although a role for calcium in the regulation of the dynamics of tip actin has yet to be demonstrated directly, several pieces of indirect evidence support this notion. In poppy pollen tubes, either artificial elevation of intracellular Ca^{2+} or S protein-induced calcium increases have been shown to cause dramatic actin depolymerization (Geitmana *et al*., 2000). Several actin-binding proteins (ABPs) have been shown to be regulated by calcium. A primary candidate for a calcium-mediated ABP to be involved in the tip actin dynamics is profilin. Profilins show calcium-dependent sequestration of G-actin (Kovar *et al*., 2000). 50% sequestration of G-actin occurred at 1 µM Ca^{2+} concentration, within the range of observed concentration of calcium at the tip. Although calcium could regulate ABPs' function, the signalling molecules that support calcium's

role in the regulation of the dynamics of tip actin are yet to be identified.

Whether or not actin depolymerizing factors (ADFs) could be involved in calcium-mediated actin dynamics at the tip of pollen tubes seems to be more controversial. ADFs have been shown to be enriched in the tip of pollen tubes and root hairs (Lopez *et al*., 1996), consistent with its potential role in actin depolymerization at the tip. However, plant ADFs have been shown to be phosphorylated at the Ser 6 residue by calcium-dependent protein kinases, and the phosphorylation inactivates its ability to depolymerize F-actin (Smertenko *et al*., 1998). It is not clear whether this phosphorylation occurs at the tip of pollen tubes or root hairs. A calcium-dependent protein kinase activity has been demonstrated at the tip of pollen tubes, but the target of this kinase has not been identified (Moutinho *et al*.,1998). Nonetheless, it remains a formal possibility that ADFs are involved in the depolymerization of tip actin either in a calcium-mediated manner or calcium-independent manner. If a potential ADF-mediated depolymerization of F-actin at the tip involves calcium, one would expect a calcium-dependent protein phosphatase to be involved in this regulation. A family of protein kinases has been shown to be activated by calcium-dependent calcinuerin-like proteins (Halfter *et al*., 2000; Kim *et al*., 2000). It would be interesting to investigate whether these protein kinases are involved in the regulation of tip actin dynamics.

The villin/gelsolin family of ABPs may be another candidate in calcium-mediated actin dynamics at the tip (Yokota and Shimmen, 2000). Animal villins have been shown to have two activities: actin bundling through the headpiece domain at Ca^{2+} concentrations of <1 μM and actin severing through a gelsolin domain at >1 μM of Ca^{2+}. Plants contain a villin homologue, but their roles in the regulation of actin dynamics/organization have not been determined. Because villin homologues have been isolated from lily pollen, they could be involved in calcium-dependent actin dynamics if they have gelsolin activity like the animal villins.

Rop1 probably activates at least two downstream pathways

In order to assess which of the above-mentioned models account for the regulation of the ROP1-mediated dynamics of tip actin and to understand how ROP1 regulates actin assembly and the formation of tip-focused calcium gradients, it is critical to identify molecular targets of ROP1. In animal and yeast, the three major subfamilies of RHO GTPases, Cdc42, Rac, and Rho, modulate a variety of cellular processes, such as the actin cytoskeleton, cell polarity establishment, axon guidance, cell cycle progression, and cell-to-cell adhesion (Bokoch, 2000; Kjoller and Hall, 1999; Kuhn *et al*., 2000; Tatsuno *et al*., 2000). To

achieve various cellular functions, GTP-bound active RHO GTPases interact with a plethora of functionally distinct target proteins or effectors. A large number of the Cdc42/Rac effectors contain the conserved Cdc42/Rac-interactive-binding (CRIB) motif that binds the effectors domain of Cdc42/Rac GTPases in a GTP-dependent manner (Aspenstrom, 1999; Burbelo *et al*., 1999). However, database searches failed to identify any plant homologue of animal Cdc42/Rac effectors including the best-studied examples of P21-activated protein kinase, Wiskott–Aldrich syndrome proteins, and the tyrosine kinases ACK-1 and ACK-2.

To identify ROP1 targets, the yeast two-hybrid method was used to screen for proteins that interact with CA-rop1 (Li *et al*., 1999; Wu *et al*., 2000). This method combined with data mining led to the identification of 11 CRIB-containing novel proteins from *Arabidopsis*. These 11 structurally divergent RICs (for Rop-interactive CRIB-containing proteins) may provide an important mechanism linking Rop GTPases to various distinct signalling pathways in *Arabidopsis*. It is shown that nine *RIC* genes are expressed in *Arabidopsis* pollen, but when GFP-RICs are overexpressed in tobacco pollen, different localization patterns and phenotypes were observed. These observations have led to the proposal that different RICs may be functionally distinct Rop targets that control distinct pathways downstream of ROPs.

Based on the overexpression phenotypes and localization patterns, it was concluded that *RIC3* and *RIC4*, two structurally distinct novel proteins, appear to be ROP1 targets that control pollen tube tip growth. Overexpression of either RIC3 or RIC4 caused depolarized growth, as does *ROP1* overexpression, and enhanced *ROP1*-induced depolarized growth, suggesting that they may be ROP1 targets. GFP-RIC3 is preferentially localized to the apical region of the pollen tube cytoplasm. Interestingly, ROP1 overexpression caused GFP-RIC3 to be preferentially localized to the cortex or the cytoplasmic region underneath the plasma membrane. This localization pattern is similar to that of GDI1, which functions to remove ROPs from the plasma membrane and sequester ROPs in the cytosol, and thus supports the notion that RIC3 only binds ROP1 transiently and thus is localized dynamically at the tip by shuttling between the PM and the cytosol. In contrast to RIC3, the localization of GFP-RIC4 is restricted to the apical region of the PM, and ROP1 OX induced more GFP-RIC4 to be localized to the wider apical region of the PM. These localization patterns support the difference in the site of action for these two RICs: RIC3 in the cytosol and RIC4 in the PM. Because RIC3 and RIC4 both contain a CRIB motif and are expected to bind the same effector domain on ROP1, it is important to have distinct kinetics during their interaction with ROP1 so that they would not compete with each other in binding to ROP1, but would co-ordinate with each other to transmit ROP1 signals.

Together with their distinct structural features, the distinct localization patterns for these two RICs suggest RIC3 and RIC4 are functionally distinct and most likely control two different pathways downstream of ROP1. Importantly, these observations provide strong support for the hypothesis that ROP1 regulates two downstream pathways that co-ordinate to control the dynamics of tip F-actin as described above. It will be interesting to determine whether these two putative ROP1 targets respectively control the assembly of tip F-actin and the formation of tip-focused calcium gradients.

Conclusions

Recent studies have shown that ROP1 GTPase is a central regulator of pollen tube growth. A ROP1-mediated signalling network that controls tip growth in pollen tubes is emerging from these studies that use both *Arabidopsis* genetics and genomics tools and the transient expression system in tobacco. This signalling network in part involves the generation of a tip-high gradient of active ROP1 through a spatially regulated positive feedback loop of ROP signalling. It is proposed that active ROP1 distributed to the apical dome of pollen tubes establishes the tip growth domain and then regulates the dynamics of tip F-actin and the oscillation of a tip-focused calcium gradient, both of which are required for tip growth in pollen tubes. ROP1 regulates these two cellular targets, most likely through two co-ordinating downstream pathways. This spatially and temporally regulated ROP signalling network may provide a central mechanism underlying tip growth in pollen tubes, in which the temporal and spatial regulation of growth must be highly co-ordinated.

Studies have revealed many exciting opportunities and invited new challenges in the elucidation of molecular and regulatory mechanisms behind pollen tube growth. There is still a need to identify the internal and external signals that regulate the ROP1 signalling network and characterize the molecular linkage between the signals and the ROP1 GTPase. The precise function of putative ROP1 targets RIC3 and RIC4 has yet to be elucidated. It will also be important to determine whether other proteins including CDPK, heterotrimeric G proteins, and actin binding proteins are connected to the ROP1-mediated signalling network. The elucidation of this signalling network is expected to increase understanding of pollination and molecular machineries that control cell polarity and cell growth in general.

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