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ROP GTPASE SIGNALING IN TIP GROWTH

DISSEPTION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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2002

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ABSTRACT

Rho family GTPase is a fundamental switch that controls many biological processes. Rop1, a pollen specific Rho family GTPase from plants (Rop) is crucial for pollen tube growth. To further understand the Rop1 signaling, I identified a putative effector protein RIC (Rop Interactive protein containing CRIB [Cdc42/Rac-Interactive Binding]) and RopGAP (Rop GTPase Activating Protein).

I showed that RIC1 only interacted with active Rop1 and was localized to the apical plasma membrane (PM) of the pollen tube. I further showed that different RICs differentially interacted with Rop1. In collaboration with Ying Gu, my study suggests that RICs may be targets of Rops.

RopGAPs contain a CRIB (Cdc42/Rac Interactive Binding) motif which is only present in the targets of Cdc42/Rac/Rop but not in any other Rho GAPs. I showed that the CRIB motif was responsible for the specific activity and strong affinity of RopGAP1 for Rop1, and demonstrated that RopGAP1 localized to the apical PM in a Rop1 and CRIB dependent manner, thus inhibiting pollen tube growth, reducing Rop1 activity, and rescuing the Rop1-induced depolarized growth of pollen tubes. However, R202L mutation mutant of RopGAP1 shows the opposite effect of RopGAP1. Our results suggests that the CRIB motif enhances the activity of the RopGAP1.

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Tip growth requires continuing vesicle delivery and fusion at the apical dome (tip growth domain). Using an active Rop1 marker, RIC1, I showed that active Rop1 localized to the tip growth domain and defined the tip growth domain. Furthermore, I showed that PM localized Rop1 was altered according to the changes of the activity of Rop1, suggesting that Rop1 PM localization is regulated by a positive feedback loop. This feedback loop is initiated locally, amplified laterally, and subject to the global inhibition of RopGAP1 and AtGDI1 (Arabidopsis Thaliana Guanine Nucleotide Dissociation Inhibitor), thus generating a tip-high gradient of active Rop1 to define the tip growth domain and regulate tip growth. Future studies will be focused on the identification and characterization of the components involved in the positive feedback loop.
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<td>Arabidopsis Biological Resource Center</td>
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<tr>
<td>Arf</td>
<td>ADP-ribosylation phosphatase</td>
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<tr>
<td>Arp2/3</td>
<td>actin-related protein 2/3</td>
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<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
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<tr>
<td>CZ</td>
<td>Clear Zone</td>
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<tr>
<td>DH</td>
<td>Dbl-homology</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine tripeptide receptor</td>
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<td>FPR</td>
<td>FMLP receptor</td>
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<td>GAP</td>
<td>GTPase activation protein</td>
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<td>GEF</td>
<td>Guanine nucleotide Exchange Factor</td>
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<td>GDI</td>
<td>GDP-dissociation inhibitors</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GFP</td>
<td>Green Fluorescence Protein</td>
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<tr>
<td>G protein</td>
<td>Guanosine triphosphate binding protein</td>
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<tr>
<td>GPRC</td>
<td>G protein coupled receptor</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<td>MLCK</td>
<td>myosin light chain kinase</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>PAK</td>
<td>P21-activated protein kinase</td>
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<td>PBD</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>Platelet-derived growth factor</td>
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<td>Phosphatidylinositol-4-phosphate 5-kinase that converts</td>
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<td>SH3</td>
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<td>WASP</td>
<td>Wiskott-Aldrich syndrome prote</td>
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CHAPTER I

INTRODUCTION

Cell polarity that is involved in almost every aspect of cell growth and development is essential for cell division, embryogenesis, neuronal development and responses, chemotaxis, and imuno-response, hormone transport, root growth, and pollen tube growth as well as fungal hyphal growth (Drubin and Nelson, 1996; Mattson, 1999; Pruyne and Bretscher, 2000; Rickert et al., 2000; Rothfield et al., 2001; Steinmann et al., 1999; Zheng and Yang, 2000b). Polarity establishment is a multi-step process regulated by intra- or extra-cellular cues (Chant, 1999). These signaling cues are thought to be responsible for the localized activation of intracellular signaling machineries at the cortical PM site defined by the cues. This localized signaling usually regulates cytoskeletal reorganization and subsequent localized growth (Chant, 1999; Gulli and Peter, 2001; Mattson, 1999; Sheu et al., 2000). Tip growth, as in pollen tube growth, root hair growth, fungal hyphal growth, and axon growth and guidance responses, is a specialized polarized growth involving a continuous delivery and deposition of membrane vesicles and cell wall components, thus resulting in the expansion of the cylindrical tube at its apex (Palanivelu and Preuss, 2000; Zheng and Yang, 2000a).
Accumulating evidence indicates that G proteins (guanosine triphosphate binding protein) are central players in the control of polar growth including tip growth (Cabib et al., 1998; Mueller, 1999; Zheng and Yang, 2000a). However, how G proteins integrate with the cellular cues to control cell polarity, especially tip growth, is far from being fully understood (Dickson, 2001; Gulli and Peter, 2001).

**G-protein signaling**

G proteins are fundamental molecular switches in eukaryotic cell signaling. Most known mammalian and yeast signaling pathways are controlled by G proteins. Two common signaling G proteins are heterotrimeric guanosine triphosphate (GTP) binding protein (trimeric G protein) and monomeric Ras superfamily small (20-30Kd) G proteins (small GTPases) (Bos, 2001; Downward, 1992a; Downward, 1992b). In animals, trimeric G proteins are probably the single most important molecular switch that transmits signals from G protein coupled receptors (GPCRs) to intracellular components. Each trimeric G protein consists of an α, a β and a γ subunit from the combination of 20 α, 5 β and 12 γ subunits in animals (Ellis and Miles, 2001; Sternweis, 1996). However, only one α, two β and one γ subunit are known in plants (Ellis and Miles, 2001). In contrast, many Ras superfamily-related GTPases are found in plants. In fungi and animals, the Ras superfamily consists of Ras, Rho, Rab, Ran and Arf/Sar. Ras controls cell growth and differentiation, whereas Rab members regulate protein trafficking and vesicular transport (Kirchhausen, 2000; Zerial and McBride, 2001). Ran controls protein...
and mRNA trafficking between the nucleus and the cytoplasm (Rush et al., 1996). Arf/Sar controls vesicle formation and regulates membrane trafficking (Schimmoller et al., 1997; Yoshihisa et al., 1993). Rho family members control more diverse biological functions, such as in the regulation of the actin cytoskeleton, membrane trafficking and vesicle transport, transcriptional activation, cell growth and development (Hall, 1998; Musch et al., 2001; Ridley, 1999; Van Aelst and D'Souza Schorey, 1997).

GTPases function through a dynamic switch between the active (GTP-bound) and inactive (GDP-bound) forms. For small GTPases, the conversion of the inactive to active form is controlled by a guanine nucleotide exchange factor while the active form returns to the inactive form through an intrinsic GTPase activity. The intrinsic GTPase activity is very weak for most small GTPases, and thus requires stimulation by GTPase-activating proteins (GAPs) under normal physiological conditions. Inactive GTPases must be recruited to the plasma membrane (PM)-bound GTPases prior to their activation by GEFs. PM-associated GTPases are recycled back to the cytosol by a guanine nucleotide dissociation inhibitor (GDI) (Hall and Nobes, 2000; Symons and Settleman, 2000).

**Rho family GTPases**

Rho family GTPases include three traditional subfamilies: Rho, Rac and Cdc42 from animals and yeast and a new subfamily, Rop (Rho family GTPase from Plants). Members within a subfamily share 75% or greater amino acid sequence identity, whereas sequence identity between subfamilies is from 45% to 70% (Li et al., 1998a; Zheng and
Yang, 2000b). In mammalian cells, members from each of the three subfamilies control a specific actin-dependent process in a hierarchical cascade (Nobes and Hall, 1995). In this cascade, Cdc42 induces filopodia formation and activates Rac, which in turns activates membrane ruffling and lamellipodia formation and Rho. Rho promotes the formation of stress fibers and focal adhesions at the bottom of the cascade (Nobes and Hall, 1995). In addition, the Rho family GTPases control other cellular processes mediated by the actin cytoskeleton, e.g. cell-cell adhesion, cell movement, axonal guidance, cytokinesis, morphogenesis and cell polarity (Van Aelst and DSouza Schorey, 1997). Furthermore, Rho family GTPases control other activities including NADPH oxidase activation, and glucan synthase activation, etc. (Arellano et al., 1996; Bokoch, 2000). In plants, Rops control a variety of actin dependent or actin independent processes i.e. pollen tube growth and root hair growth, hormone response and defense response (Li et al., 2001; Zheng and Yang, 2000b).

**Plant Rho family GTPases**

Since the discovery of first plant Rho-like GTPases in pea, many plant specific Rho-like GTPases have been identified in other plant species (Zheng and Yang, 2000b). Rop is more closely related to Rac (about 65% identity) than to Cdc42 (55% identity) or (Rho 45% - 50% identity) (Li et al., 1998b; Winge et al., 2000; Zheng and Yang, 2000b). Hence, Rop is also called plant Rac (Winge et al., 1997). However, phylogenetic
Figure 1.1 Phylogenetic tree of the Rho family GTPases.
Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; At, Arabidopsis thaliana
analysis uncovered a Rop subfamily of the Rho family GTPases that cannot be included into any of the three old subfamilies of Rho family GTPases, Rho, Rac and Cdc42. Instead, they form a distinct plant-specific subfamily in of Rho family, Rops (Figure 1.1) (Li et al., 1998b; Valster et al., 2000; Winge et al., 2000; Zheng and Yang, 2000b). Further studies showed that Rop either evolved into a distinct group prior to the divergence of Cdc42 and Rac, or applied rapid evolution at the later stage of eukaryotic evolution (Li et al., 1998a; Winge et al., 2000). There are eleven members in Rop subfamily in Arabidopsi (RopXAt, “X” equals to 1 to 11) alone (Li et al., 1998a; Winge et al., 2000). These members share more than 75% identity. Most members of Rop subfamily have the CaaL (where "a" is aliphatic amino acid) motif at their C termini, which is the typical motif responsible for geranylgeranylation in most Rho family GTPases. However, Arac7 and Arac8 have CaaX (where X is any amino acid except L) responsible for farnesylation. Arac10 does not have either motifs, thus whether it is associated with the plasma membrane is unknown (Winge et al., 2000; Zheng and Yang, 2000b).

Given the fact that only one or two trimeric G proteins and no Ras homolog are present in plants, Rop GTPases may be the single most important molecular switch in plant signaling. Emerging evidence indicates that Rops control varied developmental processes and environmental responses in plants (Ellis and Miles, 2001; Ma, 1994; Valster et al., 2000). Rop2At controls cell morphogenesis while Rop1At (Rop1) from Arabidopsis is a pollen specific member and plays an important role in pollen tube growth and polarity (Li et al., 1999b; Li et al., 2001; Li et al., 1998a). Rop is essential for
pollen tube growth since injection of anti-Rop1 antibodies and expression of dominant negative (DN) mutants of Arabidopsis \( \text{Rop1} \) and \( \text{At-Rac2/Rop5} \) as well as antisense \( \text{rop1} \) inhibited pollen tube growth (Kost et al., 1999; Li et al., 1999b). Arabidopsis with the expression of \( \text{CA-rop2} \) and \( \text{DN-rop2} \) genes affected embryo development, seed dormancy, seedling development, lateral root initiation, leaf morphology, shoot apical dominance and phyllotaxis as well as hormone responses (Li et al., 2001). Rop is also implicated in \( \text{H}_2\text{O}_2 \) production and plant defense responses, and may play a role in vacuole formation or function (Hassanain et al., 2000; Lin et al., 2001; Ono et al., 2001; Park et al., 2000).

Rho family targets

Rho family GTPases activate a variety of targets to regulate the actin organization and other biological processes. Rho kinases, p140mDia, Bni1p and Bnr1p are activated by Rho and are probably involved in actin filament assembly through their function in the recruitment of profilin (Evangelista et al., 1997; Imamura et al., 1997; Narumiya et al., 1997; Ozaki-Kuroda et al., 2001; Watanabe et al., 1997). Another Ser/Thr Rho effector, ROCK1 is associated with apoptosis (Leverrier and Ridley, 2001).

Emerging evidence suggests that the induction of actin polymerization is also mediated by the interaction of Rac with a phosphatidylinositol-4-phosphate 5-kinase (PIP5K). PIP5K converts PIP to phosphatidylinositol-4,5-bisphosphate (PIP2), which in turn affects actin filament assembly (Higgs and Pollard, 2000). A Ser-Thr kinase
p160ROCK that only interacts with activated GTP-Rho phosphorylates myosin light chain phosphatase and myosin light chain, linking Rho to the regulation of the assembly of the actin-myosin filament bundles (Hirose et al., 1998). Also a Ser-Thr kinase, PKN interacts with both Rho and Rac in a GTP dependent manner, resulting in stress fiber formation (Kawamata et al., 1998; Mukai et al., 1996).

Interestingly, a CRIB (Cdc42/Rac interactive binding) containing Ser/Thr kinase PAK or Ste20 that is an effector of both Rac and Cdc42 can relay Rho family GTPase to the MAP kinase pathways, thus regulating the actin organization (Holly and Blumer, 1999). Other CRIB motif containing proteins that interact with active GTP Rac/Cdc42 have also been documented. Besides P21-activated protein kinase (PAK), CRIB containing proteins include tyrosine kinases ACK-1, ACK-2, and non kinase protein Wiskott-Aldrich syndrome proteins (WASPs), Gic1, Gic2 and CEPs (Brown et al., 1997; Chen et al., 1997; Hirsch et al., 2001; Manser et al., 1995; Mullins, 2000; Pirone et al., 2000; Yang and Cerione, 1997). Effectors without CRIB motif including POR1 (partner of Rac1), IQGAP1 and IQGAP2, are also involved in the cytoskeleton organization (Bashour et al., 1997; Hart et al., 1996; Zhang et al., 1997). However, no effector has yet been identified for Rop in plants prior to this study. In this research, I identified and characterized a group of CRIB containing proteins, RICs. Further studies indicate that RICs may be the effectors of Rops (Wu et al., 2001).
Rho family GTPase regulators

Studies in animals and fungi show that the binding of the guanine nucleotide exchange factor (GEF) to the inactive G-proteins leads to the GTPase activation. Most known GEFs for the Rho family have a DH (Dbl homology) domain and a PH (pleckstrin homology) domain (Cerione and Zheng, 1996). GEFs include the Rho specific proto-oncoproteins, Lbc, Lfc and Lsc, Cdc42 specific Fgd1 and Vav and Dbl for Rho family (Abe et al., 2000; Glaven et al., 1996; Li and Zheng, 1997; Olson, 1996; Olson et al., 1996; Pasteris et al., 1994). In yeast, Cdc24 is a GEF for Cdc42 but not Rho, playing a critical role in the budding process (Zheng et al., 1994). Interestingly, either a null Cdc24 mutant or a strain with the overexpression of Cdc24 leads to cell cycle arrest and the loss of cell polarity (Ziman et al., 1991; Ziman et al., 1993). Cdc24 is indeed localized to the budding site suggesting a role in budding (Ziman et al., 1991; Ziman et al., 1993). Other two Rho GEFs found in yeast are ROM1 and ROM2 (Ozaki et al., 1996). However, no conventional RhoGEF is found in plants in the whole Arabidopsis genome sequence that is already completed (Valster et al., 2000; Zheng and Yang, 2000b). It is possible that there is a unique GEF in plants for Rop. Recently, Rop was found in the active complex of a receptor like kinase (RLK), CLAVATA1 (Trotochaud et al., 1999; Valster et al., 2000). Interestingly, the existence of novel protein SopE without a DH domain can act as a GEF for Rac and Cdc42, supporting the possibility that plants may have a unique GEF (Hardt et al., 1998).
While GEFs act as positive regulators, GAP and GDI are negative regulators. To inactivate G proteins, GAPs bind to active GTP bound GTPases and accelerate the hydrolysis of GTP, leading to the inactivation of the G proteins. The first identified Rho GAP is p50RhoGAP that has GAP activity for Rho, Rac and Cdc42 in vitro but only has activity for Rho in vivo (Barrett et al., 1997; Lamarche and Hall, 1994; Lancaster et al., 1994). To date, more than a dozen proteins show GAP activity for Rho family GTPases. RhoGAPs contain catalytic GAP domains that are responsible for both the binding and the activity for Rho family GTPases. Inside the GAP domain, there is an invariant Arg residue that is required for the GAP activity (Lamarche and Hall, 1994; Lancaster et al., 1994). In addition to the GAP activity, some Rho GAP proteins have effector functions. For example, overexpression of an N-chimerin lacking GAP activity results in the induction of lamellipodia and filopodia, a phenotype resembling the overexpression of Cdc42 and Rac (Kozma et al., 1996). When I initiated my research, no Rho GAPs was known from plants. One of my main objectives was to isolate Rho GAPs for the unique Rho subfamily Rop.

A more complex regulator is GDI that interacts with both GTP bound and GDP bound GTPases. Several Rho GDIs were identified from many eukaryotes, Rdi1p from yeast, RhoGDIα, RhoGDIβ and RhoGDIγ from animals, AtGDI1, AtGDI2 and AtGDI3 from Arabodopsis plants (Bischoff et al., 2000; Olofsson, 1999). Some Rho GDIs interact with all members in the Rho family while others do not, thus the specificity of GDI is not very clear (Gorvel et al., 1998). The fact that Rho GDI can bind to both active Rho and inactive Rho family GTPases further complicates its function and activity on
Rho family GTPases (Bischoff et al., 2000; Sasaki et al., 1993). Three common distinct functions have been reported for GDI. First, GDI binds to the GTP-bound GTPases and competes with GAPs, thus reducing the GAP activity (Chuang et al., 1993; Hancock and Hall, 1993). Second, GDI interacts with GDP-bound GTPases, thus competing with the GEF and stabilizing the inactive GTPase (Ohga, 1992). Third, it binds to isoprenylated membrane bound C-terminal pocket of Rho family GTPases to subtract Rho family GTPases from the membrane (Nomanbhoy et al., 1999; Sasaki and Takai, 1998; Scheffzek et al., 2000). In plants, the function of GDIs has not been reported prior to this research. In this study, I showed that AtGDI1 was involved in the global negative regulation of Rop signaling in the control of pollen tube growth (Wu et al., 2002a).

**Cell polarity control**

Essentially all cells can be polarized, and the polarization is required for virtually all aspects of living cells, i.e. cell movement, nutrition and water uptake and transport, environment responses and defense responses (Bahler and Peter, 2000; Chant, 1999; Fowler, 2000; Kropf et al., 1998; Parent and Devreotes, 1999). Polar growth plays a critical role in many fundamental biological processes in all eukaryotes as well as in prokaryotes. Several systems are used to study some typical polar processes, which may lead to the full understanding of the polarized growth. Because of the simple genetics and small genome size, the single cell budding yeast has been used as a model to study cell polarity control (Bahler and Peter, 2000; Chant, 1999). During budding and mating, two
out of the three stages of yeast life cycle, cells are highly polarized. The development processes for polar growth and polar movement are similar and all include polar site selection, followed by polar site establishment, and polar site protrusion and maintenance (Chant, 1999). These three processes are extremely complicated and involve many factors and proteins. However, in eukaryotes, it is becoming clear that polar site selection is activated and initiated by internal and/or external molecular cues while the second and third processes are controlled by Rho family GTPases (Pruyne and Bretscher, 2000).

**Polar site selection**

In yeast, the selection of a site for budding according to intrinsic spatial landmarks (cues) from previous cell divisions is regulated by a Ras GTPase, whereas the selection of a site for mating protrusion responding to the gradient of pheromones (extrinsic cues) secreted by its mating partner is regulated by trimeric G proteins (Gulli and Peter, 2001). In haploid yeast, mother and daughter cells are constrained to the bud immediately adjacent to the previous budding site, whereas in diploid cells, mother and daughter cells are restricted to the bud at birth or the distal pole. These axial spatial cues locally activate uniform PM localized Ras GTPase (Bud1), which in turn activates Cdc24 (Cdc42 GEF); Cdc24 then activates Cdc42, resulting in the activation of its downstream targets and bud formation (Bahler and Peter, 2000; Chant, 1999; Gulli and Peter, 2001; Pruyne and Bretscher, 2000).
In budding, site selection controlled by Ras signaling is supporting by the fact that mutation at either Ras GTPase \textit{BUD1}, or its GEF \textit{BUD5}, or its GAP leads to random budding (Bender, 1993; Park et al., 1993). While \textit{Bud1} is uniformly localized on the cell membrane, the localization of Ras GEF (Bud5) and Ras GAP (Bud2) are independent of each other but both are at the budding incipient site (Kang et al., 2001; Marston et al., 2001; Park et al., 1999). Since Bud5 interacts with Bud10, a budding landmark with a transmembrane domain, suggesting Bud5 may be recruited to the incipient site by this landmark (Kang et al., 2001). On the other hand, the localization of Bud2 is dependent on Bud1. Therefore, the local recruitment of Ras GEF may locally activate Ras, followed by the recruitment of Bud2, thus resulting in the rapid cycling of Ras between GTP- and GDP- forms due to the colocalization of Ras, Ras GEF and Ras GAP. This rapid cycling of Ras GTPase leads to the initiation of the budding site (Gulli and Peter, 2001). Interestingly, Cdc24 interacts with both active Bud1 and inactive Cdc42. Furthermore, the interaction of Cdc24 with Bud1 inhibits the Bud2 activity and thus reduces the GTP hydrolysis of Ras, suggesting Cdc24 may be an effector of Bud1 since many GTPase effectors inhibit GTP hydrolysis (Marston et al., 2001; Park et al., 1997; Park et al., 1993; Park et al., 1999). Moreover, the localization Cdc24 is randomized in \textit{bud1} mutants. Taken together, it has been concluded that Bud1 recruits Cdc24 to the budding sites and activates the Cdc42 through Cdc24 (Nern and Arkowitz, 1998).

Mating projection formation, however, requires a specific activation of Cdc42 in the direction of pheromone gradients. These extracellular cues have to overcome the internal signals for budding and transmit it into the intracellular signaling pathways that
control the actin reorganization and orientation, which is the prerequisite for the formation of the mating projection rather than a bud. Thus, for haploid cells to mate, they must be able to redirect their polarization axes toward their mating partners according to pheromone gradients. In this chemotropic process, mating pheromones are sensed by heterotrimeric G protein coupled receptors (GPCRs) (Nern and Arkowitz, 1998; Sah et al., 2000; Whitehead et al., 2001). The binding of pheromones to the corresponding receptors promotes the exchange of GDP for GTP of Gα subunit of the trimeric G protein. The activation of Gα leads to its dissociation from the trimeric complex (G αβγ), thus resulting in the free Gβγ. Free Gβγ in turn activates the downstream effectors to induce the mating response. In this whole process, a key component is an adapter Far1 that transmits the extracellular signals perceived by the GPCRs to Cdc42 that triggers the mating projection formation. Far1 interacts with active Gβγ and inactive Cdc42 and is regulated by a cell cycle dependent kinase, Cdc28, which is required for budding (Butty et al., 1998; Shimada et al., 2000). During budding, the phosphorylation of Cdc24 by Cdc28 is required (Gulli et al., 2000). On the other hand, the activation of Cdc28 triggers the degradation of Far1p while the inhibition of Cdc28 by pheromone stabilizes Far1p (Blondel et al., 1999; Blondel et al., 2000; Henchoz et al., 1997). These results suggest that pheromone stabilizes Far1p-Cdc24 complex that is probably required for the mating projection formation while it prevents the formation of Cdc28 assisted Cdc24-Bud1p complex that is required for the budding emergence.

In chemotaxis, in responding to the extracellular cues, growth cone attracts to or repulses from a favorable or an unfavorable gradient of guidance signals respectively.
Amoeboid movement, a motile cell migration, possesses some kind of polarity but moves randomly in the absence of the external cues. When exposed to the chemoattractant, the cells first rapidly orient themselves towards the direction of the cues, then moves quickly towards the cues. This biased movement will be persistent until a change in the direction of cues occurs, resulting in the movement towards the new source (Firtel and Chung, 2000). Similarly, leukocyte movement in response to chemokines is initiated with the polarization of itself into the leading edge (with the formation of lamellipodia) and the trailing edge (with a uropod) and followed by its migration towards the chemoattractants (chemokines) (Firtel and Chung, 2000; Rickert et al., 2000). In these movements, signaling cues received by seven membrane GPCRs are transmitted to heterotrimeric G proteins which in turn activate Rho family GTPases (Thelen, 2001). Evidence supports the direct activation of Rho family GTPases by G proteins is that G₁₃α interacts with p115RhoGEF and activate Cdc42 in vitro (Kozasa, 2001).

In animals, another mechanism that is analogous to the yeast Bud1-Cdc24 association is Rap and integrin interaction, in which Rap-like GTPase, a Bud1 homologue in animal, interacts with transmembrane receptor integrin (Caron et al., 2000; Evers et al., 2000; Price et al., 1998). This pathway is supported by the fact that the adhesion of fibroblasts to fibronectin enhances the activation of Rac and prolongs the activation of Rho, suggesting that integrin is indeed engaged in the activation of Rho family GTPases (del Pozo et al., 2000; Evers et al., 2000; Ren et al., 1999).

Interestingly, neuron responses do not go through GPCRs but heterogeneous transmembrane receptors such as tyrosine kinases, receptor phosphatases and other novel
receptors are used instead. One pathway is that Rac is activated by G protein through N-formyl-methionyl-leucyl-phenylalanine tripeptide (FMLP) receptor (FPR) and IP3 rather than GPRCs (Glogauer et al., 2000). Based on the study on Vav1, and its isoforms Vav2 and Vav3 that interact with tyrosine kinase receptors EGF or PDGF through Src-homology 2 (SH2) domain, in which the extracellular signaling cues are captured by EGF and PDGF, and transmitted to Rho family GTPase indirectly (Bustelo, 2000). The localization of Rho GEFs to the activation site may require the association of different phosphatidylinositol derivatives (PIPs) which is produced by PI kinases that are probably activated by tyrosine kinase receptors. Further studies on Vav1 has revealed that the DH domain of the GEFs possesses an autoinhibition that can be released by tyrosine phosphorylation or its binding to the transmembrane receptors, or Ras, or Far1. The releasing of such an autoinhibition leads to the activation of Rho GEFs, which in turn activates Rho family GTPases (Bustelo, 2000; Gulli and Peter, 2001; Lopez-Lago et al., 2000).

In plants, cell polarity is crucial for many processes but the mechanism for polarity development is unclear. Evidence suggests that receptor like kinases (RLKs) might play a role since Rop is found to be associated with active but not inactive CLAVATA1 complex, a receptor like kinase (RLK) (Trotochaud et al., 1999). Since no GEF has been found in plants, how Rop is regulated by RLKs remains to be seen. On the other hand, the study of pollen tube growth and root hair growth indicates that calcium gradients may be an activator or effectors or both (Fu et al., 2001; Li et al., 1999a; Molendijk et al., 2001).
Establishment and maintenance of polarity

The activated Rho family GTPases trigger their downstream effectors and control diverse events mainly through cytoskeleton organization. Polarizing the actin orientation would be the first step of polarity establishment. In yeast, mutation at Cdc42, or its GEF Cdc24, or its GAP Rga1, or the geranylgeranl transferase Cdc43, or its scaffold protein Bem1 causes the randomization of actin organization in the cell that is unable to bud and thus forms a multinucleate cell that does not divide, which is consistent with their functions in cell polarity establishment (Adams et al., 1990; Stevenson et al., 1995; Zheng et al., 1995; Zheng et al., 1994; Ziman et al., 1991).

The p21-activated kinases (PAKs), Ste20 and Cla4, are two CRIB containing protein kinases that have autoinhibitory N termini. Binding of Cdc42 to this terminus through the CRIB motif leads to the release of the autoinhibition and the activation of these kinases, which in turn activate class I myosins Myo3 and Myo5 to regulate the actin cytoskeletal organization (Lechler et al., 2000; Machesky, 2000; Tu and Wigler, 1999; Wu et al., 1997; Zenke et al., 1999). Gic1 and Gic2 are two CRIB containing proteins that have no kinase domain. Double mutant of Gic1 and Gic2 causes partial loss of the polarized growth while both double mutants of Ste20 and Cla4, and Myo3 and Myo5 lead to the complete loss of polarized growth (Pruyne and Bretscher, 2000; Richman et al., 1999; Wu et al., 1997). In mating projection formation, the signals perceived by GPCRs will be transmitted to Ste20 by Cdc42, which in turn activates MAPK pathway through
Stell (Chant, 1999). Emerging evidence suggests that Rho is also involved in the polarized growth and actin organization in yeast. It has been shown that the null double mutant of Rho3 and Rho4 loses the polarity, which can be suppressed by Cdc42 (Matsui and Toh, 1992; Robinson et al., 1999). Further study shows that Rho3 is involved in the polarized vesicle targeting (Robinson et al., 1999). On the other hand, Rhol but not Rho2 is essential to control a broad range of functions. The initial study shows that Rhol mutants produce small buds with defects at the tip of the cell wall, suggesting that Rhol is involved in the cell wall synthesis but not the actin organization (Calonge et al., 2000; Guo et al., 2001; Inoue et al., 1999). Further study reveals that some Rhol mutant alleles disturb the actin organization as some alleles of cdc42 do (Helliwell et al., 1998). Both mutants are not able to bud, suggesting a role of Rhol in the regulation of actin cytoskeleton. This regulation is likely through the interaction of Rhol with PKCl as well as the MAPK (Mpk1) (Harrison et al., 2001; Nonaka et al., 1995; Pruyne and Bretscher, 2000). Further studies reveal that the Rhol effector Bin1 and its homolog Bnr1 interact with profilin to regulate actin organization (Evangelista et al., 1997; Harrison et al., 2001; Kohno et al., 1996).

In animals, injection of activated Cdc42, Rac or Rho into quiescent fibroblasts induces the formation of distinct actin organization patterns, including filopodia and lamellipodia as well as stress fibers. Recent results show that the filopodia and lamellipodia perform contraction as the axon growth cone response to the guidance cues, indicating that Rho family GTPases may function similarly in the axons as in the fibroblasts (Katoh et al., 1998; Mueller, 1999). Rho effectors, ROKα and ROKβ are Rho
kinases that are likely directly responsible for the formation of stress fibers and focal adhesion (Leung et al., 1996). As Rho overexpression, the overexpression of ROKα promotes the formation of stress fibers and focal adhesion while the expression of dominant negative forms of this kinase blocks this process. Further study shows that myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase is a substrate of Rho kinase. Phosphorylation of MBS by Rho induced active Rho kinase reduces the activity of MLC phosphatase thus increases the phosphorylation of MLC, which in turn enhances the binding of myosin to the actin cable and subsequently the formation of stress fibers and focal adhesion (Ai et al., 2001; Fukata et al., 1998; Katoh et al., 2001; Kimura et al., 1998; Kimura et al., 1996; Kureishi et al., 1997). As a homolog of yeast Bni1, Rho specific p140mDia is colocalized with profilin, resulting in the actin polymerization (Kosako et al., 2000; Nakano et al., 1999; Watanabe et al., 1999; Watanabe et al., 1997). Other Rho effectors, such as PKN and PRK2 are also involved in the cytoskeleton organization (Mukai et al., 1996; Vincent and Settleman, 1997).

As the yeast PAK homolog, mammalian CRIB containing proteins, PAKs that interact specifically with active Cdc42 and Rac thus are important for actin organization since the overexpression of these kinases leads to the change of the actin organization. However, the expression of Rac or Cdc42 mutants that can not bind and activate PAKs still induce the formation of the membrane ruffling and lamellipodia, or filopodia respectively, suggesting that PAKs may not play direct role in these processes (Joneson et al., 1996; Lamarche et al., 1996). Similarly, another CRIB containing protein, WASP (human Wiskott-Aldrich Syndrome) that interacts with Cdc42 not Rac may not be
directly involved in the formation of filopodia since the Cdc42 mutants that fail to bind and activate WASP are still able to induce the formation of filopodia. On the other hand, POR1, a Rac specific effector, is probably in the control of the membrane ruffling since POR1 deletion mutant blocks the membrane ruffling induced by RacV12. In agreement with the role of POR1 in the Rac pathway, the Rac mutants that are unable to bind to POR1 are not able to induce membrane ruffling (Joneson et al., 1996; Van Aelst et al., 1996). Nevertheless, the overexpression of WASP does cause the formation of the cluster of polymerized actin, the opposite effect of the dominant negative Cdc42, suggesting a role of WASP in the control of actin organization (Symons et al., 1996; Van Aelst and DSouza Schorey, 1997). Further study shows that WASP functions in the actin polymerization through its interaction with Cdc42 and Arp2/3 complex to regulate the actin assembly (Higgs and Pollard, 2001).

A more complicated effector of Rho family GTPase is PI (phosphoinositide) kinase (PIK) that interacts with the active form of Rac and Cdc42 while PI4P5K interacts with Rho and Rac in a GTP dependent manner (Carpenter et al., 1999). Consistent with the effector role of PI3K is that the constitutively activated PI3K can induce membrane ruffling as Rac does and stress fiber formation as Rho does (Reif et al., 1996). Interestingly, it has been indicated that PI3K can be upstream of Rac and also can activate Rac since the PI3K specific inhibitor blocks the membrane ruffling induced by PDGF and active Ras but not active Rac (Rodriguez-Viciana et al., 1997; Schmidt and Hall, 1998). Furthermore, PI3K lipid products phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P] and phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P] are localized to the
polar sites (Rickert et al., 2000). On the other hand, PIP2, the product of PI4P5 Kinase interacts with talin, which in turn interacts with actin (Bazenet and Kazlauskas, 1994).

In plants, only very limit research has been done. It has been shown that Rops are crucial for the polarized growth in the pollen tubes and root hairs. So far the only consistent results are that Rops are involved in the regulation of actin dynamic and calcium gradient in the pollen tubes and root hairs (Fu et al., 2001; Li et al., 1999a; Molendijk et al., 2001). However, the mechanism to control these processes is completely unknown. A group of RICs, identified in this research interacts with Ropl in a GTP dependent manner and causes distinct phenotypes when they are overexpressed in the tobacco pollen tubes, shedding a light to the understanding of the Rop signaling (Wu et al., 2001)

Tip growth mechanism

Many polarized processes are only continued and maintained for a short period time before polarized signaling turns off. However, tip growth requires the continuous delivery of the vesicles to the established polar sites and thus can continue the polarized growth, resulting in a cylindrical tube capable of navigating its apical dome according to the external or internal cues (Zheng and Yang, 2000a). Typical tip growth includes pollen tubes, root hairs, algal rhizoids, fungal hyphae, and neuronal axons (Palanivelu and Preuss, 2000). It has been shown that actomyosin network accounts for the polar vesicle targeting (Fowler, 2000; Fowler and Quatrano, 1997). In pollen tubes, however,
it has been believed for a long time, that there is an “actin free” zone at the apex of the pollen tubes. How vesicles can be delivered to the apical membrane region in the free zone is a puzzle (Cai et al., 1997). Recent work from Yang's laboratory indicates that the “actin free” zone is not free of F-actin but contains many actin filaments that are regulated by Rop activity (Fu et al., 2001). Evidence suggests that actin alone can not account for the tip growth but intracellular calcium levels alone may be sufficient to establish and maintain the tip growth (Zheng, 2000). It has been shown that the tip-high calcium gradient is required for the delivery as well as for the docking and fusion of the Golgi derived vesicles (Cresti and Tiezzi, 1992; Hepler, 1997). Furthermore, the directional application of extracellular calcium alters the pollen tube orientation (Malhó et al., 1994; Malhó et al., 1995; Moutinho et al., 1998; Pierson et al., 1996). On the other hand, the dissipation of the tip high calcium gradient inhibits the pollen tube growth (Pierson et al., 1994; Schumaker, 1996). Further study shows that Rop are important for the maintenance of the tip high calcium gradient since the injection of Rop antibody in to the pollen tube dissipates the calcium gradient (Li et al., 1999b). The alteration of calcium gradient also was observed in the root hairs with the overexpression Rop4 and Rop6 (Molendijk et al., 2001). Furthermore, PH domain is localized to the apical membrane of the pollen tubes, confirming that calcium signaling is important for the tip growth (Kost et al., 1999).

In axon growth and guidance response, Rho family GTPases play an important role since Rho family GTPases link the cytoplasmic signaling to the cytoskeleton organization (Gallo and Letourneau, 1998; Shamah et al., 2001; Wahl et al., 2000).
Expression of constitutively active or dominant negative Cdc42 and Rac reduced axon guidance responses and cell migration defects (Luo, 2000; Luo et al., 1996; Luo et al., 1994; Mueller, 1999). Furthermore, the null mutants or the gain-of-function mutants of Mig-2 have defects in both guidance response and cell migration (Zipkin et al., 1997). Mig-2 shares 50-60% identity with Rho family GTPases and is most close to Rac and Cdc42 (Zipkin et al., 1997). In cultured neurons, reducing cytoplasmic calcium level abolishes the turning response of the growth cone towards the gradient of the guidance cues while the elevation of the calcium level through the releasing stored calcium in the growth cone can rescue the turning response (Hong et al., 2000; Song and Poo, 2001). In addition, the turning response induced by the gradient of guidance cues can be blocked by the PI3K inhibitors while the response of neutrophils towards chemokines is reduced in the PI3K knockout mice (Wymann et al., 2000). Emerging evidence suggests that Cdc42 and Rac regulate the growth cone advance while Rho controls the repulsion (Shamah et al., 2001). The in vivo spatial and temporal regulation of the activity of Rho family GTPases itself has not been studied (Bradke and Dotti, 2000; Gulli and Peter, 2001; Zheng and Yang, 2000a). Furthermore, the mechanism through which Rho family GTPase activity controls the tip growth is still poorly understood (Bradke and Dotti, 2000).
Objectives and Rationale

Polar growth arises as a consequence of a particular cell polarity. Genetic and biochemical study reveals that Rho family GTPases play a crucial role in polarized growth and tip growth, a special polarized growth. In plants, Rop is a central player in the tip growth of pollen tube. However, the regulation of Rop activity in vivo as well as the integration of Rop switch with the control pollen tube growth is unknown. To address these questions, I set the following objectives for my dissertation research:

1. To identify and characterize Rop effectors. In order to study the function of Rop in the control of pollen tube growth, I used a yeast two-hybrid method to identify Rop effectors using the pollen specific Rop1 as "bait". This study was to identify and characterize the first Rop target proteins.

2. To identify and characterize Rho family GTPase regulators from plants. In order to study the regulation of Rop1 activity, the regulators of Rop1 are required. Similarly, I used a yeast two-hybrid method to achieve this. This study was trying to obtain the regulators of Rop1 from plants. By characterizing these regulators, it would provide the tools for the study of the regulation of Rop signaling.

3. To investigate the function of RopGAP1 in the control of Rop activity and pollen tube growth. To unveil the mechanism of a novel RopGAP1, a regulator of Rop1 identified from objective 2, I used point mutation and deletion mutation techniques in combination with the transgenic expression or
transient expression RopGAPI and its mutants to study its role in the regulation of pollen tube growth. This study would uncover the mechanism of this novel RopGAPI in the control of Rop activity and pollen tube growth.

4. To better understand the mechanism of tip growth. To uncover the mechanism of Rop signaling in the control of tip growth, with the help of the constitutively active Rop1, and Rop negative regulators RopGAPI and AtGDI1, the regulation of the recruitment and activation of Rop1 in a spatial and temporal manner was studied in the control of pollen tube growth. This study would elucidate a model of the mechanism of tip growth.
CHAPTER 2

A GENOME-WIDE ANALYSIS OF ARABIDOPSIS ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEINS THAT ACT AS ROP GTPASE TARGETS

Abstract

The plant-specific Rop-family GTPases are versatile molecular switches in signaling to many processes during plant growth, development and responses to the environment. To understand how Rop achieves its functional versatility in signaling, I have performed a genome-wide identification of putative Rop targets using a combination of the yeast two-hybrid method, bioinformatic tools, and a robust functional assay in pollen. In this study, I have identified 11 Arabidopsis genes encoding novel proteins, termed RICs (Rop‐interactive CRIB motif‐containing proteins), which contain a CRIB (Cdc42/Rac interactive binding) motif required for their specific interaction with GTP‐bound Rop1. RICs are divergent and classified into five groups that share little sequence homology outside of the conserved Rop‐interactive domain. Overexpression of RIC1 in tobacco pollen tubes inhibits pollen tube growth. I showed that most RICs are expressed in the pollen and have distinct affinity to Rop1, suggesting that RICs
may function in the Rop1 pathway and the control of pollen tube growth.

**Introduction**

The plant-specific family of Rho GTPases, termed Rop, has emerged as a pivotal molecular switch in plant signal transduction (Initiative, 2000; Zheng and Yang, 2000). Rop is essential for pollen tube elongation, as indicated by the inhibition of pollen tube elongation by injection of anti-Rop1 antibodies and expression of dominant negative (DN) mutants of Arabidopsis Rop1 and At-Rac2/Rop5 (Lin and Yang, 1997; Kost et al., 1999; Li et al., 1999). Overexpression of wild type Rop1 or At-Rac2/Rop5 caused depolarized growth in pollen tubes (Kost et al., 1999; Li et al., 1999), as suggested by swelling of pollen tube tips or an increase in radial expansion of pollen tubes (referred to as pollen tube expansion in this paper). Expression of constitutively active (CA) mutants for these Rops induced isotropic growth, i.e., formation of a bulbous tube. These results suggest that Rop also controls the specification of a cortical site for tip growth in pollen tubes (Li et al., 1999). Evidence indicates that both polar localization of Rop and the regulation of its activity at the tip region of PM are critical for defining the site of growth in pollen tubes (Lin et al., 1996; Kost et al., 1999; Li et al., 1999; Zheng and Yang, 2000; Wu et al., 2002a). Thus, Rop controls polar growth in pollen tubes probably by integrating signals for growth with polarity signals (Li et al., 1999; Zheng and Yang, 2000). Our studies also suggest that Rop controls polar growth by regulating the
dynamics of tip F-actin and the formation of tip-focused cytosolic calcium gradients (Li et al., 1999; Fu et al., 2001).

Transgenic expression of CA-rop2 and DN-rop2 mutant genes in Arabidopsis suggests that Rop signaling may control a wide range of growth and developmental processes, including embryo development, seed dormancy, seedling development, leaf morphogenesis, shoot apical dominance, lateral root initiation, and phyllotaxis (Li et al., 2001). The expression of these mutants also alters ABA stimulation of seed dormancy, auxin stimulation of lateral root initiation and brassinolide promotion of hypocotyl elongation, suggesting a role for Rop in the regulation of hormone accumulation or responses; (Li et al., 2001). Several studies indicate that Rop activates H$_2$O$_2$ production and plant defense responses (Kawasaki et al., 1999; Potikha et al., 1999; Park et al., 2000; Ono et al., 2001). Furthermore, a Rop-like protein is localized to the tonoplast of developing vacuoles, implying a role for this Rop in vacuole development or function (Lin et al., 2001). Finally, a Rop-like protein is also associated with the active complex of the CLV1 receptor-like kinase (RLK), supporting a role for Rop in relaying RLK signals (Trotochaud et al., 1999). The Arabidopsis genome contains 11 Rop genes, and the Rop proteins are ubiquitously distributed in various organ, tissue and cell types in Arabidopsis (Li et al., 1998; Winge et al., 2000; Zheng and Yang, 2000). Hence, the Rop GTPase appears to act as a universal switch in plant signal transduction. It remains a mystery how this switch regulates these diverse cellular and physiological processes.

In animals and yeast, the three major families of RHO GTPases, Cdc42, Rac, and Rho, also modulate a wide variety of cellular processes, e.g., actin organization, cell polarity.
establishment, axon guidance, gene transcription, cell cycle progression, cell-cell adhesion, NAPH oxidase activation, glucan synthase activation (Mackay and Hall, 1998; Ridley, 2000). To achieve various cellular functions, GTP-bound active RHO GTPases interact with a plethora of functionally distinct target proteins or effectors (Mackay and Hall, 1998; Aspenstrom, 1999). Rho, Cdc42, and Rac each interact with a set of more than 10 distinct effectors in animals (Mackay and Hall, 1998; Aspenstrom, 1999). Most of the Cdc42/Rac effectors contain the conserved Cdc42/Rac-interactive binding (CRIB) motif that binds the effector domain of Cdc42/Rac GTPases in a GTP-dependent manner (Burbelo et al., 1995; Aspenstrom, 1999). The best studied examples of these types of Cdc42/Rac effectors include P21-activated protein kinase (PAK) and Wiskott-Aldrich syndrome proteins (WASPs), tyrosine kinases ACK-1 and ACK-2, and novel proteins termed CEPs (Aspenstrom, 1999; Burbelo et al., 1999; Hirsch et al., 2001). Phylogenetic analysis suggests that Rop has evolved from the same ancestor as Cdc42/Rac (Li et al., 1998; Zheng and Yang, 2000). However, our preliminary search of databases, including the completed Arabidopsis genome sequence, has failed to identify any plant homologs of these animal Cdc42/Rac effectors. This suggests that the plant-specific Rop GTPase may use novel target proteins as effectors.

In this paper, we report a genome-wide identification of a novel family of CRIB-containing proteins that interact with GTP-bound Rop GTPases from Arabidopsis. These proteins, termed RICs (Rop-interactive CRIB-containing proteins), have been identified utilizing database mining combined with the two-hybrid and other protein-protein interaction assays. RICs may be the first Rop targets found in plants.
Results

Identification of active Rop-interacting proteins using the yeast two-hybrid method

Because GTPase effectors typically interact with the GTP-bound active form of GTPases, we used a constitutively active Rop1 mutant (G15V/C188S) (Li et al., 1999; Wu et al., 2000) as a bait to screen an Arabidopsis seedling cDNA library. We identified 46 positive clones from approximately 6 million yeast transformants. Among twenty clones sequenced, four clones encode RopGAPs (Wu et al., 2000) and one clone encodes a predicted novel polypeptide containing a Cdc42/Rac-interacting binding (CRIB) motif. CRIB motifs are found in Cdc42/Rac effectors and confer their specific interaction with the active form of Cdc42/Rac GTPases in yeast and animals. Because this clone was not a full-length cDNA, we conducted a BLAST search against the Arabidopsis EST database, and identified a second clone (RZ03e09F) encoding the full-length predicted polypeptide containing a CRIB motif. This protein was termed Rop-interactive CRIB-containing protein 1 (RIC1).

To test whether RIC1 interacts specifically with the GTP-bound Rop1, we examined the interaction of RIC1 with the constitutively active rop1 (CA-rop1) and dominant negative rop1 (DN-rop1) mutant proteins using an in vitro pull-down assay. The predicted coding sequence of RIC1 was fused to maltose-binding proteins (MBP), whereas CA-rop1 and DN-rop1 were respectively fused to glutathione-S transferase (GST). The MBP fusion protein was “pulled down” with GST-CA-rop1 or GST-DN-
Figure 2.1. CRIB motif-dependent specific interaction of RIC1 with the GTP-bound active Rop. In vitro pull-down assays were performed using MBP-RIC1 and GST-CA-rop1 or GST-DN-rop1, and MBP-RICs pulled-down by GST-rop1 were detected by anti-MBP antibody as described in text. A) In vitro interaction of RIC1 with GTP-bound CA-rop1 and GDP-bound DN-rop1 mutant proteins. B) In vitro interaction of GTP-bound CA-rop1 mutant with RIC1 and RIC1 mutant proteins. H37D and H37D/H40D mutations are within the CRIB motif.
rop1 and detected using an anti-MBP antibody (Wu et al., 2000). As shown in Figure 2.1A, the RIC1-MBP fusion protein interacts specifically with GTP-bound GST-CA-rop1, but not with GDP-bound GST-DN-rop1. To confirm whether the CRIB motif is indeed critical for the interaction, we generated several mutations (H37D, H37D/H40D) in the conserved residues within the CRIB motif that are known to be important for interactions between Cdc42/Rac and their effectors. As shown in Figure 2.1B, these mutants indeed had dramatically reduced interaction with GTP-bound CA-rop1, indicating a critical role for the CRIB motif in the interaction of RICs with Rops.

Annotation and confirmation of RIC-family genes encoding CRIB-containing proteins

The CRIB-dependent specific interaction of CA-Rop1 with RIC1 is reminiscent of Cdc42/Rac interaction with effectors in animals. However, sequence comparison and BLAST searches reveal that RIC1 is a novel protein that shares no similarity with known Cdc42/Rac or Rho effectors or any proteins available in the animal and yeast genome databases. Thus, RIC1 may be a unique Rho target to transmit Rop signals to downstream events. Because the Arabidopsis genome contains 11 Rop genes that appear to participate in many distinct signaling pathways (Kost et al., 1999; Li et al., 1999; Zheng and Yang, 2000; Li et al., 2001), we sought to determine whether Arabidopsis contains multiple RICs that might be responsible for the versatile functions of the Rop family. In order to identify all Arabidopsis genes encoding CRIB motif-containing.
Figure 2.2. **Comparison of predicted amino acid sequences between 11 RICs and CRIB motifs.** A) Comparison between 11 RICs. The predicted RIC polypeptides were identified by Blast search using the CRIB motif from RIC1, and all except for RIC8 and RIC11 were confirmed by sequencing of cDNAs amplified by PCR as described in text. Sequences that are identical among 11 RICs are highlighted in black (!), and conserved consensus sequences are highlighted in gray (*). Sequence alignment was performed using the GCG program. Stretches of conserved sequences within the same group are highlighted in boxes. B) Comparison of the CRIB motif from RICs with those from RopGAPs (Wu et al., 2000) and Cdc42/Rac effectors (Burbelo et al., 1995). Asterik indicates the residues that distinguish between RopGAP CRIB (threonine) and RIC CRIB (glycine). A.t., Arabidopsis thaliana; D.d., Dictyostelium discoideum; S.c., Saccharomyces cerevisiae; H.s., human; C.e., Caenorhabditis elegans. The alignment was done with the help of Ying Gu.
(continued)
(Figure 2.2: continued)

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Motif</th>
</tr>
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<tr>
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</tr>
<tr>
<td>RopGAP2</td>
<td>A.t.</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>RIC2</td>
<td>A.t.</td>
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</tr>
<tr>
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<td>H.s.</td>
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</tr>
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</table>

proteins, we performed a BLAST search using the RIC1 CRIB motif (Figure 2.2B) or nucleotide sequences encoding this CRIB motif. We identified a total of 11 individual genomic BAC clones. Because most of these BAC clones were not annotated to contain a CRIB motif, we decided to annotate them using the Genescan software (http://genes.mit.edu/GENSCAN.html). On the basis of the conservation of intron splicing and exon numbers combined with results from the Genescan analysis, we predicted that all of these CRIB-related genomic sequences encode RIC proteins (Figure 2.2A).

To confirm that the predicted RICs are indeed functional genes, we used PCR to amplify Ric cDNAs from a flower cDNA library. We identified a total of 9 different Ric cDNAs. Sequencing of these cDNAs confirmed they encode the RIC amino acid
sequences predicted from the annotation. We were unable to amplify ric8 and ric11 from the flower cDNA library or cDNAs derived from Arabidopsis seedlings and leaves, suggesting that they are either expressed in specific stages or cell types of the plant or are pseudogenes.

One striking feature about RICs is their small molecular weights. As shown in Figure 2.2A, different RICs range from 116 to 224 amino acid residues in length. Outside of the CRIB motif, RICs share no sequence similarity with any other proteins in the database. However, different RICs share several consensus amino acid residues upstream of the CRIB motif and a consensus PSWMXDFK block downstream of the CRIB motif. RIC2 and RIC4 have distinct overall structures. They contain the CRIB motif at the C-terminus and lack the PSWMXDFK block, whereas all other RICs contain the CRIB motif near the N-terminal end.

**Overexpression of RIC1 in tobacco pollen tubes inhibit tip growth**

Because the function of Rops is currently best characterized in pollen tubes, we began to characterize the potential functions of the RIC family by overexpressing RIC1 genes in pollen tubes. Furthermore, particle bombardment-mediated transient expression has proven to be a robust method for a high-throughput characterization of gene functions in pollen (Fu et al., 2001). To facilitate monitoring RIC1 expression and RIC1 subcellular localization, we tagged RIC1 with GFP. In contrast, tubes transformed with Rop1 overexpression phenotype, the overexpression of RIC1 induced a phenotype
analogous to that caused by expression of DN-rop1 and Rop1 antisense RNA. RIC1 overexpression strongly inhibited pollen tube elongation but had less effect on pollen tube expansion.

Figure 2.3. Analysis of phenotypes in tobacco pollen tubes overexpressing RIC1 and co-overexpressing RIC1 and Rop1 and a time course series of a pollen tube expressing GFP-RIC3. Tobacco pollen grains were bombarded with different GFP-tagged constructs and germinated. The transformed tubes indicated by GFP expression were visualized using epifluorescence microscopy 5 hr after role of bombardment. Images were captured using a CCD camera and analyzed using Metamorph 4.5 software as described in text. Bars = 30 μm. A) Pollen tubes expressing different LAT52::GFP-RIC1. LAT52::GFP was used as (CK). B) Tubes co-expressing LAT52::GFP-RIC1 and LAT52::Rop1. Control (CK) was LAT52::Rop1 co-expressed with LAT52::GFP. C) A
time course series of a pollen tube expressing GFP-RIC3, showing swelling of pollen tube tips. Time 0 indicates the beginning of imaging, which was conducted 3 hr after bombardment.

Subcellular localization of RIC1 in tobacco pollen tubes

The difference of overexpression phenotypes for RIC1 to those caused by Ropl inhibition indicates that these RIC1 may act its inhibitor or adapter in the Rop1 signaling pathway. To further understand the RIC1 in Rop1 signaling, we investigated the localization of RIC1 in pollen tubes, because Rop1 has been shown to localize to the apical region of the tube plasma membrane. The intracellular localization of RIC1 in tobacco pollen tubes expressing GFP-RIC1 fusions were examined using confocal microscopy. As shown in Figure 2.4A, GFP alone was evenly distributed in the pollen tube cytoplasm. GFP-RIC1 is localized to the apical region of the tube plasma membrane (PM), a pattern similar to the localization of Rop1. These results indicate that different RIC1 may be in the Rop1 signaling pathway.

Rop1 expression alters subcellular localization of RIC1 and RIC1 overexpression phenotypes

The localization pattern and overexpression phenotype of RIC1 suggest that RIC1 may be involved in Rop1 signaling. To further test this hypothesis, we determined
whether RIC1 is localized to PM in a Rop1-dependent manner by co-expressing Rop1 with GFP-RIC1 in tobacco pollen. Results showing the effects of Rop1-coexpression on GFP-RIC1 localization and phenotype (Figure 2.3 and 2.4). We have shown that Rop1 overexpression enhances depolarized localization of Rop proteins to the apical region of tobacco pollen tubes. Tobacco pollen grains were bombarded with various constructs and germinated as described in Figure 2.3. Approximately 5 hr after bombardment, GFP localization in transformed pollen tubes was analyzed using confocal microscopy. A) GFP-RIC1 localization was analyzed either in tubes expressing GFP-RIC1 alone (upper panels) or co-expressing Rop1 (lower panels). Bar = 20 μm. All images shown are 2-μm median sections.
pollen tubes, which in turn causes depolarized growth (Li et al., 1999; Wu et al., 2002a). As shown in Figure 2.4, *Rop1* overexpression also caused enhanced and depolarized localization of GFP-RIC1 to the apical region of the pollen tube PM, suggesting that RIC1 localization to PM is dependent upon its association with Rop1. This was further confirmed by the observation that all mutations in RIC1 (H37D, H37D/H40D) abolished RIC1 interaction with Rop1 also eliminated its localization to the PM (Wu et al, 2001).

To gain further insights into the functional interaction between Rop1 and RICs, we next analyzed the phenotypes of pollen tubes co-overexpressing *RIC1* and *Rop1*. We previously showed that the level of *Rop1* overexpression is correlated with the severity of depolarized growth (Li et al., 1999). In the current experiments, we used a moderate level of *Rop1* overexpression, which causes tubes to expand ((Li et al., 1999); Figure 2.3, Figure 2.4). The level of Rop1 overexpression was manipulated in the transient expression system by using different amounts of plasmid DNA for particle bombardment (Fu et al., 2001; Wu et al., 2002a). A moderate Rop1 level of Rop1 overexpression was achieved using 0.4-0.5 μg of plasmid DNA in a typical bombardment experiment (Wu et al., 2002a).

As shown in Figure 2.3B, and Figure 2.4, *Rop1/GFP-RIC1* co-overexpression phenotype was generally consistent with the localization pattern of GFP-RIC1 and the effect of Rop1 on their localization. RIC1 which was co-localized with Rop1 to the apical PM region partially suppressed tube expansion induced by *Rop1* overexpression (Figure 2.3). However, these RIC1 only slightly rescued tube elongation (Figure 2.3).
Differential *in vitro* interaction between RICs and Rop1

To assess whether RICs bind to Rop1 with different capacities, we examined *in vitro* interaction between CA-rop1 and RICs. GST-CA-rop1 fusion and MBP-RIC fusion proteins were expressed in *E. coli*, purified by affinity chromatography, and used for pull-down assays as described in Figure 2.1. Only 7 of the 9 RICs were used for this assay, because we were unable to express the other two RICs (RIC3 and RIC10) as MBP fusion proteins in *E. coli*. The upper panel shows MBP-RIC fusion proteins that bind to GST-CA-rop1, and the lower panel shows the loading control for MBP-RIC fusion proteins used for the pull-down assay. The number in between the panels indicates relative signal intensity for CA-rop1-bound MBP-RICs that is standardized with the loading control (see text).

Figure 2.5. Differential *in vitro* interaction of Rop1 with different RICs. GTP-bound GST-CA-rop1 fusion and indicated MBP-RIC fusion proteins were expressed in *E. coli*, purified by affinity chromatography, and used for pull-down assays as described in Figure 2.1. Only 7 of the 9 RICs were used for this assay, because we were unable to express the other two RICs (RIC3 and RIC10) as MBP fusion proteins in *E. coli*. The upper panel shows MBP-RIC fusion proteins that bind to GST-CA-rop1, and the lower panel shows the loading control for MBP-RIC fusion proteins used for the pull-down assay. The number in between the panels indicates relative signal intensity for CA-rop1-bound MBP-RICs that is standardized with the loading control (see text).

were used for pull-down assays as described in Figure 2.1. GST alone did not interact with any of the MBP-RICs (data not shown). As shown in Figure 2.5, different RICs

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bind GTP-bound CA-rop1 with different capacities. These results suggest that direct binding may be involved in the differential in vivo interaction between Rop1 and RICs.

Accumulation of RIC transcripts in pollen

To determine whether the RIC1 overexpression phenotypes and RIC1 localization in pollen tubes reflected its function in pollen tubes or was caused by ectopic expression of RIC1 gene, we examined the expression of RIC genes in mature pollen. To our surprise, our RT-PCR results showed that all 9 RIC genes tested were expressed in pollen as shown in Figure 2.6. RIC1, RIC3, RIC5 and RIC7 transcripts appeared to be more abundant than those for RIC2, RIC4, RIC9 and RIC10.
<table>
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<th>Antisense Primers</th>
</tr>
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</tr>
<tr>
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<td>AGGATCCATGGCTACAAGATTACGGGGG</td>
<td>CCTAGTTAAACCCATCCAAATAA TCATTGCCG</td>
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</table>

Table 2.1. Primers for RIC cDNAs amplification and RT-PCR

**Figure 2.6.** RT-PCR analysis of RIC gene expression in Arabidopsis pollen. Total RNA were isolated from pollen or different parts of Arabidopsis plants and used for RT-PCR analysis as described in text. Showing here is the Accumulation of various RIC transcripts in mature pollen. The pilot experiment were exclusively done by myself including plant growing, pollen collecting and RT-PCR. In which every RIC tested was expressed in the pollen. This figure shows the same results as pilot expriment but the RT-PCR was performed by Ying Gu, RNA was isolated by myself and the plants were grown by Vanessa Veround and pollen were collected by Vanessa Veround.

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Discussion

In this study, we have provided evidence that RICs may act as direct targets of the versatile Rop GTPase switch. We believe that the genome-wide identification and characterization of Rop targets is significant for several reasons. First, RICs may be the first direct G protein targets known in plants. Second, the 11 structurally divergent RICs identified in this study may provide an important mechanism linking Rop GTPases to various distinct signaling pathways in plants, generating functional diversity for this universal switch. Third, RICs, being novel small proteins distinct from conventional Rho GTPases effectors from animals and fungi, may present a unique novel mechanism for Rho GTPases to regulate downstream events in plants.

RICs may act as direct Rop targets

Emerging evidence has implicated the Rop GTPase as a common signaling switch in many signaling pathways in plants (Lin and Yang, 1997; Kawasaki et al., 1999; Kost et al., 1999; Li et al., 1999; Potikha et al., 1999; Li and Yang, 2000; Zheng and Yang, 2000; Li et al., 2001; Lin et al., 2001; Ono et al., 2001). Like Ras and Rho GTPases in animals, the ability of Rop GTPases to control a plethora of processes may largely depend upon distinct families of their target proteins. A recent study suggests that a PIPK might act downstream of Rop in the control of pollen tube polar growth (Kost et al., 1999). However, no direct Rop targets had been clearly identified until this study.
Some RICs may be direct targets of the Rop GTPase in Arabidopsis. Because all 11 RICs contain a conserved CRIB motif, which was originally discovered in Cdc42/Rac effectors and allows these effectors to bind to the effector domain of the Rop-related Cdc42/Rac GTPases in animals (Burbelo et al., 1995). The CRIB motif in RICs may be functionally equivalent to the animal CRIB motif in binding to the Rop effector domain. Indeed, we have shown that RIC1 exhibits CRIB-dependent specific interaction with the GTP-bound active form of Rop1, but not GDP-bound Rop1. It is likely that other 10 RICs also specifically bind active Rop, because of the high conservation of the CRIB motif among the 11 RICs (Figure 2.2B). A variant of the CRIB motif (see Figure 2.2B) located in the N-terminus of the Rop-specific Rho GTPase-activating proteins, RopGAPs, interacts with both GDP-Rop1 and GTP-Rop1 (Wu et al., 2000). An invariable glycine residue in the CRIB motif of RICs is replaced with threonine in the CRIB variant (Figure 2.2B). In agreement with its interaction with GDP-Rop, the N-terminal domain of RopGAPs acts as a regulator of RopGAP but not as a Rop target and does not compete with RICs in binding to Rop [(Wu et al., 2000); Wu, Grotewold and Yang, unpublished]. As discussed below, the existence of 11 divergent RIC genes supports the notion that different RICs are Rop targets in distinct Rop signaling pathways.

**RICs may have evolved to control various Rop-dependent signaling pathways**

We propose that RICs may have diverged or evolved rapidly to perform distinct functions. This hypothesis was initially hinted by the analysis of predicted RIC
polypeptide sequences. Based on phylogenetic analysis and comparison of their predicted amino acid sequences, the 11 RIC genes are divided into four groups and an orphan member (Figure 2.2 and 3). Apart from the CRIB motif (Figure 2.2B), most RICs share a PSWMxDFK domain immediately downstream of the CRIB motif and several conserved residues immediately upstream of the CRIB motif. However, outside of this conserved region, different RIC groups have no sequence similarity, and homology is low even within members of the same group.

The potential functional of RIC1 has been tested by our functional analysis of RIC1 using overexpression in tobacco pollen tubes. We were able to detect RNA transcripts for 9 out of the 11 RIC genes, and all of the 9 genes are expressed in Arabidopsis pollen.

RIC1 gene inhibits pollen tube growth when it was overexpressed in tobacco pollen tubes. Since RICs have differential affinity to Rop1, RICs may evolve or diverge to perform distinct functions by interacting with distinct partners. It is tempting to speculate that various RICs may function as Rop targets to control different Rop-dependent pathways during other parts of the plant life cycle. RICs would be analogous to the plethora of functionally distinct effectors for Cdc42, Rac, and Rho in animals and yeast (Aspenstrom, 1999). As discussed below, the functional specificity of RICs may involve both their specific interactions with distinct downstream effectors via the variable C- or N-termini of RICs and differential interactions with different Rops.
Our *in vitro* pull-down assays suggest that differential binding between Rop1 and different RICs partly contributes to the differential interactions between RICs and Rops. Rop1-RIC binding capacity is largely correlated with the in vivo Rop1 interaction with different RICs that was assessed based on their co-localization with Rop1, the dependency of their localization on Rop1, and their ability to affect *Rop1* overexpression phenotypes as discussed above (Wu, et al., 2002a). It has been shown that the CRIB motif is required and is sufficient for Cdc42/Rac effectors to interact with Cdc42/Rac, however, amino acid sequences immediately adjacent to this motif are also involved in the interaction (Thompson et al., 1998). Thus, the CRIB motif and its flanking sequences of RICs likely have a major role in determining their interaction specificity with Rops.

**RICs may act as adaptors to link Rops to specific effector proteins**

Apart from the conserved CRIB motif, the RIC family of proteins is characterized by their high sequence variability, sequence uniqueness, and low molecular weights. For example, predicted polypeptides for some RICs are only 116 to 167 amino acid residues in length. It seems unlikely that the variable regions of RICs can function as an enzyme to regulate downstream events. Nonetheless our results suggest that at least some RICs, if not all of them, function as Rop target proteins. Thus, it is reasonable to speculate that RICs act as adaptor proteins linking Rops to effector proteins that in turn activate specific downstream events. Such a linker function would allow the generation of a greater functional diversity for the Rop GTPase switch, and would present a novel mechanism.
for the activation of G protein effectors. Typically, G proteins directly activate an effector that is an enzyme producing second messengers, a protein kinase, an ion channel, or a regulator of actin-binding proteins. This potential non-conventional mechanism of Rop GTPase signaling is consistent with the fact Arabidopsis apparently lack homologs of animal and yeast RHO effectors (see Introduction).

All three types of Cdc42/Rac effectors [kinases (PAK or ACK), WASPs, and CEPs] have been shown to regulate Cdc42-dependent actin assembly (Aspenstrom, 1999; Burbelo et al., 1999; Hirsch et al., 2001), whereas no homologs for these Cdc42 efectors have been identified in plants. Our recent studies suggest that Rop1 also promotes actin assembly in the control of polar growth in pollen tubes (Fu et al., 2001). Rop1 regulation of actin organization appears to be dependent on a novel mechanism distinct from the Cdc42/Rac regulation of actin organization in animals and yeast. Identification of RIC functional partners should provide insights into the mechanism by which Rop regulates actin organization and other cellular processes in plants.

Materials and Methods

The yeast two-hybrid method

The constitutively active Rop1At mutant (G15V/C188S, pDP1S) was used as bait to screen an Arabidopsis seedling cDNA library, as described previously (Wu et al., 2000). The plasmids from putative positive clones were rescued in Escherichia coli and
reintroduced into the yeast strain containing pDP1S to confirm the interaction. The confirmed clones were then sequenced, and the sequences were used for the Blast search of the GenBank or Arabidopsis databases. One clone encoding a novel polypeptide containing a CRIB motif was identified. The polypeptide was designated as RIC1 (Rop-interactive CRIB-containing protein).

Database search and bioinformatics

To identify additional genes encoding CRIB-containing proteins, the CRIB motif or the RIC1 cDNA sequence was used as a query for BLAST search of NCBI database and TAIR database. From this search, we identified one EST clone and many BAC clones from Arabidopsis and several EST sequences from other plant species. Because most of the RIC1-related genomic sequences were not annotated, we used a combination of several Web-based programs and visual examination to predict CRIB-containing proteins. Genomic sequences were extracted and analyzed using DNAStridel.2 to predict the frame encoding the CRIB. We then predicted the full-length open reading frames encoding CRIB motif-containing polypeptides from genomic sequences using GENESCAN. Motif search was performed using scan prosite tools (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html). Sequence alignment and phylogenetic analysis was performed using the GCG Pile-up programs. GenBank accession numbers for various annotated RICs are: RIC1, AC002332; RIC2, AC004557;
DNA manipulation and plasmid construction

A BamHI site was created at the predicted translation initiation codon of RIC-coding sequences for annotated RIC1. Amplified RIC1 cDNA was then cut with BamHI, and ligated into pBluescript BamHI/Smal site. The RIC1 clones were then sequenced. All plasmids used for transient expression in pollen were constructed in a derivative of the pBI221 vector (CloneTech), termed pLAT52, in which CaMV 35S::GUS was replaced with the LAT52 promoter (Twell et al., 1991). The enhanced GFP mutant (S65C) gene was cloned downstream of the LAT52 promoter in pLAT52 to create pLAT52::GFP as previously described (Li et al., 1999). To generate various pLAT52::GFP-RIC1 constructs, RIC1 DNA fragments were cut using BamHI/KpnI from the corresponding pBluescript constructs and cloned as a translational fusion to the C-terminus of GFP in pLAT52::GFP. To generate pLAT52::RIC1 constructs, the GFP cDNA sequence was removed from pLAT52::GFP-RIC1 by BamHI digestion followed by self-ligation.

Site-directed mutagenesis of RIC1

To create point mutations for RIC1, we used PCR-based site-directed
mutagenesis. The primer RIC1F (5'- AAGATCTATGGCGACGACAATGAAGGG TCTTCTTAAGGGCCTT-3') and a mutant primer containing H37D mutation (5'- CGGATCCAATGTCGGCAACATGdTTACATCGG-3') were used to amplify mutant RIC1 fragment A from pBS-RIC1. RIC1 fragment A was ligated into pZErO™-1.1 (Invitrogen) EcoRV sites and was sequenced to confirm the proper mutation. RIC1 fragment B was amplified using primer 42GF (5'-TGGATCCGACGGTCCAACCAA-3') and RIC1R (5'-GTTTCTCAGATAATATCGTTACAGG-3') and then ligated into pZErO™-1.1 EcoRV sites (p42GF). RIC fragment B was cut with BamHI/KpnI and then ligated with individual RIC1 fragment A BamH/KpnI fragments. H37D/H40D was similarly generated.

In vitro protein-protein interaction assays

To demonstrate direct interaction between RICs and Rops, we used RICs fused with maltose-binding protein (MBP) and Rops fused with glutathione S-transferase (GST) for pull-down assays as described previously (Wu et al., 2000). Approximately 10 μg of GST-CA-rop1 or GST-DN-rop1 fusion proteins were bound to glutathione-conjugated agarose beads, and similar amounts of MBP-RIC fusion proteins were used in each assay. The MBP-fusion proteins were detected using a polyclonal antibody against MBP (New England Biolab) and the BM Chemiluminescence Western Blot Kit (Boehringer Mannheim, Basel). Loading controls were performed by western blotting analysis of 2 μl of a total of 500 μl of reaction mixtures from each interaction assay using
the anti-MBP antibody. To compare the relative ability of different MBP-RICs to interact with GST-CA-Rop1, interaction signals were standardized with the loading control. Films were scanned, and the total intensity of signals from each band was measured using the MetaMorph v 4.5 software. The ratio of the intensity from the pull-down assay to the intensity from the corresponding loading control was calculated. The lowest ratio was arbitrarily designated as 1, and all other values shown in Figure 2.5 are relative to this value.

Reverse transcription and PCR analysis of RIC transcripts

Total RNA was isolated from Arabidopsis mature pollen using the TRIZOL Reagent (GIBCO-BRL). Reverse transcription and PCR amplification were carried out as described previously (Li et al., 1998). For all 9 RIC genes, 30 cycles of PCR amplification (94 °C for 30s, at 54 °C for 30s, and at 72 °C for 1 min) were carried out using primers shown in Table 2.1. As PCR amplification and loading controls, the same template cDNA was amplified using primers for the constitutive Act2 gene (An et al., 1996). Five microliters of each PCR product was loaded on a 0.8% Agarose gel to visualize the amplified cDNAs.

Particle bombardment-mediated transient expression in tobacco pollen

Nicotiana tabacum plants were grown in growth chambers at 22 °C under a light
regime of 12 hr darkness and 12 hr light. Pollen grains collected from these plants were used for transient expression using a particle bombardment procedure described previously (Fu et al., 2001). All plasmid DNAs were amplified in the *E. coli* strain Top 10 and purified using QIAGEN plasmid Midi or Mini Kits according to Manufacture's instruction (QIAGEN, Valencia, CA). Routinely, 0.5 mg gold particles were coated with 0.5 µg of pLAT52::GFP-RIC1 DNA or a mixture of 0.5 µg of pLAT52::GFP-RIC1 DNA with 0.5 µg of pLAT52::Rop1. As control, the pLAT52::GFP vector DNA was bombarded into tobacco pollen. Bombarded pollen grains were washed into petri dishes with 0.5 ml germination medium right after the bombardment (Fu et al., 2001). For expression of RIC1, pLAT52::GFP was co-bombardment with pLAT52::RIC1 to facilitate the identification of transformed tubes. The pollen grains were then incubated for 4-6 hr before observation under a Nikon TE300 inverted microscopy equipped with a Hamamatsu cooled CCD camera (Hamamatsu C4742-95) or confocal microscope as described below.

**Analyses of RIC Localization and Morphology**

Tubes expressing pLAT52::GFP-RIC were identified using epifluorescence microscopy and observed as described previously (Fu et al., 2001). We measured the width and length of pollen tubes exactly 5 h after pollen grains were bombarded. Images of fluorescent tubes were rapidly recorded through a cooled CCD camera (model C4742-95; Hamamatsu) attached on an Eclipse inverted microscope (model TE300; Nikon). The
images were analyzed using the MetaMorph v4.5 measurement function. The degree of depolarized growth was determined by measuring the diameter of the widest region of the tube, and the degree of polar growth was determined by measuring the length of pollen tubes. For each treatment, data were collected from three independent experiments (40–80 tubes).

To determine subcellular localization, tubes expressing GFP fusion proteins were analyzed using laser scanning confocal microscope under a Nikon OPTIPHOT upright microscope equipped with a Bio-Rad MRC 600 confocal laser scanning device (Bio-Rad, Hercules, CA). One-micrometer optical sections were scanned and captured using the Comos software. Confocal images were analyzed using the Metamorph 4.5 software (Universal Imaging, West Chester, PA) and processed using Photoshop 5.5.

§ I appreciate Ying Gu’s help in writing and data generating of this chapter of my thesis.
CHAPTER 3

ARABIDOPSIS ROPGAPS ARE A NOVEL FAMILY OF RHO GTPASE-ACTIVATING PROTEINS THAT REQUIRE THE CDC42/RAC-INTERACTIVE BINDING MOTIF FOR ROP-SPECIFIC GTPASE STIMULATION

Abstract

The plant-specific Rop subfamily of Rho GTPases, most closely related to the mammalian Cdc42 and Rac GTPases, plays an important role in the regulation of calcium-dependent pollen tube growth, H$_2$O$_2$-mediated cell death, and many other processes in plants. In a search for Rop-interactors using the two-hybrid method, we identified a family of Rho GTPase-activating proteins (GAP) from Arabidopsis, termed RopGAPs. In addition to a GAP catalytic domain, RopGAPs contain a Cdc42/Rac-interactive binding (CRIB) motif known to allow Cdc42/Rac effector proteins to bind activated Cdc42/Rac. This novel combination of a GAP domain with a CRIB motif is widespread in higher plants and is unique to the regulation of the Rop GTPase. A critical role for CRIB in the regulation of in vitro RopGAP activity was demonstrated using both point and deletion mutations. Both types of mutants have drastically reduced capacities to stimulate the intrinsic Rop GTPase activity and to bind Rop. Furthermore, RopGAPs
preferentially stimulate the GTPase activity of Rop, but not of Cdc42 in a CRIB-dependent manner. In vitro binding assays show that the RopGAP CRIB domain interacts with both GTP-bound and GDP-bound forms of Rop as well as the transitional state of Rop mimicked by aluminum fluoride. Finally, the CRIB domain promotes the association of the GAP domain with the GDP-bound Rop, as does aluminum fluoride. These results reveal a novel CRIB-dependent mechanism for the regulation of the plant-specific family of Rho GAPs. We propose that the CRIB domain facilitates the formation of or enhance GAP-mediated stabilization of the transitional state of the Rop GTPase.

**Introduction**

Existing as cycling GTP-bound "on" and GDP-bound "off" forms, G proteins are pivotal switches in eukaryotic signal transduction. Two major classes of signaling G proteins are known: heterotrimeric G proteins and the Ras superfamily of monomeric small GTPases. Among the five families within the Ras superfamily (RAS, RHO, RAB/YPT, ARF, and RAN), RAS and RHO GTPases are considered *bona fide* signaling proteins. In animals, trimeric G portsins, RAS, and RHO all play an important role in signaling. For example, mammals possess a large number of trimeric G proteins that are formed from the combinations of 20 α, 5 γ and 7 δ subunits (Sternweis, 1996), and thus more than one third of mammalian pathways are dependent on trimeric G proteins (Sternweis, 1996). In contrast, only two Gβ homologs and one Gα homolog and no RAS

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orthologs have been identified in plants (Ma et al., 1990; Weiss et al., 1994; Lee and Assmann, 1999). Loss-of-function Ga mutants in rice are retarded in stem elongation and have reduced seed sizes, but specific pathways controlled by this G protein are unclear (Ma et al., 1990; Weiss et al., 1994; Lee and Assmann, 1999).

Plants, however, possess a large family of RHO-related small GTPases, termed Rop (Yang and Watson, 1993; Delmer et al., 1995; Winge et al., 1997; Li et al., 1998; Zheng and Yang, 2000). Studies using constitutively active and dominant negative Rop mutants suggest a pivotal role for Rop in signaling to many important processes in plants, including tip growth, cell polarity formation, cell morphogenesis, \( \text{H}_2\text{O}_2 \) production and programmed cell death, cell wall synthesis, and probably hormone responses (Kawasaki et al., 1999; Potikha et al., 1999; Li and Yang, 2000; Zheng and Yang, 2000). The role of Rop in pollen tube growth is best studied. Rop acts as a central switch in the pathway leading to tip growth in pollen tubes (Lin et al., 1996; Lin and Yang, 1997; Li et al., 1998; Kost et al., 1999; Li et al., 1999; Zheng and Yang, 2000). Evidence suggests that Rop signaling controls the formation of tip-focused intracellular calcium gradient and tip-localized calcium entry in pollen tubes and may also control the organization of the actin cytoskeleton (Lin and Yang, 1997; Kost et al., 1999; Li et al., 1999). Both the tip-localized calcium signaling and the actin cytoskeleton are crucial for tip growth (Malhó et al., 1995; Yang, 1998; Franklin-Tong, 1999; Franklin-Tong, 1999; Gibbon et al., 1999; Li et al., 1999). Phosphoinositol phosphate kinase and phosphoinositol 4,5-bisphosphate likely act downstream of Rop to control tip growth (Kost et al., 1999). Nonetheless, little
is known about the signals that control Rop-dependent pathways and the mechanisms for
Rop regulation and action.

In animals and yeast, the RHO GTPase switch is controlled by multiple factors
(Whitehead et al., 1997). The conversion of the inactive to active form is promoted by
guanine nucleotide exchange factors (Whitehead et al., 1997). Following its activation of
an effector, the active form returns to the inactive form through an intrinsic GTPase
activity, which is stimulated by Rho GTPase-activating proteins (Rho GAPs). Rho GAPs
are indispensable for Rho-dependent signal transduction (Ridley et al., 1993; Ridley,
1994), e.g., mutations in the round locus encoding a Rac GAP cause cell death in
imaginal disk and appendage shortening in Drosophila (Agnel et al., 1992). The
importance of Rho GAPs in Rho signaling is also reflected by the large number and the
structural diversity of Rho GAPs identified from animals and fungi (Lamarche and Hall,
1994). Various Rho GAPs are characterized by the presence of a GAP catalytic domain
composed of three conserved sub-domains (Lamarche and Hall, 1994; Lancaster et al.,
1994). In addition, many Rho GAPs contain multiple signaling domains such as those
involved in guanine-nucleotide exchange, nucleotide binding, and protein kinase activity
(Tan et al., 1993; Lancaster et al., 1994; Homma and Emori, 1995; Lamarche-Vane and
Hall, 1998; Tatsis et al., 1998). Roles for these domains in GAPs are not clear.

There are hints that the mechanism for Rop signaling may be distinct from that for
yeast and animal RHO GTPases. First, no homologs for conventional Rho GEFs have
been identified to date, even though all the the Arabidopsis genome has been sequenced.
Instead, evidence suggests that Rop directly associates with and may be directly regulated
by receptor-like Ser/Thr kinases (Trotochaud et al., 1999). Furthermore, few homologs of RHO effectors found in yeast and animals are known in plants. Finally, two lotus Rho GAP-like proteins with a novel structural feature (i.e., the presence of the Rho GTPase-binding CRIB domain in their N-terminal region) were recently reported (Borg et al., 1999), and similar sequences are present in various plant ESTs and Arabidopsis databases. We have also identified several of these proteins in our yeast two-hybrid screen for Rop-interacting proteins.

Here, we describe biochemical characterization of one of these Rho GAPs from Arabidopsis, designated as RopGAPs. In vitro GAP assays show that RopGAPs specifically stimulate GTP hydrolysis of Rop GTPases but not Cdc42 GTPases. This Rop-specific GTPase stimulation is dependent on the CRIB domain. Furthermore, the CRIB domain appears to facilitate the formation of or stabilize the transitional state of Rop GTPases. These results provide strong evidence that the signaling by the plant-specific Rop GTPase involves a unique GTPase-regulatory mechanism.

Results

Identification of Arabidopsis genes encoding Rho GAP-like proteins

To identify proteins that interact with the GTP-bound form of Rop, we used a constitutively active rop1At mutant (G15V) as bait in the yeast two-hybrid system (Li et al., 1999). Using this mutant to screen an Arabidopsis seedling library (Kim et al., 1997),
Figure 3.1. Comparison of predicted amino acid sequences between three RopGAPs and their conserved domains and motifs. Amino acid sequences for RopGAP1, RopGAP2 and RopGAP3 are predicted from a combination of two-hybrid clones, EST clones and genomic sequences from the Arabidopsis database as described in text; RopGAP4 and RopGAP5 are annotated by the Arabidopsis Genome Sequencing Project. The lotus RopGAP as described previously (Borg et al., 1999). Sequence alignment was performed by using the Clustalw program. The conserved motifs or domains shown were obtained from the GenBank database by using the BLAST search. Abbreviations: A.t., Arabidopsis thaliana; D.d., Dictyostelium discoideum; S.c. Saccharomyces cerevisiae; H.s., Homo sapiens; C.e., Caenorhabditis elegans.

A. Alignment of predicted amino acid sequences of RopGAPs.
B. Alignment of the GAP-like domain from RopGAP1 with various Rho GAPs.
C. Alignment of the CRIB-like motif with known CRIB motifs from Cdc42/Rac effector proteins.
D. Alignment of SH3-binding motifs from RopGAPs and other signaling proteins.
(Figure 3.1: continued)

B

RhoGAP1 (A. t.)
p50rhoGAP (H. a.)
Chimerins (H. a.)
BCR (H. a.)
RhoGAP1 (A. t.)
p50rhoGAP (H. a.)
Chimerins (H. a.)
BCR (H. a.)
RhoGAP1 (A. t.)
p50rhoGAP (H. a.)
Chimerins (H. a.)
BCR (H. a.)

C

RhoGAP1 (A. t.)
RhoGAP2 (A. t.)
RhoGAP3 (A. t.)
Ste20 (D. a.)
Pak65 (H. a.)
CePak (C. e.)
WASP (H. a.)

D

RhoGAP1 (A. t.)
RhoGAP2 (A. t.)
p50rhoGAP (H. a.)
p85alpha (H. a.)
p85beta (H. a.)
p85alpha (H. a.)

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we obtained 46 positive clones from approximately 6 million yeast transformants. Eight clones were sequenced and predicted amino acid sequences were used to search the GenBank database. Four of them encode amino acid sequences that exhibit significant similarity to Rho GAPs. These clones fall into three distinct genes designated as *RopGAP1*, *RopGAP2* and *RopGAP3*.

Because none of the cDNA clones are full-length sequences, we searched the Arabidopsis database for predicted complete coding sequences. *RopGAP1* is identical to the EST clone 142H15T7 and the hypothetical gene MWD9.12 encoding a predicted polypeptide of 466 amino acid residues. The *RopGAP1* clone obtained from the two-hybrid screen lacks the N-terminal 89 residues. The full-length sequence for the *RopGAP2* gene encoding a predicted polypeptide of 424 amino acid residues is found in the BAC clone T4I9.2. *RopGAP2* from two-hybrid screen lacks the N-terminal 115 amino acids. Two overlapping cDNA clones obtained from the two-hybrid screen encode *RopGAP3*. The predicted *RopGAP3* polypeptide shown in Figure 3.1A is derived from these two cDNA clones and the BAC clone T4A3.7. The BLAST search also identify two additional RopGAPs that we designate *RopGAP4* (BAC clone F24K9.16) and *RopGAP5* (BAC clone T27G7.4).

The alignment of the predicted amino acid sequences for the five RopGAPs shows several conserved structural domains (Figure 3.1). The central region contains a GAP-like domain (residues P179 to E341 in *RopGAP1*) that shares 70% identity among different RopGAPs and about 27% identity with various Rho GAP domains from animals and yeast (Figure 3.1B). It contains the typical three sub-domains in all Rho GAPs and it
has the invariant Arg (residue 202 for RopGAP1) required for GAP catalytic activity (Rittinger et al., 1997; Leonard et al., 1998; Scheffzek et al., 1998). The GAP-like domain is most similar to the p50 rhoGAP, which preferentially activates Cdc42 GTPase (Lancaster et al., 1994).

Interestingly, all RopGAPs contain a Cdc42/Rac-interactive binding (CRIB) motif found in several Cdc42/Rac effector proteins (Burbelo et al., 1995) (Figure 3.1C). None of the known animal and fungal GAPs contains this motif. The CRIB motif and the GAP domain are joint by another conserved region having a consensus sequence for SH3 (src homology domain 3)-binding motifs (PxxxxPxxP or PxxPR) (Ren et al., 1993) (Figure 3.1D). However, both C-terminal and N-terminal regions of RopGAPs are quite divergent among different RopGAPs. Two homologs of RopGAPs from lotus have been recently reported (Borg et al., 1999), and sequences related to RopGAPs are also present in rice and maize EST databases. RopGAP1 and Lotus Rac GAP1 (L-GAP1) share the first and the last five amino acid residues in the N termini and C termini respectively, suggesting that they might be orthologs.

**CRIB motif enhances RopGAP-mediated stimulation of Rop GTPase activity**

We were interested in the potential function of the CRIB-like domain in RopGAPs, because CRIB motifs are found in Cdc42/Rac effector proteins in yeast and animals (Burbelo et al., 1995). Mammalian CRIB motifs are known to inhibit Rho GAP activity by competing with GAPs for binding to Rho GTPases (Zhang et al., 1997).
Figure 3.2. Effects of removing the CRIB domain from RopGAP1 on its GTPase stimulation and interaction with Rop1At.

A. RopGAP1 and deletion mutants used in GAP assays. All deletion constructs except for CRIB are derived from RopGAP1. We used a CRIB domain from RopGAP3 because the corresponding region from RopGAP1 was unstable when expressed as a fusion protein with MBP in *E. coli*. However, we have shown that the CRIB domain from either RopGAP has very similar properties of interaction with Rop1At using yeast two-hybrid interaction assays (data not shown). Numbers above each construct indicate amino acid residues as shown in Figure 3.1A.

B. The activation of Rop GTPase by RopGAP1 and deletion mutants. The full-length RopGAP1 and deletion mutants shown in A were fused to the C-terminus of MBP, and the fusion proteins were expressed in *E. coli*, purified through maltose-conjugated agarose, and used for GAP activity assays as described in text. GST-Rop1At fusion was used as a GTPase substrate for RopGAPs. Each reaction contains 500 nM MBP fusion proteins and 1 μM GST fusion proteins. The release of phosphate from the GST fusion proteins was monitored by spectroscopy every min after the single turnover reaction was initiated.

C. Analyses of RopGAP-Rop1At interactions by in vitro binding assays. The MBP fusion protein containing RopGAP1 and deletion mutants shown in A were used for in vitro binding assays to test their interactions with GST-Rop1At fusion proteins as described in text. The MBP fusion proteins were "pulled down" with GST-Rop1At fusion proteins that were bound to glutathione-conjugated beads, used for western blotting, and detected with anti-MBP antibodies. Abbreviations: CA, constitutively active rop1At mutant; DN, dominant negative rop1At mutant.
Table C

<table>
<thead>
<tr>
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<th>RopGAP1</th>
<th>CRIB</th>
<th>GAP</th>
<th>C-terminus</th>
<th>MW</th>
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<tr>
<td>CA DN</td>
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<tr>
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<td></td>
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-100KD
-80KD
-60KD
Figure 3.3. Effects of point mutations within the CRIB motif of RopGAP1 on its GTPase stimulation and interaction with Rop1At. A). The conserved histidine 125 residue within the CRIB motif was replaced with tyrosine by site-directed mutagenesis as described in text. The point mutation (H125Y) was generated from the MBP-RopGAP1 fusion construct (see Figure 3.2A). B). For GAP activity assays, 350 nM of MBP-RopGAP1 and mutant fusion proteins was used in each GTP hydrolysis reaction. C). Binding of the RopGAP1(H125Y) mutant with the constitutively active rop1At was compared with the full-length RopGAP1 and its deletion mutant lacking the CRIB domain (see Figure 3.2A). Both in vitro binding and GAP activity assays were performed as described in Figure 3.2; however, a different protein preparation and 350 nM of MBP fusion proteins were used instead. Western blot analyses using anti-MBP antibody (lower panel) confirmed that equal amounts of each MBP fusion protein were used in the pull-down assays. These analyses also indicate that the GAP domain is partially degraded and that some degraded forms remain capable of interacting with Rop1At. Lane 1, RopGAP1; lane 2, H125Y; lane 3, GAP; lane 4, C-terminus.
A.

CRIB 117 IGWPTNVRHVAVHVTDF 132

B

(continued)
(Figure 3.3: continued)

C

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<th>RopGAP1</th>
<th>H125Y</th>
<th>GAP</th>
<th>C-terminus</th>
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Pull-down Assay

Loading Control
Thus, we sought to determine whether the CRIB-like domain in RopGAPs has a similar role in inhibiting GAP activity. First, we examined the effects of CRIB domain deletion on the GTPase-stimulating activity of RopGAPs (Figure 3.2A). The activation of Rop GTPase activity by RopGAP was determined by measuring the rate of phosphate release from the GTP-bound GST-Rop1At fusion protein in the presence of RopGAP fused with maltose-binding protein (MBPs). All of our GAP assays including those for various RopGAP1 mutants described below were repeated 2-3 times using the same preparation of E. coli-expressed and affinity-purified proteins. Consistent results were obtained in each repeat.

As shown in Figure 3.2B, GST-Rop1At fusion proteins have very weak intrinsic GTPase activity. However, 500 nM of the full-length RopGAP1-MBP fusion protein stimulates Rop1At GTPase activity by at least 70 fold within 30 min. Equal amounts of E. coli-expressed MBP protein by itself have no effects on Rop1At GTPase activity (data not shown). These results clearly demonstrate that RopGAP1 function as an active GAP for Rop GTPase, as expected from the presence of the Rho GAP-like domain. Similar results have also been shown for a lotus RopGAP (Borg et al., 1999). Surprisingly, a truncated RopGAP1 mutant lacking the CRIB domain had greatly reduced GAP activity on Rop1At compared to the full-length RopGAP1. This suggests that the N-terminal region containing the CRIB motif does not inhibit RopGAP activity, instead, is required for the full GAP activity of RopGAP1 on Rop1At. Furthermore, the CRIB-containing domain by itself had no GAP activity on Rop1At (Figure 3.2B). Similar deletion mutations for RopGAP3 produced the same effects on GAP activity (data not
Figure 3.4. Comparison of GTPase stimulation by RopGAP1 and deletion mutants between Rop1At and Cdc42. GAP activities of RopGAP1 and deletion mutants were compared between Rop1At and Cdc42 using GTP hydrolysis assays. Rop1At and human Cdc42 fused to GST and RopGAP1 and its deletion mutants fused with MBP were isolated and used for GAP activity assays as described in Figure 3.2.
shown). Because the N-terminal region outside of the CRIB motif has limited sequence similarity between RopGAP1 and RopGAP3, these results suggest that the CRIB motif in the N-terminal region is likely required for the full GAP activity in RopGAPs.

To confirm that the CRIB motif is critical for the regulation of GAP activity, we created point mutations on the highly conserved histidine 125 residue within the CRIB motif of RopGAP1 (Figure 3.3A). The corresponding residue has been shown to be critical for the interaction of Cdc42/Rac effectors with GTPases (Abdul-Manan et al., 1999; Mott et al., 1999). As shown in Figure 3.3B, a single histidine mutation (H125Y) caused dramatic reduction of GAP activity on Rop1 GTPase. A double mutation and several triple mutations within the CRIB motif produced a similar effect as the single mutation (data not shown). The extent of GAP activity reduction caused by the point mutations is less than that caused by the CRIB deletion mutants described above. This may be due to some protein degradation that occurred for the deletion mutant (see Figure 3.2C described below) or an additional requirement of sequences outside of the CRIB motif in the N-terminal region for the full GAP activity. Nonetheless, these results clearly establish a critical role for the CRIB motif in the positive regulation of RopGAP activity.

We next asked whether the CRIB-dependent mechanism for GAP activity regulation is specific for the Rop subgroup of Rho GTPases or is general for different Rho GTPases. We investigated the ability of RopGAP1 to stimulate Cdc42 GTPase, because the GAP domain is most similar to p50 rhoGAP, which preferentially activates Cdc42 GTPase. As shown in Figure 3.4, the full-length RopGAP1 fusion protein had a
weak GTPase-stimulating activity on a human Cdc42. MBP alone does not alter Cdc42 GTPase activity (data not shown). The removal of the CRIB-containing domain from RopGAP1 enhanced RopGAP1-mediated Cdc42 GTPase activation by at least two folds, whereas the deletion RopGAP1 mutant lacking the CRIB domain had the identical GAP activity on Cdc42 and Rop1At. Furthermore, the GAP domain, when mixed with an equal molar ratio of the CRIB domain, had identical GAP activity on Cdc42 as the full-length RopGAP1, indicating that the Arabidopsis CRIB motif competes with the GAP domain for binding to Cdc42, as the mammalian CRIB motif does (Zhang et al., 1998). Therefore, these results indicate that the CRIB domain provides a mechanism that underlies the specific stimulation of Rop GTPase activity by RopGAPs.

The CRIB domain interacts with both GTP- and GDP-bound Rop and enhances binding of RopGAPs to Rop

To gain insights into the mechanism by which the CRIB motif regulates RopGAP activity, we investigated the role of the CRIB motif in the modulation of RopGAP-Rop interaction. We examined the interaction of Rop with the full-length RopGAP1 and different deletion mutants fused with MBP (see Figure 3.2A). The MBP fusion proteins were "pulled down" with constitutively active or dominant negative rop1At mutants fused with glutathione S-transferase (GST) and detected with anti-MBP antibodies. Two micrograms of each fusion protein was used in this assay. Western blot analyses
using anti-Rop antibody or anti-MBP antibody confirmed that equal amounts of fusion proteins were used in each assay (data not shown).

As shown in Figure 3.2C, both of the full-length RopGAP1-MBP (~105 kDa) and GAP-MBP (~90 kDa) interact specifically with constitutively active rop1At but not dominant negative rop1At. Similarly, most mammalian Rho GAPs specifically interact with the GTP-bound Rho GTPases (Lamarche and Hall, 1994). However, GAP-MBP has a drastically lower capacity to interact with ropAt than the full-length RopGAP1-MBP does. In the GAP-MBP reaction, constitutively active rop1At was also associated with two lower molecular weight proteins, which probably resulted from partial degradation of the GAP-MBP fusion protein. Surprisingly, CRIB-MBP interacts with both constitutively active and dominant negative Rop1At mutants, although the interaction with dominant negative rop1At is somewhat weaker. This finding is in contrast with the CRIB motif in Cdc42/Rac effectors, which specifically binds the active form of GTPases (Manser et al., 1994; Burbelo et al., 1995). The C-terminal divergent region lacking both the GAP domain and the CRIB domain did not bind either form of Rop1At. Furthermore, GST alone does not interact with any of the MBP protein fused with either the full-length RopGAP or any truncated versions, demonstrating that GST does not contribute to the interaction of Rop1At with RopGAPs (data not shown). Our results from yeast two-hybrid interaction assays are consistent with these in vitro binding assays (data not shown).

To confirm that the CRIB motif is responsible for the interaction between CRIB-MBP and Rop1At, we examined in vitro binding of constitutively active Rop1At to
RopGAP1 mutants in which the specific conserved residues within the CRIB motif are mutated (see Figure 3.3A). As shown in Figure 3.3C, the H125Y point mutation in the CRIB motif drastically reduced the Rop-RopGAP interaction, as did a combination of two or three point mutations within the CRIB motif (data not shown). These mutants interact with Rop only somewhat better than the GAP domain lacking the N-terminal region. Western blot analyses using anti-MBP antibody confirmed that equal amounts of each MBP fusion protein were used in the assay (Figure 3.3C). The difference in Rop-binding capacity between the deletion and point mutations may be due to protein instability for the deletion mutant or a requirement for additional sequences within the N-terminal region of RopGAPs. Importantly, the reduced interaction is tightly correlated with reduced GAP activity in these mutants (see figs. 3.2B and 3.3B). These results suggest that the CRIB motif is required for the Rop-specific stimulation of RopGAP activity through its enhancement of RopGAP binding to Rop.

The CRIB domain of RopGAPs binds to the transitional state of Rop GTPases.

How is the CRIB-mediated interaction of RopGAPs with Rop involved in the stimulation of RopGAP activity? Our observation that the CRIB-containing domain of RopGAP1 interacts with both GTP- and GDP-bound forms of Rop1At suggests that this domain might bind to the transitional state of Rop GTPases. This is supported by our two hybrid interaction assays showing that the CRIB domain interacted with the wild type Rop1At much more strongly than with the constitutively active rop1At mutant (data not
Figure 3.5. Effects of aluminum tetrafluoride on the interaction between Rop and RopGAP. To determine the interaction of RopGAP and its deletion mutants with the transitional state of Rop, the dominant negative rop1At mutant was used for in vitro binding experiments in the presence (+AIF4) or absence (-AIF4) of 20 mM aluminum tetrafluoride. The binding assays were as described in Figure 3.3C except for the addition of AIF4 to the binding buffer. All fusion proteins except for RopGAP3 fused with MBP are described in Figure 3.2. Near full-length (residue 16-388) RopGAP3 was used in this assay for comparison with the CRIB domain, which is derived from RopGAP3 (see Figure 3.2). Bands marked with * are most likely degraded (to be continued next page) forms of the GAP domain. Similar products were observed when the interaction was performed using the constitutively active rop1At mutants (see Figures 2C and 3C). Because signals for the CRIB domain treated with AlF4 were higher by several orders of magnitudes than the signals for the reaction without AlF4, three different exposures of the film were necessary to compare these signals. A short exposure is shown in the upper panel, and medium and long exposures are shown in the middle and lower panels, respectively. Only lane 4 through lane 8 are shown for the longer exposure. The long exposure also detected an extremely weak signal for the GAP domain and RopGAP3 in the absence of AlF4.
shown). To further test this hypothesis, we created a transitional state from GDP-bound Rop1At using aluminum tetrafluoride (Scheffzek et al., 1997; Hoffman et al., 1998). As shown in Figure 3.5, treatments with aluminum tetrafluoride allowed both RopGAP3 and the GAP domain to interact with the GDP-bound rop1At. This was expected because GAPs are known to bind the transitional state of small GTPases. Similarly, the aluminum tetrafluoride treatment dramatically enhanced the interaction of the GDP-bound rop1At with the CRIB domain, indicating that the CRIB domain indeed binds the transitional state of Rop. The addition of an equal molar ratio of the GAP domain reduced the interaction of the CRIB domain with the GDP-bound rop1At (lanes CRIB + GAP), suggesting that the GAP domain associates more strongly with the transitional state of Rop than the CRIB domain does. Interestingly, the CRIB and GAP domains associated with the GDP-bound rop1At synergistically in the absence of aluminum tetrafluoride, implying that the CRIB domain either is able to create a transitional state or stabilize the interaction of the GAP domain with the transitional state.

Discussion

Our in vitro studies clearly indicate that RopGAPs belong to a unique class of Rho GAPs that may act as negative regulators in Rop GTPase signaling. Furthermore, these studies reveal a CRIB motif-dependent novel mechanism for the regulation of Rho GAPs and a role for this mechanism in defining substrate specificity for Rho GAPs. To
our knowledge, this report is the first to demonstrate a role for CRIB motifs in the regulation of GAPs.

**RopGAPs belong to a novel family of Rho GAPs containing a CRIB motif**

All five Arabidopsis RopGAPs share a unique structural characteristic that is critical for the Rop-specific regulation of GAP activity, i.e., the presence of a CRIB motif near the GAP domain in their N-terminal region. RopGAPs with similar structural features are also found in other plant species such as lotus (Borg et al., 1999), rice and maize (EST databases). Many Rho GAPs possess multiple signaling domains besides the GAP domain (see Introduction). However, RopGAPs are the only GAPs known to contain a CRIB motif.

Cdc42/Rac effectors such as Ste20, p65PAK, p120ACK and WASP are the only other group of proteins known to contain CRIB motifs, which mediate the specific interaction of effectors with Cdc42/Rac GTPases in a GTP-dependent manner (Burbelo et al., 1995; Symons et al., 1996; Tapon and Hall, 1997). The CRIB motif in these Cdc42/Rac effectors binds to the effector domain of the GTP-bound Cdc42 or Rac. The Rho GAP domain also binds to the effector domain of GTPases in a GTP-dependent manner, and thus mammalian CRIB motifs inhibit GAP activity by competing with the GAP domain for the effector-binding site (Zhang et al., 1997). We found that the CRIB domain of RopGAPs similarly inhibits the RopGAP-stimulated GTP hydrolysis by Cdc42 (Figure 3.4). Thus, a possible explanation for the presence of the CRIB motif in RopGAPs is that
the CRIB-containing N-terminal region functions as a Rop effector. Another explanation is that the CRIB motif has a role in RopGAP regulation. Although our current studies could not rule out a possible effector functioning for RopGAP through the CRIB motif, our results provide concrete evidence that the CRIB motif acts as a positive regulator of GAP activity in RopGAPs as discussed below.

The CRIB motif defines the specificity of RopGAPs by regulating the activity of the GAP catalytic domain through its interaction with Rop

We have shown that RopGAPs preferentially stimulate GTPase activity on the Rop subgroup of Rho GTPases but only weakly promote GTP hydrolysis catalyzed by Cdc42. These results indicate that RopGAPs are Rop-specific GAPs, similar to several mammalian Rho GAPs, e.g., n-chimearin specifically stimulates GTPase activity of Rac but not Cdc42 or Rho, and p50rhoGAP preferentially activates Cdc42 and TC10 (Lancaster et al., 1994; Leung et al., 1998; Neudauer et al., 1998). The molecular and structural basis for member-specific activation of GTPase for these Cdc42 and Rac GAPs is unknown (Lancaster et al., 1994).

Our studies provide convincing evidence that the CRIB motif plays an essential role for the Rop-specific stimulation of GTP hydrolysis by RopGAPs, because deletion of the CRIB motif or mutations of conserved residues within this motif abolishes or dramatically reduces Rop-specific stimulation of GTPase activity by RopGAPs. For the reasons summarized below, we believe that the elimination or dramatic reduction of Rop-
specific GAP activity in these mutants is not due to instability of the GAP domain or disruption of its active 3-D structure. First, western blot analyses show that all RopGAP1 mutation mutant proteins used for GAP assays, except for the CRIB domain deletion mutant, did not show significant degradation. Although the deletion mutant showed some degradation, it could not have accounted for the drastic reduction of GAP activity. Furthermore, the CRIB deletion mutant had a greater GAP activity on Cdc42 than the full-length RopGAPs, demonstrating that this mutant retains the GAP catalytic activity. Finally, compared to the point mutations within the CRIB motif, the removal of the N-terminal region containing this motif caused similar effects on GAP activity although the effect was somewhat weaker. This suggests that the 3-D structure of the GAP domain in RopGAPs is independent of the N-terminal region. That the modification of RopGAP activity by a CRIB-dependent mechanism does not involve altering the structure of the GAP catalytic domain is consistent with the fact that the CRIB domain directly binds different forms of Rop GTPases to alter the interaction of Rop with RopGAPs as discussed below. Because the CRIB-containing domain has no GTPase-stimulating activity on its own, we conclude that the CRIB motif functions to regulate the activity of the GAP catalytic domain. It is possible that additional sequences in the N-terminal region of RopGAPs are also involved in the regulation of the GAP activity.
CRIB motif allows high affinity binding of RopGAPs to Rop and facilitates the stabilization of the transitional state of Rop GTPases

How does the CRIB motif mediate the Rop-specific activation of RopGAPs? One role for the CRIB motif in RopGAPs is to enhance their affinity for the GTP-bound form of Rop, as suggested by our results showing that both CRIB deletion and point mutations dramatically reduce the interaction of RopGAPs with the GTP-bound Rop (see Figs. 3.2C and 3.3C). The reduced interaction of various RopGAP mutants with the active Rop is tightly associated with their reduced GAP activity on Rop, suggesting that the CRIB-mediated RopGAP-Rop interaction may be critical for the regulation of RopGAP activity. Furthermore, with the aid of the CRIB motif, RopGAPs would have a much greater capacity to compete with Rop effectors for binding to the Rop effector domain. It is known that Rho GAPs and effectors both bind the effector domain of Rho GTPases, and that Rho effectors are known to inhibit Rho GTPase activity (Manser et al., 1994; Zhang et al., 1997). Thus, the CRIB motif in RopGAPs is expected to be critical for an effective in vivo de-activation of Rop.

How can the CRIB motif-dependent interaction of RopGAPs with Rop contribute to the regulation of RopGAP activity? Our results strongly support the hypothesis that the CRIB domain regulates GTPase activity of RopGAPs via its interaction with the transitional state of Rop GTPase during GTP hydrolysis. This was first hinted by two observations: 1) that the CRIB domain interacts much more strongly with wild type Rop1At, which likely exists in different (GTP- or GDP-bound, and transitional) forms,
than with either constitutively active or dominant negative rop1At mutant; 2) that the CRIB domain interacts with both constitutively active and dominant negative rop1At mutants, whereas the GAP domain specifically interact with the constitutively active rop1At. More importantly, we showed that the binding of the CRIB domain to the GDP-bound Rop is dramatically enhanced by aluminum fluoride. Aluminum fluoride is known to enhance the binding of the GAP catalytic domain to GDP-bound Rho GTPases by mimicking the GTPase transisitional state (Vincent et al., 1998). Based on the crystal structure of the complex of the aluminum fluoride-mimicked GTPase transitional state and the GAP domain, it is proposed that Rho GAPs stimulates GTPase activity through stabilizing the transitional state of GTPases (Vincent et al., 1998). The interaction of the CRIB domain with the Rop transitional state is further supported by the ability of the GAP domain to compete with the CRIB domain for their interaction with the GDP-bound Rop in the presence of aluminum fluoride (Figure 3.5). Interestingly, the CRIB and GAP domains of RopGAPs bind the GDP-bound rop1At synergistically in the absence of aluminum fluoride. These results imply that the CRIB domain either promotes the formation of the transitional state, which is then stabilized by the GAP domain, or facilitate the GAP-mediated stabilization of the transitional state. Thus, it is conceivable that the CRIB motif could regulate the GAP activity via either of these two mechanisms. Resolution of the three-dimensional structure of RopGAPs and elucidation of the mechanism for the CRIB-Rop interaction should further our understanding of the mechanism by which the CRIB motif regulates RopGAP activity.
Materials and Methods

Materials and chemicals

The yeast two-hybrid screen system including an Arabidopsis seedling cDNA library constructed in the prey vector pACT, the bait vector pAS2, and the yeast strain Y190 was obtained from The Ohio State University Arabidopsis Biological Resources Center (Columbus). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coraville, IA). DNA sequencing was performed on an automated sequencer (ABI model 373A). All chemicals, unless specified, were purchased from Sigma (St. Louis, MO).

Construction of constitutively active and dominant negative Rop1At mutants

Dominant rop1At mutant genes were generated by site-directed mutagenesis as described previously (Li et al., 1999). Briefly, a NcoI site was created at the translation initiation codon of the Rop1At coding sequence (Li et al., 1998) using polymerase chain reaction (PCR). The modified coding sequence was then cloned into pSelect for site-directed mutagenesis to create constitutively active (G15V) and dominant negative (D121A) mutations, respectively. An isoprenylation-defective mutation (C188S) was subsequently introduced into each of the dominant mutants by PCR. These
rop1At mutants were used for the two-hybrid screen or in vitro-binding assays described below.

**Two-hybrid screen**

The constitutively active rop1At mutant (G15V/C188S) was fused into the C-terminus of GAL4 DNA-binding domain encoded in pAS2. The resulting construct pDP1S was used as a "bait" to screen the Arabidopsis seedling library (Kim et al., 1997). The yeast strain Y190 containing pDP1S was transformed with the library plasmid DNA (Ausubel et al., 1998). The transformants were selected on a yeast drop-out (-his-trp-leu) synthetic dextrose medium supplemented with 100 mM 3-aminotriazole. After incubation at 30 C for 1-2 weeks, the plates were used for the β-galactosidase filter assay to detect positive clones (Breeden and Nasmyth, 1985). The plasmids from putative positive clones were first rescued in *E. coli* and then reintroduced into the yeast strain containing pDP1S to confirm the interaction. The confirmed clones were then sequenced, and the sequences were used for the Blast search of the GenBank or the Arabidopsis database.

**Site-directed mutagenesis of RopGAP1**

To create point mutations on RopGAP1, we used PCR-based site-directed mutagenesis. The MalE primer (5'-ggtcgctcagagtctggtgctgatgagcc) and a mutant primer
containing H125Y mutation (5'-gatacgtagcgacgtcttaattcagc) were used to amplify a mutant
RopGAP1 fragments from pMAlc2-RopGAP1. This mutation also creates a SnaB1 site
in the fragment. The PCR fragment was subcloned into BamH1 (a vector cloning site)
and PmlI (compatible with the SnaB1 site) to replace the corresponding wild type N-
terminal 89 residues. (Other mutations including H125YH128R, P120G&H125Y,
P120G&H125Y&T130G and P120G&H125Y&H128R were similarly generated. The
effect of these mutations on both RopGAP activity and interaction of Rop is very similar
to that of the single site mutation (H125Y) and thus is not described in this report.

Fusion protein preparations

The constitutively active mutant, dominant negative mutant, or wild type Rop1At
gene was cloned in frame with the glutathione S-transferase (GST) gene in pGEX-KG
using NcoI and SstI sites (Guan and Dixon, 1991). GST-Cdc42 fusion is a gift from Y.
Zheng (Li et al., 1997). GST fusion proteins were purified using glutathione-agarose
beads. Briefly, E. coli cells were lysed by sonication in a Hepes buffer (50 mM Hepes,
pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DDT, 200 mM PMSF, 0.1% β-
mercaptoethanol, 0.1% Triton -100). Following centrifugation (10,000g, 10 min, 4 C),
the supernatants of lysates were mixed with glutathione-agarose beads, which were
washed 5 times with 10 ml Hepes buffer. Fusion proteins were eluted with 10 mM
glutathione solution in the Hepes buffer. Protein concentrations were measured by using
Dot Metric protein detection kit (Geno Technology, Inc.). Purified proteins were routinely stored in 40% glycerol at -20 C.

The full-length RopGAP1 coding sequence and various deletion mutants (see Figure 3.2A) were fused in frame with the maltose binding protein (MBP) gene in pMAL-c2 vector (New England Biolab). MBP fusion proteins were expressed in *E. coli* and purified using a similar procedure described above for the GST fusion proteins except that the amylose resin (50%, V/V) was used instead of glutathione agarose beads. The MBP fusion proteins were eluted with a 10 mM maltose solution in the Hepes buffer.

**GTPase activity assay by spectroscopy**

GTPase activity assays were performed by using Enzcheck™ Phosphate Assay Kit (E-6646, Molecular Probes) to monitor the rate of the phosphate release from GTP bound to the GST-Ropi At fusion protein. For these assays, we used a previously described protocol with minor modification (Li et al., 1997). Briefly, 1 nmole of purified GST-Ropi At fusion protein in a volume of 15 μl was mixed in an one-ml crystal cuvette with 10 μl of 0.2 mM GTP, 0.2 ml of 2-amino-6-mercapto-7-methylpurine ribonucleoside, and 10 μl (1 unit) of purine nucleotide phosphorylase, and 0.78 ml of Hepes buffer (pH 7.5). The cuvette was immediately placed in the spectrophotometer (AT UNICAM UV/VIS Spectrometer UV4) to monitor absorbance at 360 nm. When the multiple turnover reached an equilibrium, 5 μl of 1 M MgCl₂ solution containing MBP-RopGAP fusion proteins were added to initiate the single turnover reaction. The
absorbance at 360 nm was recorded every 5 min. The data were standardized to the same starting point.

**In vitro protein-protein interaction assays**

For in vitro binding assays, approximately 10 µg of GST-Rop1At fusion proteins in the glutathione agarose beads were mixed with a binding buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DDT, 10 mg/ml BSA and 5 mM EDTA) containing 3 mM GTP or GDP. Following 30-min incubation with shaking at 30 C, the beads were aliquoted into 4 parts. Each part was mixed with 2 µg of each of the 4 different GAP-MBP fusions (Figure 3.2A) in the binding buffer Figure 3.1D). The beads were washed with the binding buffer without BSA for 4 times. The proteins associated with the agarose beads were resuspended in 10 µl of SDS-PAGE loading buffer, separated on a 10% PAGE/SDS gel by electrophoresis, and transferred to nitrocellulose membranes (Schleicher & Schuell). The MBP-fusion proteins were then detected using a polyclonal antibody against MBP (New England Biolab) and the BM Chemiluminescence Western Blot Kit (Boehringer Mannheim).
CHAPTER 4

ROP GAP1 IS LOCALIZED TO THE TIP OF POLLEN TUBES AND ACTS AS A NEGATIVE REGULATOR OF ROP1 SIGNALING IN THE CONTROL OF POLLEN TUBE GROWTH

Abstract

Switching between "On" and "Off" is the essence for the functions of Rho family GTPases. The factors that turn "off" the switch are the Rho GAPs. Arabidopsis possess a novel family of Rho GAPs, termed RopGAPs, which uniquely contain a CRIB (Cdc42/Rac interactive binding) motif. RopGAP1, a member of this family, is expressed in pollen. I showed that GFP-RopGAP1 strongly localized to the apical region of the plasma membrane (PM) in tobacco pollen tubes as a tip high gradient. I further demonstrated that RopGAP1 reduced Rop activity, inhibited the pollen tube growth, enhanced pollen tube polarity and rescued Rop1-induced pollen tube depolarized growth, suggesting RopGAP1 is a negative regulator of Rop in vivo. These effects, however, were abolished by the R202L-mutation in the GAP catalytic domain. I further showed that R202L mutant had a better PM localization wild type RopGAP1 in the pollen tubes. These results indicate that R202L mutant of RopGAP1 is a dominant negative mutant in
vivo, which is distinct from the similar mutation in other Rho GAPs since in the other Rho GAPs, the Arg mutation leads to the loss of the activity, and function of those Rho GAPs. Interestingly, these effects were also abrogated by the expression of several mutants in which the CRIB motif is mutated. These results suggest a critical role for the CRIB motif in the regulation of Rop activity. In agreement with this hypothesis, CRIB-motif mutant (101-143) and H125Y of RopGAP1 was unable to localize to the PM in wild type pollen tubes. Co-expression of Rop1 restored the PM localization of (101-143)RopGAP1 and H125Y-RopGAP1 mutants, suggesting a weak PM association in wild type pollen tubes, similar to most other Rho GAPs in animals and fungi. However, the PM localization for these mutants in Rop1 overexpression pollen tubes was abolished when RIC1 was coexpressed, a Rop-interacting protein containing a CRIB motif. In contrast, in pollen tubes, RIC1 did not compete with RopGAP1 as indicated by GFP-RopGAP1 PM localization. Interestingly, our in vitro studies show that RIC1 did eliminate the interaction of RopGAP1 with active Rop1 as CRIB containing pros described above. Rho GTPase. Taken together, our results suggest that a novel CRIB dependent mechanism provides a tight regulation to the activity of Rop by RopGAP1 in the control of tip growth in pollen tubes.

Introduction

Rho family GTPases play a critical role in a diverse array of biological processes,
including defense responses, hormone responses and cell polarity control (Li et al., 2001; Drubin and Nelson, 1996; Van Aelst and D'Souza Schorey, 1997). Rho, Cdc42, Rac and Rop are four subfamilies of Rho family GTPases (Li et al., 1998; Zheng and Yang, 2000b). Emerging evidence indicates that Rops are involved in the tip growth of pollen tubes and root hairs through the regulation of calcium gradient and actin dynamic (Fu et al., 2001; Li et al., 1999b; Molendijk et al., 2001). However, how Rop is regulated during tip growth is unclear (Zheng and Yang, 2000a; Zheng and Yang, 2000b).

The regulation of the cycling between active GTP-bound and inactive GDP-bound of guanine nucleotide binding proteins (G protein) is crucial for Rho family GTPase signaling. The conversion of the inactive to the active form is controlled by GEFs (guanine nucleotide exchange factor), while the active form returns to the inactive form through GTP hydrolysis. The GTP hydrolysis is stimulated by GAPs. The inactive GTPases then dissociate from the plasma membrane (PM) by a mechanism involving a guanine nucleotide dissociation inhibitor (GDI), while the association with the cell membrane by isoprenylation is a prerequisite for the activation of Rho GTPases (Mackay and Hall, 1998). The activity of the Rho family GTPases is therefore regulated by the combinatorial effect of Rho GEFs, Rho GAPs and Rho GDIs (Gulli and Peter, 2001).

In animals and yeast, more than a dozen proteins show GAP activity for specific members of the Rho family (Schmidt and Hall, 1998). Rho GAPs contain a catalytic GAP domain with an invariant Arg residue that is required for GAP activity (Lamarche and Hall, 1994; Li et al., 1997). However, mutations of Arg to Leu or Met do not affect the interaction of Rho GAPs with the corresponding Rho family GTPases (Geiszt et al.,
2001; Li et al., 1997). Besides the GAP domain, different Rho GAPs may contain other functional regions. For example, Cdc42-GAP contains a Pro-rich domain that may bind to actin binding proteins; p122 GAP contains a Ser-rich region that binds to PLCδ; myr-5 contains domains that include a myosin head, an IQ motif and a Zinc finger motif (Homma and Emori, 1995; Sekimata et al., 1999); p190 Rho GAP also contains a GTPase domain, and a SH2 binding domain (Brouns et al., 2001; Fincham et al., 1999; Settleman et al., 1992). In plants, a unique family of Rho GAP identified for Rop is RopGAP (Borg et al., 1999; Wu et al., 2000). Interestingly, apart from the normal Rho GAP domain, there is a Cdc42/Rac interactive binding (CRIB) motif which is present in the putative effectors of Cdc42 / Rac or Rop (Borg et al., 1999; Wu et al., 2000). The variable N and C termini encompass the conserved regions that consist of the CRIB motif and the GAP domain, connected by a conserved SH3 binding domain (Wu et al., 2000). RopGAPs are particularly conserved in the plant kingdom. So far, there are 5 members of RopGAPs in Arabidopsis and three members in Lotus reported. Many EST clones are found in soybean, rice and other plant species. It has been shown that Lotus RopGAPs have specific activity for lotus Rops (Borg et al., 1999). Our previous in vitro studies show that RopGAP1 requires the CRIB motif for its specific GAP activity for Rop1 (Wu et al., 2000). Furthermore, we previously showed that Arabidopsis RopGAP1 specifically interacted with GTP-bound active Rop1, as well as the mimic transitional-state of Rop1 induced by AlF4 in vitro (Wu et al., 2000).

The distinct structure of the different Rho GAPs suggests that Rho GAPs are functionally divergent and may have different mechanisms for the regulation of their
activity (Harada et al., 2000; RIChnau and Aspenstrom, 2001). It has been reported that other regions, besides the GAP domain, may regulate the specific function of Rho GAPs. In p190 Rho GAP, the SH3 binding domain binds to an actin-binding protein, which in turn regulates the GAP activity of p190 Rho GAP (Brouns et al., 2001; Chang et al., 1995; Fincham et al., 1999; Roof et al., 1998). Interestingly, microinjection of the GAP domain from N-chimerin represses the Rac and Cdc 42 induced lamellipodia and filopodia while either full-length N- chimerin or chimerin mutant lacking GAP activity induces lamellipodia and filopodia, suggesting that Rho GAPs have an effector function (Kozma et al., 1996). In most cases, the GAP domain is as active as their wild type full-length Rho GAPs are, i.e. mry-5, Cdc42 GAP (Leonard et al., 1998; Muller et al., 1997; Nassar et al., 1998; Zhang et al., 1997; Zhang and Zheng, 1998). Interestingly, our in vitro study shows that a CRIB-deficient mutant of RopGAP1 has a 15-fold less GAP activity than that of wild type RopGAP1 on Rop1, but has a similar activity on both Rop1 and Cdc42, indicating that the CRIB motif is critical in the regulation of the specific activity of RopGAP1 for Rop1 through its interaction with Rop1 (Wu et al., 2000). However, the role of the CRIB motif in the regulation of the activity of RopGAP1 for Rop1 in vivo is unknown. Rho GAP activity may also be regulated by the Rho family GTPase effectors since the binding of the effectors of Rho GTPases to the corresponding GTPases generally reduce the hydrolysis of GTP to GDP of the GTPases (Zhang et al., 1998). It has been proposed that Rho GAPs bind to the transitional state of Rho GTPases, while CRIB- containing effectors bind to the effector loop (Hoffman et al., 1998). PAK (p21 activated kinase), a typical PBD (PAK binding domain)/CRIB motif
containing effector for Cdc42/Rac, regulates actin reorganization in animals (Holly and Blumer, 1999). PBD and IQGAP1 inhibit the intrinsic GTPase activity as well as the Rho GAP-enhanced GTPase activity of Cdc42 but binding to different region in the effector domain (Li et al., 1999c; Zhang et al., 1998; Zhang et al., 1997). However, IQGAP1 which has an even higher affinity than PBD has for Cdc42 does not compete with Cdc42 GAP while PBD does (Leonard et al., 1997). Interestingly, PBD competes with IQGAPs (McCallum et al., 1996).

Plants possess a group of CRIB-containing putative Rop effectors, the RIC (Rop interacting proteins containing CRIB) proteins. RIC1, which is expressed in mature pollen, interacts specifically with active Rop1, and has been used as a marker for the localization of Rop activity (Wu et al., 2002a; Wu et al., 2001). To gain insights into the function of Rho GAPs in the regulation of Rho GTPases in vivo, I investigated the function of RopGAP1 in the control of Rop activity and pollen tube growth. Using GFP-tagged RopGAP1, I showed for the first time that a Rho GAP is polarly localized to the apical PM region in tobacco pollen tubes. RopGAP1 overexpression inhibited tip growth and suppressed Rop1-induced depolarized growth in pollen tubes, suggesting that RopGAP1 acts as a negative regulator of tip-localized Rop1 in the control of pollen tube growth. More importantly, the PM localization and the activity of RopGAP1 were dependent on its unique CRIB motif but were not competed by another CRIB containing protein RIC1, indicating a novel regulation of RopGAPs for the unique Rop GTPases in plants.
Results

RopGAP1 is expressed in pollen and is localized to the apical region of the pollen tube plasma membrane

My previous in vitro study shows that RopGAP1 promotes the GTPase activity of the pollen-specific Rop1 from Arabidopsis (Wu et al., 2000). As a first step in assessing whether RopGAP1 is a negative regulator of Rop1 in vivo, I investigated whether RopGAP1 is expressed in pollen. Using RT-PCR with mRNA from mature Arabidopsis pollen, RopGAP1 was found indeed to be expressed in mature pollen (Figure 4.1).

Because Rop1 has been shown to polarly localize to the site of growth in pollen, I investigated whether RopGAP1 localized to the tip of the pollen tubes as well. Using GFP-tagging, RopGAP1 was found to be strongly localized to the apical PM when this construct was expressed in the growing tobacco pollen tubes (Figure 4.3A) (Kost et al., 1999; Li et al., 1999b; Lin et al., 1996). As shown in the Figure 4.3A, the PM localization RopGAP1 has a tip-high gradient in pollen tubes, similar to the localization GFP-Rop1 and GFP-RIC1 (Wu et al., 2001). Interestingly, GFP-RopGAP1 was also able to localize to the germinal pore from which a pollen tube might emerge (Figure 4.3B),
Figure 4.1. RopGAP1 transcripts are expressed in Arabidopsis mature pollen. Total RNA was isolated from pollen as described in methods. Reverse transcription and polymerase chain reaction were also performed as described in methods. **Left lane** is positive control for the full-length *RIC1* with the same pair of primers as for RIC1 cloning described in the methods (Table 4.3); **Middle lane** is *RopGAP1* (1011-1353) with primers (MWD912R and 16-1011F) (Table 4.3); and **right lane** is the negative control (*RopGAP2*).

suggesting that RopGAP1 may play a role in pollen germination. Consistent with this is that we observed the reduction of germination rate for transgenic Arabidopsis pollen overexpressing RopGAP1 (data not shown). Interestingly, the reduction of germination rate for transgenic Arabidopsis pollen expressing dominant negative (DN) rop1 mutant was also observed (Li and Yang, personal communication). In about 20 lines of the transgenic plants with homozygous *RopGAP1* and heterozygous GFP-DN-rop1 examined, all pollen grains with GFP florescence did not germinate while the ones without did (Wu, Vernoud and Yang, unpublished data). In contrast, weak overexpression of Rop1 in the Arabidopsis pollen promoted pollen germination (Wu, Vernoud, Li and Yang, unpublished data).
Figure 4.2. Diagrams for wild type RopGAP1 and its mutant constructs that were expressed in the pollen tubes. Numbers above each construct indicate the position of the amino acid residues. The names of the constructs were listed on the left side of the diagrams. The different domains were indicated inside the blocks that represent the coding sequences of RopGAP1. The names with "\" in front of numbers indicate that those amino acid residues were deleted, and the deleted sequences of RopGAP1 were indicated by the straight lines in the diagram. Point mutation was highlighted with arrows and the point that was mutated was indicated on top of the arrow. H125Y is the His to Tyr mutation in the CRIB motif. R202L is the Arg to Leu mutation in the GAP domain. Diagrams were not drawn on scale. The block shape diagrams represent the coding sequences.
Figure 4.3. The localization of RopGAP1 to the plasma membrane (PM) of the growing site. All constructs were constructed under the Lat52 promoter and transiently expressed in the tobacco wild type pollen tubes as described in methods. A) GFP-RopGAP1 in a pollen tube. B) GFP-RopGAP1 in a germinating pollen C) GFP only. Bar=10μm.

The localization of RopGAP1 is Rop-dependent

Because both RopGAP1 and Rop1 localized to the apical PM of the pollen tubes as a tip high gradient (Figure 4.3A) (Lin et al., 1996; Wu et al., 2002a), I next assessed whether the PM localizations of both RopGAP1 and Rop1 were dependent or
Figure 4.4. The localization of RopGAP1 to the plasma membrane (PM) is Rop1 dependent. All the constructs were constructed under the Lat52 promoter and transiently expressed in the tobacco wild type pollen tubes as described in the method. Upper panel, GFP-RopGAP1 was expressed wild type pollen tubes. Lower panel, GFP-RopGAP1 was coexpressed Rop1. Left, with AtGDI1. Right, vector plasmid. Bar=10μm.

independent of each other. To address this question, I altered the localization of Rop1 in the pollen tubes by using AtGDI1 that can remove the Rop1 from the PM into the cytosol (Isomura et al., 1990). As expected, GFP-RopGAP1 was not detected on the PM when AtGDI1 was co-overexpressed, suggesting that the PM localization of Rop1 is the prerequisite of the PM localization of RopGAP1 (Figure 4.4). To test whether RopGAP1
is in the Rop1 pathway, we co-overexpressed Rop1 with GFP-RopGAP1. Indeed, the PM localization of GFP-RopGAP1 was increased by Rop1 overexpression (Figure 4.4). In fact, PM localization of GFP-RopGAP1 was not only to the apical PM, but also to the expanded subapical PM as PM localized Rop1 and the active Rop1 in Rop1 overexpression pollen tubes (Figure 4.4) (Li et al., 1999a; Wu et al., 2002a). Furthermore, addition of AtGDI1 to the Rop1 overexpression pollen tubes reduced the expanded PM localization of GFP-RopGAP1 (Figure 4.4). These results indicate that the localization of RopGAP1 is dependent on the localization of Rop1. Since our previous study showed that RopGAP1 interacted with Rop1 in vitro and had preferential activity for Rop1, I concluded that the Rop1 increased localization of RopGAP1 to the PM is due to the interaction of RopGAP1 with Rop1 in vivo (Wu et al., 2000). This conclusion is further supported by the reduced PM localization of RopGAP1 mutants with the deletion of either the CRIB motif or the GAP domain or both [(101-143)RopGAP1, (166-340)RopGAP1, (101-340)RopGAP1], both of which are critical for the interaction of RopGAP1 with Rop1 as shown below.

Strong PM localization of RopGAP1 requires a CRIB motif

In contrast to RopGAP1, other Rho GAPs poorly localized to the PM and their PM localization could only be detected using highly sensitive membrane fraction methods (Geiszt et al., 2001). Because RopGAPs uniquely contain the CRIB motif critical for the interaction between RopGAP1 and Rop1 in vitro, we reasoned that the
Figure 4.5. CRIB motif mutation reduced strong PM localization of RopGAP1. All the constructs were under the control of Lat52 promoter and transiently expressed in the tobacco wild type pollen tubes as described in the methods. Upper panel, GFP fusion constructs were expressed in wild type pollen tubes, the mutation of CRIB motif abolished the localization of RopGAP1 in the wild type pollen tubes. Lower panel, GFP-fusion constructs were coexpressed with Rop1. Rop1 restored the localization of mutants with CRIB motif mutation. Left, GFP-RopGAP1; Middle, GFP-RopGAP1(H125Y); Right, GFP-(166-340)RopGAP1. Bar=10μm.

strong PM localization of RopGAP1 is mainly due to the enhanced interaction of RopGAP1 with Rop1 via the CRIB motif. In order to address this question, we expressed
a GFP-tagged mutant (H125Y-RopGAP1) in wild type tobacco pollen tubes. Indeed, this mutant reduced the PM localization of RopGAP1 to an undetectable level in wild type pollen tubes (Figure 4.5). The question is whether these mutants could not localize to the PM or they have weak PM association. Since the localization of RopGAP1 was enhanced when Rop1 was co-expressed (Figure 4.4 and Figure 4.5), we investigated whether Rop1 could restore the localization of the H125Y-RopGAP1 mutant. In fact, in pollen tubes overexpressing Rop1, the H125Y-RopGAP1 mutant was localized to the PM, suggesting that even in the absence of the CRIB motif, RopGAP1 is capable of associating with the PM probably via its weak interaction with Rop1 through its GAP domain (Wu et al, 2000). Similar result was obtained using the CRIB domain deletion mutant [GFP- (101-143)RopGAP1] (Figure 4.7 and unpublished data). Because H125Y mutant of RopGAP1 reduced the affinity and activity of RopGAP1 for Rop1 in our previous in vitro assays, we conclude that CRIB motif is responsible for the strong PM localization of RopGAP1 through its interaction with Rop1. However, the localization of GAP domain alone [GFP-(166-340)RopGAP1] was abolished in the Rop1 overexpression pollen tubes, suggesting N and C termini may have a certain role in the regulation of the localization of RopGAP1. Interestingly, the N terminal deletion [GFP-(1-101) RopGAP1] localized to the PM similar to wild type RopGAP1 in Rop1 overexpression pollen tubes and wild type pollen tubes (Data not shown). Further studies are needed to define the additional structural requirement for the interaction of RopGAP1 with Rop1.
RIC1 expression did not alter RopGAP1 localization

Effectors usually bind to the effector loop of Rho GTPases and reduce the Rho GAP activity for Rho GTPases. PAK and IQGAP are two members of Cdc42 and Rac.

Figure 4.6. RIC1 competed with RopGAP1 for binding to Rop1 in the in vitro binding assay. The competition for the binding of RIC1 and RopGAP1 was performed using a pull-down assay with GST-CA-rop1, and MBP-RIC1 and MBP-RopGAP1. **Left lane**, MBP-RopGAP1 with GST-CA-rop1; **Middle lane**, MBP-RIC1 and MBP-RopGAP1 with GST-CA-rop1; **Right lane**, MBP-RIC1 alone with GST-CA-rop1. Protein purification and the pull-down assay were performed as described in the method and previously (Wu et al., 2000). MBP fusion proteins were pulled down by GST-CA-rop1 fusion protein that was first conjugated to glutathione-conjugated agarose. Anti-MBP antisera were then used to detect the MBP fusion protein using the Western blot.
Figure 4.7. RopGAP1 coexisted with RIC1 on Rop in vivo. All the constructs were constructed under the Lat52 promoter and transiently expressed in the tobacco wild type pollen tubes as described in methods. 0.4µg of Rop1 plasmid, and 0.8µg RIC1 and 0.8µg GFP-RopGAP1 were used in the bombardment. Upper panel, GFP fusion constructs were coexpressed with Rop1 only. Lower panel, GFP-fusion constructs were coexpressed with Rop1 and RIC1. Left, GFP-RopGAP1; Middle, GFP-(166-340)RopGAP1; Right, GFP-(101-143)RopGAP1. Bar=10µm.

effectors which competed with each other for the corresponding GTPases in the in vitro binding assays (McCallum et al., 1996; Zhang et al., 1998). However, only PAK but not
IQGAP competes with a Cdc42 GAP (Zhang et al., 1998). Our previous studies show that RICs are a group of CRIB-domain containing proteins that may function as the effectors of Rops (Wu et al., 2001). I first tested whether RICs competed with RopGAPs in vitro. As expected, an equal molar amount of MBP-RIC1 abolished the binding of MBP-RopGAP1 to the constitutively active GST-Rop1, suggesting RIC1 has higher affinity than RopGAP1 for Rop1 (Figure 4.6). I next wanted to investigate whether they competed with each other in vivo. To our surprise, RIC1 overexpression did not affect the localization of RopGAP1 in Rop1-overexpressing pollen tubes (Figure 4.7), suggesting that RIC1 does not compete for RopGAP1 in vivo or RopGAP1 has higher affinity than RIC1 for Rop1 in vivo. Furthermore, RopGAP1 together with RIC1 suppressed the Rop1 induced depolarized growth better than either RIC1 or RopGAP1 alone, further suggesting that RopGAP1 and RIC1 are acting independently on Rop1 (Figure 4.7 and data not shown).

How can these apparently contradictory in vivo and in vitro results be explained? Since RopGAPs distinguish themselves from other Rho GAPs by having a CRIB motif, we tested whether the deletion of such a domain would alter the competition between RopGAP1 and RIC1. Indeed, RIC1 overexpression abolished the PM localization of the CRIB deletion p(101-143)RopGAP1 in the Rop1 overexpressing pollen tubes (Figure 4.7). Furthermore, the PM localization of the GAP-domain deletion in the Rop1-overexpressing pollen tubes was also eliminated by the addition of an equal amount of RIC1. Thus, RIC1 apparently is able to compete with the CRIB motif or the GAP
domain of RopGAP1 alone. These results may also imply that pollen tubes contain a factor that directly or indirectly prevent RIC1 from competing with RopGAP1. -

**RopGAP1 overexpression inhibited pollen tube growth and suppressed Rop1-induced depolarization of pollen tubes**

Our previous studies suggest that Rop1-overexpression promotes pollen tube growth (Li et al., 1999b; Wu et al., 2002a). To further understand the role of RopGAP1 in Rop1 signaling and in the control of pollen tube growth, we also overexpressed RopGAP1 in pollen tubes. As expected, pollen tube growth was inhibited both in transgenic Arabidopsis plants overexpressing RopGAP1 under the pollen-specific LAT52

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Length (mean±SD, μm)</th>
<th>Width (mean±SD, μm)</th>
</tr>
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<tbody>
<tr>
<td>GFP+Vector</td>
<td>222±97 (n*=116)</td>
<td>11±2</td>
</tr>
<tr>
<td>GFP+RopGAP1</td>
<td>203±98 (n=68)</td>
<td>8±1</td>
</tr>
<tr>
<td>GFP+R202L-RopGAP1</td>
<td>306±126 (n=42)</td>
<td>11±2</td>
</tr>
<tr>
<td>GFP+Rop1</td>
<td>204±121 (n=40)</td>
<td>21±5</td>
</tr>
<tr>
<td>GFP+Rop1+RopGAP1</td>
<td>246±106 (n=53)</td>
<td>14±2</td>
</tr>
</tbody>
</table>

Table 4.1 RopGAP1 inhibited wild type pollen tube growth and suppressed the Rop1 induced depolarized growth.
*n is the number of pollen tubes measured.
Table 4.2. RopGAPl but not its deletion mutants of CRIB motif and/or GAP domain inhibited pollen tube growth in transgenic Arabidopsis.

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>RopGAPl (101-340)</th>
<th>(101-143)</th>
<th>(166-340)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mean±SD)</td>
<td>(mean±SD)</td>
<td>(mean±SD)</td>
<td>(mean±SD)</td>
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<td>µm</td>
<td>µm</td>
<td>µm</td>
<td>µm</td>
</tr>
<tr>
<td>216±93</td>
<td>90±78</td>
<td>247±116</td>
<td>186±92</td>
</tr>
<tr>
<td>(n=158)</td>
<td>(n=272)</td>
<td>(n=150)</td>
<td>(n=117)</td>
</tr>
<tr>
<td>d</td>
<td>(n=164)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. RopGAPl but not its deletion mutants of CRIB motif and/or GAP domain inhibited pollen tube growth in transgenic Arabidopsis.

a, Both CRIB motif and GAP domain deletion
b, CRIB motif deletion
c, GAP domain deletion
d, n is the number of pollen tubes measured

promoter and in tobacco pollen tubes transiently expressing RopGAPl (Table 4.1 and Table 4.2). Furthermore, the overexpression of RopGAPl suppressed the depolarized growth induced by Rop1 overexpression (Table 4.1). To test whether the above effects were due to the GAP activity only, R202L-RopGAPl mutant that loses the GAP activity but not affinity to Rop1 was transiently expressed in tobacco pollen tubes (Li et al., 1997). As expected, this mutant did not inhibit pollen tube growth; instead, it slightly promoted the growth of wild type pollen tubes (Table 4.1). In addition, this mutant was not able to rescue the Rop1 induced depolarized growth (data not shown) (Wu et al., 2002a). To further investigate the role of CRIB motif and GAP domain in the regulation of RopGAPl, we studied the transgenic Arabidopsis pollen tubes with different deletion mutants. Consistent with the PM localization, mutants with the deletion of either CRIB motif or GAP domain or both abolished the inhibition effect of RopGAPl in the transgenic Arabidopsis pollen tubes (Table 4.2). All these mutants with the deletion of
either CRIB motif, or GAP domain, or both were not able to rescue the Rop1 induced depolarized growth (data not shown).

R202L mutant of RopGAP1 may function as a dominant negative mutant of RopGAP1 in pollen tubes

As stated above, R202L-RopGAP1 slightly promoted the wild type pollen tube growth similar to the weak Rop1 overexpression phenotype (Table 1) (Wu et al., 2002a). This result is interesting because other Arg to Leu mutation mutants of the invariant Arg residue of the Rho GAPs only lead to the loss of its activity and function (Lamarche and Hall, 1994; Muller et al., 1997). Therefore, we suspected that R202L-RopGAP1 functioned as a dominant negative mutant while Arg mutation mutants in other Rho GAPs functioned as null mutants. If R202L-RopGAP1 acts as a dominant negative mutant, we expected it to compete with WT RopGAP1 for Rop1 and thus to localize to the apical PM region. Indeed, the localization of GFP-R202L-RopGAP1 to the apical PM is better than GFP-RopGAP1 in the wild type pollen tubes (Figure 4.8B&D). The localization of GFP-R202L-RopGAP1 to the apical PM region remained even it was coexpressed with AtGDII while AtGDII abolished the localization of GFP-RopGAP1 to the PM (Figure 4.8 A&C). This result is similar to GFP-RopGAP1 in the Rop1 overexpression pollen tubes with AtGDII or GFP-RopGAP1 in wild type pollen tubes (Figure 4.4C and Figure 4.8B&C). Furthermore, I showed that R202L-RopGAP1 did increase the activity and PM localization of Rop1 (see chapter 5) (Wu et al., 2002a).
Taken together, these results strongly support the notion that R202L-RopGAP1 acts as a dominant negative RopGAP1 and functions similar to the overexpression of Rop1. The

Figure 4.8. R202L-RopGAP1 acted as a dominant negative RopGAP1 in Rop signaling. GFP-R202L-RopGAP1 and GFP-RopGAP1 were both constructed under the Lat52 promoter and transiently expressed in the tobacco pollen tubes. A) GFP-RopGAP1 with AtGDI1. B) GFP-RopGAP1 alone. C) GFP-R202L-RopGAP1 with AtGDI1. C) GFP-R202L-RopGAP1 alone. Bar=10μm.
effect of R202L-RopGAP1 on the localization of RopGAP1 will more directly test this hypothesis.

Discussion

Knowledge of in vivo regulation of the Rho GTPase switch is essential for the understanding of the function of Rho GTPase and the mechanism of the regulation of Rho GTPase (Gulli and Peter, 2001). The Rho GTPases have been shown to be critical for polar growth in several systems (Luo, 2000; Molendijk et al., 2001; Nobes and Hall, 1999; Pruyne and Bretscher, 2000; Zheng and Yang, 2000a). The pollen-specific Rop1 is crucial for pollen tube tip growth (Li et al., 1999a). In vitro studies show that a novel RopGAP1 possesses a preferential activity for Rop1 (Wu et al., 2000). However, the mechanism by which RopGAP1 regulates Rop1 activity in vivo and in the control of the pollen tubes is unknown. In this research, we uncovered a PM-localized Rho GAP, RopGAP1. Using this novel Rho GAP, we were able to dissect the in vivo activity and function of a Rho GAP in detail. Here, we revealed a crucial role of a novel CRIB dependent mechanism in the regulation of the function of RopGAP1 in the control of Rop activity and pollen tube growth.
RopGAP1 is a negative regulator for Rop1

In this study, I have obtained convincing evidence that RopGAP1 acts as a negative regulator of Rop1 in the control of pollen tube tip growth, consistent with my previous in vitro study showing that RopGAP1 is a potent GAP that promotes the GTPase activity of Rop1 (Wu et al., 2000). First of all, I showed that RopGAP1 was localized to the apical PM region as Rop1 was. Second, I demonstrated that the PM localization of RopGAP1 was dependent on Rop1. Because RopGAP1 did not localize to the PM when Rop1 was removed from PM by AtGDI1, and the localization was increased by the overexpression of Rop1. Furthermore, the interaction of RopGAP1 with Rop1 was dependent on CRIB motif and GAP domain. Together, these results suggest that the PM localization of RopGAP1 involves direct interaction with Rop1. Third, the overexpression of RopGAP1 inhibited wild type pollen tubes both in transgenic Arabidopsis plants and tobacco pollen tubes. This effect is similar to that of DN-rop1 or antisense Rop1 expression, but opposite to that of Rop1 overexpression. Fourth, RopGAP1 overexpression suppressed depolarized growth caused by Rop1 overexpression. Furthermore, this effect of RopGAP1 or its PM localization was abolished by deletions on RopGAP1 that either affect the catalytic activity of RopGAP1 or its interaction with Rop1 (Table 4.1).

RopGAP1 overexpression also produced an effect opposite to that of Rop1 overexpression in pollen tubes but its mutants with GAP domain or CRIB motif did not. These results suggest that RopGAP1 may act as a negative regulator of Rop1 signaling by
down-regulating Rop1 activity through the interaction of its CRIB motif and GAP domain with Rop1 in vivo. Indeed, in contrast to the tip-high gradient of GFP-RIC1 alone in pollen tubes, no observable PM localized GFP-RIC1 was detected when GFP-RIC1 was co-expressed with RopGAP1, even though the fluorescence level is high (data not shown) (Wu et al., 2001b). On the other hand, GFP-RIC1 was increased by the addition of Rop1, and this increase was suppressed by the addition of RopGAP1 in the Rop1 overexpression pollen tubes (Wu et al., 2002a). Consistent with the localization and phenotypes of the mutants with either CRIB motif deletion or GAP domain deletion or both deletions, all mutants have little or no effect on Rop activity (data not shown). Taken together, the activity of Rop1 is down-regulated by RopGAP1.

In animals, it has been shown that the overexpression of myr5, a RhoGAP in NRK and HeLa cells inhibited the formation of actin stress fibers and focal contacts by inactivating Rho (Muller et al., 1997). This is analogous to the effect of RopGAP1 overexpression in pollen tubes. A GAP activity deficient mutant (R1695M) did not have any effects when overexpressed, which is in contrast to the R202L-RopGAP1 mutant, whose overexpression even promoted pollen tube growth, similar to the effect of Rop1 overexpression (Table 1) (Muller et al., 1997). There are at least two possible explanations for the effect of this mutant. 1) RopGAP1 has dual functions: GAP for Rop1 and effector for Rop1. Thus, the GAP activity-deficient mutant would act as an effector only. 2) The R202L-RopGAP1 mutant acts as a dominant negative mutant of RopGAP1 by competing with a RopGAP1 interacting protein (either Rop1 or a factor that inactivates the GAP activity of RopGAP1). Several lines of evidence strongly
support the latter explanation. First, the former explanation is not consistent with the localization of the R202L-RopGAP1 mutant tagged with GFP, which was distributed throughout an extensive apical PM region of pollen tubes, but only caused a weak Rop1 overexpression phenotype. Furthermore, GFP-R202L-RopGAP1 still could localize to the PM even coexpressed with AtGDI1 in the pollen tubes (Figure 8). More importantly, the expression of the R202L-RopGAP1 mutant enhanced the activity of Rop1 at the apical PM region (Wu et al., 2002a). Interestingly, a loss-of-function mutant of a Rho GAP in filamentous fungi displayed the loss of polarity at the hyphal tip, similar to the Rop1 overexpression in the pollen tubes (Wendland and Philippsen, 2000). A more definite conclusion about the function of RopGAP1 awaits the isolation of its knockout mutants and the investigation of potential functional redundancy of other RopGAPs with RopGAP1 in the control of the Rop-dependent signaling to pollen tube tip growth.

The CRIB motif is required for the normal function of RopGAP1

Many Rho GAPs contain multiple identifiable functional regions in addition to the catalytic GAP domain (Lamarche and Hall, 1994). Therefore, the functions of Rho GAPs are quite divergent. Overexpression of Rho GAP N-chimerin with or without GAP domain causes the same phenotype, leading to the conclusion that other regions are important for the Rho GAP function and Rho signaling (Kozma et al., 1996). For example, p190 RhoGAP possesses other domains, such as an SH2 binding domain, and a GTPase domain (Brouns et al., 2001; Settleman et al., 1992). Mry5 also has a myosin
domain, a Zinc finger and an actin-binding domain (Muller et al., 1997). Interestingly, the GAP domains from above two Rho GAPs have the similar activity as their corresponding Rho GAPs (Barfod et al., 1993; Muller et al., 1997). RopGAPs are novel plant Rho GAPs which distinguishes themselves from other Rho GAPs by having a CRIB motif (Wu et al., 2000).

In order for Rho GAPs to function, they have to localize to the place where the Rho GTPases are active. Since the localization to the PM is a prerequisite for the function of Rho GAPs, thus Rho GAPs have to be localized to the PM. Indeed, many Rho GAPs localize to the PM but with weak PM localization that was only detected by highly sensitive membrane fractionation methods (Geiszt et al., 2001). In fact, no live labeling of Rho GAPs to the PM has been shown (Geiszt et al., 2001). Here, using live GFP marker, not only we showed that a plant Rho GAP, RopGAP1, localized to the PM but also with a strong tip-high PM localization in the pollen tubes. Since plant RopGAPs contain a CRIB motif while others do not, a simple explanation is that CRIB motif makes the distinct differences between the RopGAP1 and other Rho GAPs. If that is true, then the RopGAP1 without the CRIB may function similar to other Rho GAPs. Indeed, the CRIB deletion mutant acts similar to other Rho GAP domains or other full-length Rho GAPs in some aspects: First, a CRIB-motif deletion mutant is localized to the PM in a way similar to other Rho GAPs (Figure 4.5&4.7). Second, CRIB containing protein RIC1 competes with the PM localization of CRIB deletion mutant in a way also similar to PAK with GAP domain from Cdc42 GAP (Figure 4.7). Third, a CRIB-motif deletion mutant has a capacity of increasing the GTP hydrolysis for several folds for both Rop1
and Cdc42 from yeast (Wu et al., 2000). This level of capacity is similar to many other Rho GAPs (Lamarche and Hall, 1994; Lancaster et al., 1994). However, the mutant with CRIB motif deletion had much less (15 fold less) than wild type RopGAP1 for Rop1 and was ineffective to inhibit the pollen tube growth and reduced the activity of Rop1 in vivo (Wu et al., 2000). Consistent with the reduction of the PM localization and activity of the CRIB deletion mutant, this mutant did not inhibit the pollen tube growth when it was expressed in the transgenic Arabidopsis and transiently expressed in pollen tobacco pollen tubes (Table 2) (Wu et al., 2002a). Taken together, CRIB is critical for its normal activity of RopGAP1 for Rop1.

Since the many known CRIB containing proteins such as PAK reduce the Rho GAP activity, this enhancement of the GAP activity of RopGAP1 by its CRIB motif is interesting. However, the CRIB motif from RopGAPs does have some unique features: First, it has a synergetic effect with CRIB minus mutant when both added to the transitional state Rop1 (Wu et al., 2000). Second, it binds to the transitional state, but CRIB-containing effectors do not (Wu et al., 2000). Third, it binds not only to both constitutive active Rop1 and dominant negative Rop1 but also strongly binds to the wild type Rop1 (Wu et al., 2000). Fourth, it has a consensus sequence (IxxPxxxxHxxHVT) different from the one in PAK (IxxPxxxxHxxHVG) or RICs (IxxPxxxxHxxHIG). Interestingly, all 6 RopGAPs and all 11 RICs in Arabidopsis have the above conserved sequences respectively, suggesting the CRIB in the RopGAPs is unique, thus may perform distinct functions (Wu et al., 2001b; Wu et al., 2000).
In wild type pollen tubes, CRIB motif in RopGAP1 probably binds to the Rop1 to create or stabilize the transitional state to which RopGAP1 can binds. This idea is supported by the fact that CRIB motif can bind to the transitional state and has a synergetic effect with the RopGAP1 mutant that is with GAP domain but without CRIB motif (Wu et al., 2000). In this way, more stable and strong association of RopGAP1 and Rop1 is established, thus resulting in the better PM localization. Since higher activity for PM Rho GAP than for cytoplasmic Rho GAP has been reported, we proposed that the possession of a CRIB motif in RopGAPs enhanced the PM localization of RopGAPs, thus increasing the activity of RopGAPs for Rops. Thereby, CRIB motif is required for the normal function of RopGAPs. Why Rop1 requires strong down-regulation by RopGAPs is unknown. One possibility is probably the existence of unique effectors such as RICs in the Rop signaling pathway in plants (see discussion below).

RopGAP1 occupies a niche in Rop1 signaling

The paradox for the regulation and the action of small GTPases is that the GDP-bound form is converted to the active GTP form, which then activates one or more effectors prior to being cycled back to the GDP-bound form with the help of a GAP. Thus, both effectors and GAPs bind the active form of GTPases. Therefore, how the effectors and Rho GAPs can accommodate with each other is a very interesting question. Several in vitro studies suggest that animal Ras and Rho GTPases contain a GAP binding site that is identical to or overlapping with the effector-binding sites (Diekmann et al.,
For example, PAK, an effector of Rac/Cdc42 containing CRIB (PBD), competes with Cdc42 GAP (Leonard et al., 1997). In the completed sequence of Arabidopsis, no PAK homolog is found. However, a group of CRIB-containing small molecules (RICs) that only interacted with active Rop1 has been identified (Wu et al., 2001). The in vivo study in tobacco pollen tubes revealed that these RICs perform distinct function, thus are putative effectors of Rops. Like PAKs, which competed with a Cdc42 GAP in the in vitro interaction with Cdc42, RIC1 also competed with RopGAP1 for in vitro binding to the GTP-bound constitutively active Rop1 mutant (Figure 6). If effectors and GAPs compete for the same binding site on GTPases, their interaction with GTPases is expected to be changing and is therefore transient. The fact that Rho GAPs are only weakly associated with PM is consistent with this hypothesis. Surprisingly, our in vivo studies indicate that RopGAP1 is strongly associated the PM in a Rop-dependent manner and that this association is not affected by RIC1 overexpression (Figure 4.7). I have shown that the tight PM association of RopGAP1 is dependent on the CRIB motif that is unique to the plant RopGAPs. This tight PM association may represent a unique regulation of Rops by RopGAPs. It was shown that the PM-localized Rac GAPs have 5-fold higher GAP activity comparing to their cytosolic species (Geiszt et al., 2001). In pollen tubes, the CRIB-dependent PM association of RopGAP1 allows an effective suppression of positive feedback in the Rop signaling to maintain polar tip growth (Wu et al., 2002a).

There are several possibilities regarding the apparently contradictory results between the in vitro to in vivo studies. First, the interaction of RopGAP1 with
constitutively active Rop in vitro may not represent the in vivo situation in which the relationship of the RopGAP1 is established with the wild type RopGAP1. In vivo, the Rop1 can have a transitional state but the CA-Rop1 cannot. Since RopGAP1 prefers to bind to the transitional state but RIC1 might not, thus the RopGAP1 would have the advantage over the RIC1 in the in vivo. Therefore, RopGAP1 cannot be competed out by RIC1. Second, both can bind to the transitional state but RopGAP1 has the advantage. In fact, RIC1 did bind the aluminum fluoride mimic transitional state in vitro (Wu and Yang, unpublished data). Whereas other Rho family effectors do not bind to the transitional state Rho GTPases (Hoffman et al., 1998). Even though we did observe competition between them for transitional state Rop1 but both signals were seen in the pull-down assay in contrast to the results from the competition between RIC1 and Rop1 for CA-rop1 (Figure 4.6 and Wu and Yang, unpublished data). These results strongly imply that RopGAP1 may compete better with RIC1 in the in vivo, probably due to the novel functions and regulations of both RopGAP1 and RIC1 on Rop1 signaling. Third, RIC1 or RopGAP1 may have a distinct confirmation in vivo that allows them to bind two non-overlapping sites on Rop1. Last but not the least, pollen tubes contain another factor affects the in vivo Rop-RopGAP interaction. This possibility is not necessarily mutually exclusive with the third possibility, because this factor may alter RopGAP1 confirmation.

Interestingly, RIC1 is able to compete with either the CRIB or the GAP domain mutant of RopGAP1 to eliminate the PM localization of both mutants in the Rop1 over expressing pollen tubes (Figure 4.7). The fact that RopGAP1 competed with both
mutants both in vivo and in vitro does not support the presence of two different binding sites on Rop that are distinct from the binding site for the full-length RopGAP1 in vivo (Wu and Yang, unpublished data). Furthermore, the positive effect of the C terminus on the localization of the CRIB deletion mutant suggests that the C terminus plays a role in the in vivo regulation of RopGAP1 action on Rop1 (Figure 4.5). Therefore, we are in favor with the hypothesis that a third component binds to the C terminus, which ensures a right conformation of RopGAP1. This conformation of RopGAP1 can be partitioned into binding site after the binding of RICs or other effectors to Rop in vivo. Furthermore, the additive suppression effect of RopGAP1 and RIC1 on Rop1 overexpressed pollen tubes strongly implies that RopGAP1 and RIC1 can act on Rop1 independently. This potential RopGAP-interacting factor may provide a unique mechanism for the regulation of RopGAP1 in vivo. Furthermore, as stated above, it is possible that both RopGAP1 and RIC1 can coexist with the transitional state Rop1. The high resolution of three dimensional structure of the complex with Rop1, RIC1 and RopGAP1 would provide more insight into the regulation of the Rop1 signaling.

Materials and Methods

Reverse transcription and polymerase chain reaction (PCR) for RopGAP1 transcripts

Total RNA was isolated from mature Arabidopsis pollens using TRIZOL Reagent

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following the instructions provided by the manufacture. About 1μg of total RNA was used for each RT reaction in a total volume of 20μl. Then 5 μl of this RT product was then used as template for each PCR reaction. Reverse transcription was performed as described previously (Li et al., 1998). PCR reaction was carried out using the following condition: 94 °C for 4 min, at 55 °C for 1min, and at 72 °C for 90 s for 40 cycles, followed by 7 min extension time and cooled at 4 °C. Five micromilliter of each PCR product was loaded onto each lane of a 1% agarose gel to visualize the cDNA fragment.

DNA manipulation and plasmid constructs

An NcoI site was created into the 5' end of the predicted RopGAP1 coding sequences by PCR amplification using primers MWD912F and MWD912R (Table 4.3) with EST clone 142H15T7 as the template. The PCR product was first cloned into the SmaI site of pBluescript SK+ and generated pBS16. pBS16 was cut by NcoI/Sall and then cloned into pGEX with NcoI/Xhol and obtained clone pG16. The maltose binding protein (MBP) fusion was created by cloning the BamHI/PstI fragment from pG16 into pMAL-C2 vector. For RIC1 construct, product from PCR amplification using EST clone (RZ03e09F) as template with primers F4P9.23F and F4P9.23R at both end of the predicted coding sequence were cloned in to pBluescript SK+ as RopGAP1 to generate pBS-F4P9 (Table 4.3). Only the clone in which a fragment with BamHI/PstI site can be
<table>
<thead>
<tr>
<th>Primer's Name</th>
<th>Primer's Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWD912F</td>
<td>CCC CAT GGC CAT GAC TGA AGT TCT TCA</td>
</tr>
<tr>
<td>MWD912R</td>
<td>CAT CAC CGC CAA GCT TCA GTA CGC</td>
</tr>
<tr>
<td>R202LF</td>
<td>CTC ACT GCA GAG AAT AGT GAG GAA G</td>
</tr>
<tr>
<td>R202LR</td>
<td>ATT CTC TGC AGT GAG TAG AAA AAT GCC CTC TGC</td>
</tr>
<tr>
<td>16-414F</td>
<td>GGT TTG CCT GTT GGA TCC GAG CCT GAA</td>
</tr>
<tr>
<td>16-481R</td>
<td>TAA TTG GAT GGA TCC GGT TGA TAC CCC</td>
</tr>
<tr>
<td>16-1011F</td>
<td>ACT TTA AGA TCT AGG CAA GAC TCA GTG</td>
</tr>
<tr>
<td>16-1021R</td>
<td>GCT TGC TCG ACC AAC TAG TCT TAC CTTC TCT</td>
</tr>
<tr>
<td>F4p9.23F</td>
<td>GGG GGA TCC ATG GCG ACG ACA ATG AAG GG</td>
</tr>
<tr>
<td>F4p9.23R</td>
<td>GTT TCT CAG ATA ATA TCG TTA CAG G</td>
</tr>
</tbody>
</table>

Table 4.3. Primers used for mutant construction and RT-PCR analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primers</th>
<th>PCR Fragments</th>
<th>Mutant Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-1011F</td>
<td>M13R</td>
<td>A</td>
<td>(101-340) RopGAP1</td>
</tr>
<tr>
<td>16-414F</td>
<td>M13R</td>
<td>B</td>
<td>(101-143) RopGAP1</td>
</tr>
<tr>
<td>R202LR</td>
<td>M13R</td>
<td>C</td>
<td>R202L-RopGAP1</td>
</tr>
<tr>
<td>R202LF</td>
<td>M13R</td>
<td>D</td>
<td>R202L-RopGAP1</td>
</tr>
<tr>
<td>16-1011F</td>
<td>M13R</td>
<td>E</td>
<td>(166-340) RopGAP1</td>
</tr>
<tr>
<td>16-481R</td>
<td>MWD912F</td>
<td>F</td>
<td>(166-340) RopGAP1</td>
</tr>
<tr>
<td>16-414F</td>
<td>16-1021R</td>
<td>G</td>
<td>(143-340) RopGAP1</td>
</tr>
</tbody>
</table>

Table 4.4. List of PCR fragments used for construction of different RopGAP1 mutants

a, “F” and “R” indicate the sense and antisense primers respectively, or forward and reverse primers respectively.
obtained was selected, and this fragment was subsequently cloned into pMAL-C2 vector to generate MBP fusion construct. All plasmids used for transient expression in pollen were constructed in a derivative of the pBI221 vector (CloneTech), termed pL, in which CaMV 35S::GUS was replaced with the LAT52 promoter (Twell et al., 1991). The enhanced GFP mutant (S65C) gene was cloned downstream of the LAT52 promoter in pL to create pLG as previously described (Li et al., 1999a).

For transient expression of all other genes, including Rop1 (Li et al., 1999a), RopGAP1 (Fu et al., 2002a), and RIC1 (Wu et al., 2001), the full-length coding sequence for each gene was constructed behind the LAT52 promoter in pL. Rop1 was digested from pBI101-Lat52-GFP-Rop1At plasmid (Li et al., 1999a) using BglII/SstI sites and subcloned into pL with BamHI/SstI, whereas RIC1 was digested with BamHI/Kpnl from pBS-F4P9 plasmid and cloned into the same sites in pL. For RopGAP1, the full-length RopGAP1-coding sequence was amplified from pMAL-C2-RopGAP1 using the MalE primer in the MBP region and a reverse primer (MWD912R, see Table 4.3) (Wu et al., 2000). The PCR product was then cut by BamHI to produce a fragment with BamHI site at 5' only, and was subcloned into the BamHI/EcoRV sites in pL. AtGDI1 constructs were constructed similar to Rop1.

For transient expression of GFP fusions, each gene was subcloned as a translational fusion behind GFP C-terminus in pLG using the same fragments described above. Briefly, Rop1 were subcloned using Bgll/SstI sites, whereas RIC1 digested with BamHI/Kpnl was subcloned into Bgll/ Kpnl sites in pLG. For the GFP-RopGAP1 fusion, the above PCR fragment of the full-length RopGAP1-coding sequence with
BamHI at 5’ only was fused into BglII/EcoRV sites in pLG. All plasmids used for the bombardment were purified from *E. coli* bacteria using QIAGEN Mini purification Kits according to Manufacture's instruction (QIAGEN, Valencia, CA).

**RopGAP1 Mutant construction**

Constructs for different RopGAP1 point mutations and deletions used in this study are shown in Figure 2. The generation of His mutation (H125Y) of RopGAP1 was described previously (Wu et al., 2000). The (H125Y) RopGAP1 mutant was cloned into pL and pLG as described for wild type RopGAP1 using pMAL-H125Y-RopGAP1 as template for the PCR amplification. All PCR products, including A, B, C, D, E, F and G, were amplified using pBS16 as the template with primers listed in Table 4.4. To create mutant (101-340)RopGAP1 and (101-143)RopGAP1 mutants, PCR products A and B were cut by BglII/KpnI and BamHI/KpnI respectively and used to replace the BglII/KpnI fragments of pL-RopGAP1 and pLG-RopGAP1 respectively (Table 4.3 and Table 4.4). R202L mutant was created by PCR based point mutation by introducing a PstI site with two pairs of primers as shown in Table 4.3 and Table 4.4 to create an Arg to Leu mutation. The PCR fragment C was cut by BamHI/PstI and first cloned into the pBluescript SK+ to generate pBS-C, followed by the cloning of D fragment into pBS-C with PstI. Then, the corresponding fragments of pL-RopGAP1 and pLG-RopGAP1 were replaced with same region of pBSR202L-RopGAP1 using BglII/KpnI sites. (143-340)RopGAP1 mutant was cloned using PCR product G fragment.
cutting by BamHI/BgII to insert into pL and pLG vector with BamHI and BgII respectively. For (166-340)RopGAP1 mutant, E was cut by KpnI to create one sticking end and one blunt end fragment, and then cloned into pBS with KpnI/Smal to create pBS-E; then F fragment was cut by BamHI to create a fragment with BamHI at each end and subsequently cloned in to pBS-E with BgII site created at the 5' with primer 16-1011F to generate pBS-EF (Table 4.3.4 and Table 4.3.5). Finally, pBS-EF was cut by BamH/KpnI and cloned in to pL and pLG with the same restriction sites. All clones were sequenced to confirm the accurate of the mutations.

**Generation of Arabidopsis transgenic plants**

The coding sequence of RopGAP1 in pBS16 was cut by Ncol/KpnI and subcloned into the pCAMBIA1300 with a LAT52 promoter with Ncol/KpnI sites. Using the same vector, all other RopGAP1 mutant constructs were cloned from the mutants that were in the pL vector constructed above with Ncol/KpnI sites. All constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation and transformed into Arabidopsis wild type (Columbia ecotype) plants using the vacuum infiltration method. Transgenic plants were selected on Murashige and Skoog medium (Gibco BRL) containing 30 µg/ml hygromycin (Sigma, St. Luis). Hygromycin-resistant seedlings were transferred to soil and grown at 22°C in a growth room with 12-hr-light and 12-hr-dark cycles. Several independent T1 plants were selfed, and T2 that was
generated from the single insertion T1, or later progeny with homozygous transgenes were chosen by antibiotic selection for pollen tube phenotype analyses.

In vitro Arabidopsis pollen germination and growth measurement

Flowers collected from transgenic plants or WT plants 1 to 3 weeks after bolting were used for the examination of pollen tube phenotypes. Flowers from plants were dehydrated at room temperature for about 2 hr before pollen grains were dipped onto a pollen germination medium as previously described (Li et al., 1999a). Briefly, mature pollen grains from homozygous transgenic and Columbia wild-type plants were germinated on agar media containing 18% sucrose, 0.01% boric acid, 1 mM MgSO4, 0.5% agar, pH 6.5-7.0 and 2 mM of Ca2+ (with an equal molar ratio of CaCl2 and Ca [NO3]2). Pollen tubes were incubated at room temperature for 5 hr before they were killed and the images were taken through a cooled CCD camera (Hamamatsu C4742-95, Japan) attached on an inverted microscope (Nikon ECLIPSE TE300, Japan). The images were analyzed using the measurement function of Metamorph 4.5. For each transgenic plant, at least 100 pollen tubes were chosen randomly from the images for length measurements. Average length and standard deviations among 100 pollen tubes were calculated.
Transient expression in tobacco pollen

Tabacco (*Nicotiana tabacum*) plants were grown in growth chambers at 22 °C under a condition of 12 hrs light a day. Pollen grains were collected immediately prior to each experiment and suspended in a pollen germination medium (GM) which was the same as above for Arabidopsis pollen except that the calcium concentration is 10 μM and without agar. Pollen grains were then immediately bombarded with DNA-coated gold particles using a PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA) as described (Fu et al., 2002; Wu et al., 2001). Routinely, 0.5 mg particles were coated with 0.4 μg of a plasmid DNA containing a gene of interest or otherwise as indicated amount. Bombarded pollen grains were being washed into petri dishes with 0.5 ml GM right after bombardment and incubated in the room temperature.

Imaging and analysis of the subcellular localization of GFP-tagged RopGAP1 and its mutants

To determine the overexpression effect of RopGAPs and its activity on the pollen tube growth, we measured length of pollen tubes bombarded with same amount of plasmids containing RopGAP1 or the mutants of RopGAP1 respectively with or without Rop1. Five hours after bombardment, images of fluorescent tubes were rapidly recorded through a cooled CCD camera. The images were also analyzed using the measurement function of Metamorph 4.5. In order to observe the localization of RopGAP1 and its
mutants, The GFP-tagged RopGAP1 and its mutants would expressed alone, or with Rop1, or with Rop1 and RIC1, the image of the median section of pollen tubes was taken using a laser scanning confocal microscope (MRC 600 laser).

**In vitro competition of RIC1 and RopGAP1 in the protein pull-down assays**

To determine whether RIC1 inhibits RopGAP1 interaction with Rop1 in vitro, we fused RIC1 and RopGAP1 with maltose-binding protein (MBP) and CA-rop1 with glutathione S-transferase (GST) for pull-down assays as described previously (Wu et al., 2000). The equal molar of MBP-RIC1 and MBP-RopGAP1 were mixed separately or together with about 10 μg of GST-CA-rop1 fusion proteins together with glutathione-conjugated agarose beads. The procedure for pull-down assay was described previously (Wu et al., 2000). The western blot signals were detected using a polyclonal antibody against MBP (New England Biolab) and the BM Chemiluminescence I stern Blot Kit (Boehringer Mannheim, Basel).
CHAPTER 5

CONTROL OF POLAR GROWTH BY A SPATIALLY REGULATED FEEDBACK LOOP OF RHO GTPASE SIGNALING

Abstract

Polarized tip growth generates cylindrical cells capable of navigating their growth according to external cues, e.g., neuronal axons, pollen tubes, and fungal hyphae. This process requires formation and continuous regeneration of an apical plasma membrane (PM) domain (termed herein tip growth domain) to which Golgi vesicles are targeted and fuse for growth. How tip growth domain is defined and regenerated remains unclear. The Arabidopsis Rop1 Rho GTPase has been localized to the apical PM domain and is critical for tip growth in pollen tubes. Here we show that Rop1 is activated at the apical PM domain and that active Rop1 promotes Rop1 recruitment to the PM, forming a positive feedback loop of Rop1 activation and recruitment. This loop is initiated locally, amplified laterally and inhibited globally, generating a tip-high gradient of active Rop1. Our results suggest that this active Rop1 gradient defines the tip growth domain, whereas the positive feedback loop provides a mechanism for continuous regeneration of this
growth domain. This model may provide a general mechanism for the control of polar and directional growth and chemotaxis in eukaryotic cells.

Introduction

Polar growth is fundamentally important for morphogenesis and development in all eukaryotic organisms. Cells undergoing polar or directional growth require a mechanism for defining a specialized domain of the plasma membrane (PM) for growth (Bradke and Dotti, 2000; Chant, 1999; Drubin and Nelson, 1996; Fowler and Quatrano, 1997; Parent and Devreotes, 1999; Zheng and Yang, 2000). In various eukaryotes including yeast, plants, and animals, it is believed that a signaling machinery that controls localized growth needs to be recruited to the dynamic growth domain (Bradke and Dotti, 2000; Drees et al., 2001; Drubin and Nelson, 1996; Guo et al., 2001; Zheng and Yang, 2000). As a nascent PM domain is being generated during localized growth, the signaling machinery is continuously removed and must be replenished. Thus, a central enigma of polarized or guided cell growth is how a localized or vectorial growth cue controls the recruitment and subsequent replenishment of the signaling machinery that regulates localized growth. Many studies have demonstrated that GTPases of the conserved Rho family, including Rho, Cdc42, Rac, and Rop, are localized to the site of growth and act as a universal switch in the signaling machinery that control polar growth in different systems (Chant, 1999; Dickson, 2001; Guo et al., 2001; Parent and Devreotes, 1999; Ridley, 1999; Zheng and Yang, 2000). The Rho GTPase switch controls polar
growth through cortical F-actin dynamics, localized accumulation of cytosolic calcium, and polar recruitment of exocytosis machinery (Fu et al., 2001; Guo et al., 2001; Hall, 1998; Li et al., 1999b; Li et al., 1995). In this report, we test the hypothesis that the Rho GTPase switch also controls the recruitment and replenishment of the Rho GTPase-dependent signaling machinery to the site of growth using the pollen tube model system.

As in animal neuronal axons and fungal hyphae, pollen tubes extend their length by tip growth, an extreme form of polar growth in which growth is restricted to the cell apex (Franklin-Tong, 1999; Palanivelu and Preuss, 2000; Zheng and Yang, 2000). Because of extremely rapid growth rate (up to 1 cm/hr), pollen tubes represent an excellent model to study the mechanism for the regeneration of polar growth sites or replenishment of signaling machinery at the growth site (Franklin-Tong, 1999; Palanivelu and Preuss, 2000; Zheng and Yang, 2000). Several recent studies have shown that Arabidopsis Rop GTPases of the Rho family are localized to the apical PM region and are essential for tip growth in pollen tubes (Fu et al., 2001; Kost et al., 1999; Li et al., 1999b; Lin et al., 1996; Lin and Yang, 1997). Furthermore, expression of constitutively active mutant for Rop1 (CA-rop1) caused a complete loss of cell polarity (i.e., bulbous pollen tubes), and overexpression of wild type (WT) Rop1 gene caused less severe depolarization of pollen tubes (i.e., increased radial expansion of pollen tubes or swelling of pollen tube tips) (Li et al., 1999a). The Rop1-induced depolarized growth is associated with increased Rop1 distribution (i.e., the apical PM region containing Rop1) and abundance (i.e., the amount of PM-localized Rop1) in pollen tubes. Finally, PM localization of Rop1 is a prerequisite for its function in tip growth (Li et al., 1999a).
Figure 5.1. Relationship between polar growth and localization of GTP-bound active Rop1 at the apical PM region of pollen tubes.  A) Hypotheses being tested in this study: 1) Apical PM-localized active Rop1, indicated by a red-to-yellow color scale, defines tip growth domain; 2) Active Rop1 promotes Rop1 recruitment to the apical PM, forming a positive feedback loop.  B) The effect of Rop1 overexpression on the localization of GFP-RIC1 to the apical PM region of tobacco pollen tubes. To test the hypotheses, the distribution and abundance of GTP-bound active Rop1 at the apical PM was examined in tobacco pollen tubes expressing different levels of Rop1. The PM localization of GTP-bound Rop1 was visualized by co-expressing Rop1 with GFP-RIC1. Co-expression was performed using a particle bombardment-mediated transformation method (Fu et al., 2001). In each transformation, 0.4 μg of pL:GFP-RIC1 plasmid DNA and indicated amount of pL:Rop1 were used. Four hr after transformation, fluorescent tubes were imaged using confocal microscopy and the vertical medium laser sections of pollen tubes were analyzed using MetaMorph v4.5. C) Correlation of pollen tube depolarization with abundance and distribution of GFP-RIC1 at the apical PM region. The degree of pollen tube depolarization is indicated by increased maximum tube width. Distribution of GFP-RIC1 refers to the apical PM region containing GFP-RIC1 (indicated by arrows in B) in the median sections of pollen tubes. Abundance of GFP-RIC1 at the apical PM refers to amount of GFP-RIC1 localized to the apical PM region, as indicated by average fluorescence intensity in the PM region containing GFP-RIC1. All data shown in this Figure were the average values measured from 35 pollen tubes. Bar=10 μm.
Tip growth domain and Rop1 activity:
- Red, high Rop1 activity and fast growth
- Yellow, low Rop1 activity and slow growth
- Green, no Rop1 activity and no growth

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These observations led us to propose the following two hypotheses (see Figure 5.1A). First, the activity of Rop1 localized to the apical PM region defines tip growth domain and controls tip growth of pollen tubes. Second, active Rop1 localized to the apical PM region promotes the recruitment of Rop1 to this region, forming a positive feedback loop that allows a rapid regeneration of the tip growth domain.

Results

To test these hypotheses, we first analyzed the effect of Rop1 overexpression on the Rop activity localized to the apical PM region in pollen tubes. To monitor Rop activity, we utilized a novel Arabidopsis protein containing a CRIB (Cdc42/Rac interactive binding) motif, RIC1, which interacts specifically with GTP-bound active Rop1 in a CRIB-dependent manner (Wu et al., 2001). Assays based on the interaction between the CRIB motif and GTP-bound Cdc42/Rac have been widely used to monitor the activation of Cdc42 and Rac GTPases in living cells and in cell extracts (Benard et al., 1999; Burbelo et al., 1995; Kraynov et al., 2000). We previously showed that GFP:RIC1 expression does not alter pollen tube morphology (Wu et al., 2001). Furthermore, only PM-localized Rop1 can be activated (Li et al., 1999a). GFP-RIC1 displays a CRIB motif- and Rop1-dependent localization to the apical domain of the pollen tube PM with a tip-high gradient (Wu et al., 2001). Thus, GFP-RIC1 serves as a
useful marker to localize active Rop1 in the PM of pollen tubes (Wu et al., 2001). As shown in Figure 5.1B and

<table>
<thead>
<tr>
<th>pLRop1 (µg)</th>
<th>Length (µm)(^{b}) (Mean ± SD)</th>
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<tr>
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<td>14.4 ± 2.8</td>
<td>22.6</td>
</tr>
<tr>
<td>0.4</td>
<td>223.2 ± 105.3</td>
<td>19.7 ± 5.0</td>
<td>11.3</td>
</tr>
<tr>
<td>1.6</td>
<td>164.6 ± 83.3</td>
<td>28.5 ± 7.4</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Table 5.1 Rop1 affects pollen tube growth and polarity.
\(^{a}\) In each experiment, pollen grains were bombarded with 0.4 mg pLAT52:GFP and indicated amount of pLRop1.
\(^{b}\) Measurement was conducted from images taken from fluorescent tubes approximately 5 hr after bombardment as described in Methods.
Figure 5.2. Enhanced recruitment and activation of Rop1 at the apical PM region is the direct cause of Rop1 overexpression but not of depolarized growth. The localization and activation of Rop1 were visualized using GFP-Rop1 and GFP-RIC1, respectively. A) shows the apical PM localization of GFP-Rop1 in tubes expressing low (0.1 µg pL:GFP-Rop1 DNA used for transformation) or high (0.4 µg pL:GFP-Rop1 DNA used) levels of GFP-Rop1, and the effect of 5 nM latrunculin B (LatB) treatment or AtGDII co-expression on the localization. B) shows the apical PM localization of active Rop1 (indicated by GFP-RIC1) in tubes overexpressing Rop1 (0.4 µg pL:Rop1 DNA used for transformation), and the effect of LatB treatments or AtGDII co-expression on the localization. Both A and B show tubes that exhibited the widest PM distribution of GFP-Rop1 or GFP-RIC1 in a typical experiment. LatB had no effects on the distribution of either GFP-Rop1 or GFP-RIC1, whereas AtGDII suppressed enhanced distribution of GFP-Rop1 or GFP-RIC1 induced by Rop1 overexpression. Bar=10 µm.
1C, the abundance and distribution of GFP-RIC1 at the apical PM were tightly correlated with the level of Rop1 overexpression, as does the PM-localization of GFP-Rop1 shown previously (Li et al., 1999a). Furthermore, the Rop1-induced changes in GFP-RIC1 localization were tightly correlated with the changes in polar growth of pollen tubes (Figure 5.1C, Table 5.1). Consistent with a role for tip-localized Rop1 in promoting growth, a moderate increase in the abundance and distribution of the PM-localized GFP-RIC1 was associated with an increase in both tube elongation and radial expansion, although tube elongation increased slightly more than radial expansion (Table 5.1). A more dramatic increase in the distribution of GFP-RIC1 to the apical PM was associated with a much greater increase in radial expansion (Figure 5.1C and Table 5.1). These results strongly support the hypothesis that Rop1 overexpression first causes increased abundance and distribution of Rop1 in the apical PM region, leading to a corresponding change in its activation, and then depolarized growth of pollen tubes.

We sought to directly test whether the increased abundance, distribution, and activation of Rop1 in the apical PM domain are the result or the cause of Rop1 overexpression-induced depolarized growth. We first treated Rop1-overexpressing tubes with 5 nM latrunculin B (LatB), which suppresses Rop1-induced depolarized growth by promoting the disassembly of tip F-actin (Fu et al., 2001). As shown in Figure 5.2A and 2B, enhanced abundance and distribution of both GFP-Rop1 and GFP-RIC1 at the apical PM were not affected by LatB treatments. We next determined the effect of expressing AtGDII, an Arabidopsis homolog of guanine nucleotide dissociation inhibitor (GDI) that dissociates Rho GTPases from membranes (Bischoff et al., 2000; Fukumoto et al., 1990;
Hoffman et al., 2000). As shown in Figure 5.2A and 2B (the far right panels), AtGDI1 dramatically reduced the localization of both GFP-Rop1 and GFP-RIC1 at the apical PM and suppressed depolarized growth induced by \textit{Rop1} overexpression. From these results, we conclude that AtGDI1 does not interact with another protein in the polar pole establishment and the depolarized growth is not the cause but the result of ectopic Rop1 localization and activation induced by \textit{Rop1} overexpression. Thus, the most reasonable interpretation of these results together with those shown in Figure 5.1 is that the apical PM-localized active Rop1 defines the tip growth domain as well as controls polar growth.

To test whether active Rop1 promotes recruitment of Rop1 to the apical PM region, we altered Rop1 activity at the tip. To do so, we used Arabidopsis RopGAP1 and its dominant negative mutant. RopGAP1 promotes the conversion of GTP-bound Rop1 to GDP-bound Rop1 (Wu et al., 2000). RopGAP1 is expressed in Arabidopsis pollen, is co-localized with Rop1 to the apical PM domain, and acts as a negative regulator of Rop1 in Arabidopsis (Wu et al., 2001). The R202L mutation on RopGAP1 eliminates the GTPase-promoting activity without alteration of its interaction with Rop1, generating a dominant negative RopGAP1 mutant (DN-RopGAP1) (Li et al., 1997; Wu et al., 2001; Wu et al., 2000). As shown in Figure 5.3A (upper panels), expression of WT RopGAP1 in pollen tubes suppressed Rop1-induced increase in GFP-RIC1 localization to the apical PM domain. On the contrary, expression of DN-RopGAP1 mutant greatly enhanced the Rop1-induced increase in GFP-RIC1 distribution to the apical PM region. These changes in GFP-RIC1 localization were associated with the suppression and the enhancement of Rop1-induced depolarized growth (i.e., tube expansion), respectively. Thus, these results
Figure 5.3. Effects of WT RopGAP1 or DN-RopGAP1 mutant expression on Rop1 activity and localization at the apical PM. To determine the effects of WT RopGAP1 or the DN-RopGAP1 mutant on PM-localized GTP-bound Rop1, GFP-RIC1 was co-expressed with Rop1 alone (control), Rop1 plus RopGAP1, or Rop1 plus DN-RopGAP1 (A, upper panels). PM-localized GFP-RIC1 was visualized using confocal microscopy 3 hrs after transformation. The effect of RopGAP1 or DN-RopGAP1 on Rop1 localization to the apical PM was determined by co-expressing GFP-Rop1 with RopGAP1 or DN-RopGAP1 (A, lower panels). Control was GFP-Rop1 alone. RopGAP1 and DN-RopGAP1 respectively reduced and enhanced the distribution of both GFP-RIC1 and GFP-Rop1 to the apical PM region. B) shows the quantitative effect of RopGAP1 and DN-RopGAP1 on the distribution of PM-localized GFP–Rop1. Shown is the average apical PM region containing GFP-Rop1 measured from more than 80 pollen tubes for each treatment. Bar = 10 μm.
confirm that overexpression of WT RopGAP1 and DN-RopGAP1 mutant indeed alters the tip-localized Rop1 activity. We then determined whether the alteration of Rop1 activity affects Rop1 localization. As shown in Figure 5.3A (lower panels), the localization of GFP-Rop1 to the apical PM became weaker and more restricted to the apex in tubes co-overexpressing RopGAP1. RopGAP1 overexpression caused approximately 30% (from 220 nm to 150 nm) reduction in the apical PM distribution of GFP-Rop1 (Figure 5.3B). In contrast, DN-RopGAP1 enhanced the ectopic localization of GFP-Rop1 to the apical PM, causing more than a 2.5-fold (from 220 nm to 570 nm) increase in the apical PM distribution of GFP-Rop1 (Figures 3A and 3B). These results provide strong direct evidence that active Rop1 promotes the recruitment of Rop1 protein to the apical PM region in pollen tubes. Thus, we conclude that the activation and recruitment of Rop1 in the apical PM domain form a positive feedback loop.

This positive feedback regulation of Rop activity underscores the similarity and differences in phenotypes between WT Rop1 and CA-rop1 expression (Li et al., 1999b). Rop1 overexpression causes more Rop1 to stay associated with the apical PM region probably by sequestering AtGDI1. The additional PM-associated Rop proteins are activated and participate in the positive feedback loop. This causes gradual amplification of this loop and the typical club-shaped tubes (gradual tip expansion and swelling). In contrast, CA-rop1 expression induces much more rapid amplification of positive feedback loop and abrupt tip expansion and swelling, leading to bulb-shaped tubes (Li et al., 1999b).
We hypothesized that the positive feedback loop is initiated locally at the tip and then amplified laterally. This hypothesis is supported by the observation that Rop1 and RIC1 localization to the PM is restricted to the apex in normal tubes but is extended to the subapical region and tapers off in the flank of Rop1-overexpressing tubes. To directly test the lateral amplification of the feedback loop, we performed a time-course

Figure 5.4. Lateral amplification of Rop1 activation at the apical PM region of pollen tubes expressing CA-rop1. GFP-RIC1 was co-expressed with CA-rop1 in tobacco pollen tubes, and GFP-RIC1 localization was analyzed using confocal microscopy every 2 min after GFP-RIC1 expression was first clearly detected (approximately 2 hr after transformation). Time 0 indicates the point GFP-RIC1 was first detected at the apical PM region. Arrows indicate the boundary of the PM region clearly containing GFP-RIC1. Bar = 15 μm.
### Table 5.2. GAP and GDI coordinate to suppress CA-Rop1 induced depolarized growth.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Length (µm)(^a)</th>
<th>Width (µm)(^b)</th>
<th>Length/Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-Rop1 (^1)</td>
<td>132.4±43.8</td>
<td>29.2±4.2</td>
<td>4.5</td>
</tr>
<tr>
<td>CA-Rop1+RopGAP1 (^1)</td>
<td>187.2±95.7</td>
<td>17.2±3.3</td>
<td>10.9</td>
</tr>
<tr>
<td>CA-Rop1+AtGDI1 (^1)</td>
<td>153.2±57.6</td>
<td>27.6±6.1</td>
<td>5.6</td>
</tr>
<tr>
<td>CA-Rop1+RopGAP1+AtGDI1 (^1)</td>
<td>280.5±119.2</td>
<td>13.9±2.3</td>
<td>20.2</td>
</tr>
<tr>
<td>Control (^1)</td>
<td>254.2±105.3</td>
<td>12.2±1.7</td>
<td>20.7</td>
</tr>
</tbody>
</table>

\(^a\) All constructs were in the pLAT52 vector and co-bombarded with pLAT52:GFP. Control is pLAT52:GFP alone.  
\(^b\) Measurement was conducted on images taken as described in Table 5.1.

### Table 5.3. Coordinate effects of GAP and GDI.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Length (µm)(^a)</th>
<th>Width (µm)(^b)</th>
<th>Length/Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (^1)</td>
<td>453±170.1</td>
<td>13.9±3.0</td>
<td>32</td>
</tr>
<tr>
<td>RopGAP1 (^1)</td>
<td>398±191.9</td>
<td>9.2±1.7</td>
<td>43</td>
</tr>
<tr>
<td>AtGDI1 (^1)</td>
<td>223±96.1</td>
<td>10.2±1.8</td>
<td>22</td>
</tr>
<tr>
<td>RopGAP1+AtGDI1 (^1)</td>
<td>214±62.9</td>
<td>7.7±0.8</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) All constructs were in the pLAT52 vector and co-bombarded with pLAT52:GFP. Control is pLAT52:GFP alone.  
\(^b\) Measurement was conducted on images taken approximately 6 hr after bombardment.
Figure 5.5. A coordinate action of RopGAP1 and AtGDI1 to globally inhibit the amplification of the Rop1 positive feedback loop. A) Suppression of CA-rop1 induced depolarized growth by RopGAP1 and AtGDI1. pL:CA-Rop1 (0.2 μg) was co-bombarded with 1 μg pL:RopGAP1, 1 μg pL:AtGDI1, or a mixture of pL:RopGAP1 and pL:AtGDI1 (1 μg each). Control (the far right panel) was pL:GFP alone. Arrows indicate the pollen grains. Bar = 50 μm. Quantitative data for this experiment are shown in Table 5.2. B) The morphology of the tips of pollen tubes overexpressing RopGAP1, AtGDI1, or both. Amounts of plasmid DNA used were identical to those in A. Bar = 15 μm. See Table 5.3 for quantitative data on the effect of these constructs on pollen tube morphology. C) The effect of RopGAP1 and AtGDI1 on the localization of GFP-RIC1 in pollen tubes expressing CA-rop1. GFP-RIC1 was co-expressed with indicated constructs as described in A, and its localization was analyzed using confocal microscopy. Bar = 15 μm.
A

\begin{align*}
\text{CA-Rop1} & : + & + & + & + & - \\
\text{RopGAP1} & : - & + & - & + & - \\
\text{AtGDI1} & : - & - & + & + & - \\
\end{align*}

B

\begin{align*}
\text{RopGAP1} & : - & + & - & + \\
\text{AtGDI1} & : - & - & + & + \\
\end{align*}

C

\begin{align*}
\text{GFP-RIC1} & : & & & & \\
\text{CA-Rop1} & : + & + & + \\
\text{RopGAP1} & : - & + & + \\
\text{AtGDI1} & : - & - & + \\
\end{align*}
analysis of PM localization of GFP-RIC1 in tubes transiently expressing CA-rop1 (Figure 5.4). At time 0 (the first detectable appearance of GFP-RIC1 at the PM), GFP-RIC1 was detected at the extreme apex of the tube PM. The GFP-RIC1 distribution to PM increased by approximately 3 fold after 4 min and by approximately 6 folds 4 min later. The increase in PM distribution of GFP-RIC1 was associated with tip swelling, suggesting that the unrestricted lateral amplification resulted in depolarized growth.

The lateral amplification must be inhibited to maintain polar tip growth in wild type pollen tubes. We tested whether RopGAP1 inhibits the lateral amplification of the positive feedback loop. As shown in Figure 5.5A and Table 5.2, co-overexpression of RopGAP1 with CA-rop1 greatly suppressed CA-rop1 induced depolarized growth, demonstrating a crucial role for RopGAP1 in restricting the lateral amplification of the feedback loop. It is well established that GTP-bound constitutively active Rho mutants, such as CA-rop1 (Li et al., 1999b), are unable to undergo the GAP-mediated conversion to the GDP-bound inactive form. How could then RopGAP1 suppress CA-rop1 induced depolarized growth? The most reasonable explanation is that RopGAP1 deactivates WT Rop1 that is activated by CA-rop1 through the positive feedback loop. By doing so, RopGAP1 suppresses the amplification of this feedback loop in CA-rop1 expressing tubes. Thus these results further confirm the positive feedback loop hypothesis. Interestingly, RopGAP1 overexpression in WT pollen tubes not only inhibited elongation but more strongly inhibited radial tube expansion, causing tips to become pointed ("pipette tip" phenotype) (Figure 5.5B and Table 5.3). This "pipette tip" phenotype can be explained by a localized cue that continuously activates Rop1 and initiates the positive
feedback loop, while the subsequent lateral amplification of this loop is inhibited by RopGAP1 overexpression. Thus, this "pipette tip" phenotype implies the existence of a localized cue that activates Rop1 and initiates the positive feedback loop in WT pollen tubes.

RopGAP1-induced "pipette tip" phenotype also suggests the involvement of additional factor(s) in the spatial control of the feedback loop in order to generate the dome-shape tips in WT pollen tubes. We suspected that AtGDI1 might also be a factor that inhibits the feedback loop. As shown in Figure 5.5B and Table 5.3, AtGDI1 overexpression caused greater inhibition in pollen tube elongation than radial expansion, i.e., length/width was smaller in AtGDI1-overexpressing tubes than in WT pollen tubes. Furthermore, pollen tubes overexpressing AtGDI1 tended to have a flatter dome than WT tubes do.

The complementary effects of RopGAP1 and AtGDI1 on tip morphology and pollen tube growth suggest that these two negative regulators of the Rop1 positive feedback loop may coordinate to spatially regulate Rop1 activity in the apical dome of pollen tubes. To test this hypothesis, we investigated the effect of AtGDI1 and RopGAP1 co-overexpression. As shown in Figure 5.5B and Table 5.3, co-overexpression of AtGDI1 and RopGAP1 in WT pollen tubes completely recovered the normal shape of the apical dome and proportionally inhibited elongation and expansion of pollen tubes. The length:width ratio (approximately 30) in pollen tubes co-overexpressing AtGDI1 and RopGAP1 is similar to that (approximately 32) in WT pollen tubes. The coordinate action of AtGDI1 and RopGAP1 was confirmed by their effects on
CA-rop1 induced depolarized growth (Figure 5.5A and Table 5.2). Co-expression of AtGDII and RopGAP1 completely suppressed CA-rop1 induced bulbous tubes, whereas AtGDII alone only slightly suppressed the CA-rop1 phenotype (Figure 5.5A and Table 5.2). As expected, the localization of GFP-RIC1 to the apical PM domain was also recovered in tubes co-overexpressing CA-rop1 with AtGDII and RopGAP1 (Figure 5.5C). Our interpretation of these results is that the coordinate action of RopGAP1 and AtGDII globally inhibits Rop1 activation at the apical PM region.

**Discussion**

Based on these observations, we propose that global inhibition of the Rop1 recruitment-activation feedback loop that is activated locally and amplified laterally allows the generation of a tip-high Rop1 activity gradient. This gradient then defines the tip growth domain and controls polar growth. An unknown localized cue or 'tip-growth signal' appears to initiate the positive feedback loop. The feedback loop may act as an "engine" to amplify the signal and to continuously regenerate the tip growth domain (and thus the signaling machinery that promotes polar exocytosis), allowing rapid and efficient tip growth. However, the global inhibition of the feedback loop functions to "brake the engine" and to assure that growth occurs in a polar manner according to the polarity defined by the internal tip-growth signal during in vitro pollen tube growth. During in vivo guided growth, the Rop1 feedback loop might also be activated by external signals that guide pollen tubes toward the ovule (Palanivelu and Preuss, 2000). Similarly, the
global inhibition of the feedback loop would assure growth in the direction defined by the guidance signals. It would be interesting to determine what these localized signals are and what other components are in the feedback loop.

Polar growth, directional growth, chemotaxis, and directional movement in various eukaryotic cells are all controlled by the Rho-family GTPase switch (Chant, 1999; Dickson, 2001; Drees et al., 2001; Drubin and Nelson, 1996; Firtel and Chung, 2000; Hall, 1998; Mackay and Hall, 1998; Parent and Devreotes, 1999; Redmond and Ghosh, 2001; Ridley, 1999; Song and Poo, 2001; Wendland and Philippsen, 2001; Zheng and Yang, 2000; Zipkin et al., 1997). Polar recruitment of Rho GTPases to the growth site or the leading edge has also been demonstrated in yeast and animal cells (Ayscough et al., 1999; Gulli and Peter, 2001; Guo et al., 2001; Yamochi et al., 1994; Ziman et al., 1993). It was also proposed more than 20 years ago that chemotaxis may be controlled by a chemical gradient-initiated positive feedback loop coupled with a global inhibition (Meinhardt and Gierer, 1974; Meinhardt and Gierer, 2000). Our results reported here for the first time demonstrate that localized Rho GTPase activity is regulated by a Rho GTPase activation-recruitment feedback loop that is initiated locally and inhibited globally. The Rho GTPase feedback loop-dependent mechanism for the continuous regeneration of polar sites may provide a paradigm for the control of polar growth, directional growth, and chemotaxis in different eukaryotic systems.
Materials and Methods

DNA manipulation and plasmid constructs

All constructs were expressed under the control of the pollen-specific LAT52 promoter using the pLAT52 (pL) vectors described (Fu et al., 2001). pL:GFP-RIC1, pL:Rop1, pL:RopGAP1, and pL:AtGDI1 were described previously (Fu et al., 2001; Wu et al., 2001). pL:CA-rop1 was constructed using the CA-rop1 (G15V) mutant and BgIII/SstI sites (Li et al., 1999). pL:GFP-Rop1 was constructed using BgIII/SstI sites. The DN-RopGAP1 mutant (R202L) was generated by a PCR-based method as described in chapter 4. All plasmids were transformed into E. coli TOP 10 strain (Invitrogen) and purified using QIAGEN plasmid purification Kits according to Manufacture's instruction (QIAGEN, Valencia, CA).

Transient expression in tobacco pollen

All constructs were transiently expressed in tobacco pollen tubes using a particle bombardment method as previously described (Fu et al., 2001). Briefly, 0.4 µg of plasmid DNA (or amounts indicated in the legend of each figure) was used to coat 0.5-mg gold particles, which were used to bombard pollen grains freshly harvested from 2-3 flowers. Bombarded pollen grains were immediately washed into petri dishes with pollen
germination medium. Three to six hrs after bombardment, transfected tubes indicated by GFP or GFP fusion expression were recorded and analyzed as described below (Fu et al., 2001). For pollen tube morphological analysis, all data shown were collected from more than 50 tubes from one typical experiment, and similar results were obtained from at least three independent experiments.

**Analysis of pollen tube polar growth and GFP-Rop1 and GFP-RIC1 localization to the pollen tube PM**

The effect of various constructs on pollen tube growth and polarity was determined by measuring the length of transfected pollen tubes and the width of their tips. Three to six hrs after bombardment, images of fluorescent pollen tubes were rapidly recorded through a cooled CCD camera (model C4742-95; Hamamatsu) and analyzed using the MetaMorph v4.5 measurement function as previously described (Fu et al., 2001). Polar growth was indicated by the length of pollen tubes, while depolarized growth was indicated by the maximum width of pollen tube tips, which were measured at the widest region near the apex of the pollen tube.

To measure the abundance and distribution of GFP-Rop1 and GFP-RIC1 in the apical region of the pollen tube plasma membrane, fluorescent pollen tubes were analyzed using an MRC 600 laser confocal microscope. The median sections of laser scanning were collected and analyzed using the MetaMorph v4.5 measurement function. All images were taken 3 hr (GFP-Rop1) or 5 hr (GFP-RIC1) after bombardment for a
period of one hr in a fixed setting of the confocal microscope. The apical PM distribution of GFP-Rop1 and GFP-RIC1 was determined by measuring the apical PM region whose PM fluorescence was clearly distinct from the cytoplasmic fluorescence. The abundance of PM-localized GFP-RIC1 was determined by measuring the average fluorescence intensity of GFP distributed to the apical PM region containing GFP-RIC1.
CHAPTER 6

CONCLUSION AND FUTURE DIRECTION

Rho GTPase signaling provides a fundamental molecular switch that controls a broad spectrum of biological processes including polar directional growth and tip growth. Tremendous effort has been put on the identification of the mutants of Rho GTPases, and the isolation of their effectors and regulators. Biochemical characterization of these components and the functional characterization of genetic mutants have profoundly enriched our understanding of Rho GTPase signaling. On the other hand, how Rho GTPases themselves are being regulated in vivo and how Rho GTPase activation integrates with signaling cues in the control of diverse functions, especially in the control of tip growth, is not well studied and unclear (Gulli and Peter, 2001). Previous studies reveal that Ropl, a pollen specific Rop (RhGTPase of plant), is crucial in the control of tip growth in pollen tubes. However, how this Rop switch is regulated in tip growing pollen tubes is unknown (Li et al., 1999; Zheng and Yang, 2000). In this study, I first identified an active Rop marker, RIC1 and two negative regulators, RopGAP1 and AtGDI11, I then used the combination of these regulators and marker to study the regulation of Rop. I have uncovered a novel mechanism for Rop signaling in the
control of tip growth in pollen tubes, in which the localized activation of Rop by signaling cues is amplified by a positive feedback loop. This positive feedback loop is globally inhibited by the coordinating action of RopGAP1 and AtGDI. The combinatorial effect of the amplification by positive feedback loop and the global inhibition by RopGAP1 and AtGDI1, a tip-high gradient of the Rop1 activity is established. The tip-high Rop1 activity then defines the tip growth domain and controls the tip growth.

To further understand this mechanism, we have to knock out the negative regulators (RopGAP and AtGDI) and identify the factors involved in the positive feedback loop. To knock out the negative regulators would lead to the amplification of Rop localization and activity on the PM, thus will lead to the enhancement of growth and depolarization. In agreement with this is that a Rho GAP knock-out caused the depolarized growth in filamentous fungi (Wendland and Philippsen, 2000). Currently, two methods are very effective in knocking out genes in Arabidopsis: double strand RNA interference (RNAi) and T-DNA insertion line screen. However, due to the redundancy, RNAi method should be tried first.

A parallel study of this research shows that Rop effectors RICs control distinct functions correlating with their unique localization patterns in pollen tubes, in which RIC4 promotes pollen tube growth with expanded PM localization and RIC1 inhibits pollen tube growth with normal PM localization, implying that RIC4 may be in the positive feedback loop (Wu et al, 2002a). Indeed, RIC4 promoted the GFP-Rop1 localization and RIC1 did not, suggesting RIC4 is an activator or recruiter of Rop1 (Wu
and Yang, unpublished data). Further study is to knock out RIC4 and RIC1 to confirm whether RIC4 is indeed as an activator or recruiter of Rop.

On the other hand, in animal and fungi, Rho GEFs play a crucial role in the regulation of the special and temporal control of the activation of the Rho GTPases. Most GEFs have the conserved DH domain. However, no GEF with DH domain are found in the whole sequenced Arabidopsis genome or any other plant species. Therefore, how the Rop is being activated is completely unknown. Interestingly, Rop is found to be associated with CLAVATA 1, a receptor like kinase (RLK) containing transmembrane. While whether RLKs are the activators of Rop remains to be seen, a serine/threonine/tyrosine like kinase (STYLK), STYLK1, was identified from the yeast two hybrid screen with either constitutively active (G15V) and dominant negative (D121A) Rop as baits, suggesting that this kinase interacts with both GTP- and GDP- bound Rop. Interestingly, GFP-STYLK1 was localized to the PM of the whole pollen tubes but was probably only activated at the growing region, since the PM localization of other regions except the tip growing apical domain was spotty that is probably due to the degradation of the active complex (Wu et al, unpublished data). This active PM domain of STYLK1 increased when the Rop activity increased, and reduced when the Rop activity reduced by adding RopGAP1 and AtGDI, suggesting a close correlation between Rop activity and STYLK1 activation (Wu et al, unpublished data).

In animals, a variety of receptor kinases are shown to have a critical role in the regulation of Rho GTPases directly or indirectly. Interestingly, STYLK1 is closer to the Pti1, the protein that interacts with avirulent gene Pto that is similar to RLKs but has no
transmembrane domain, suggesting this kinase might interact with RLK (Bogdanove and Martin, 2000). The finding of Rop in the active CLAVATA1 complex further implies STYLK might be associated with RLKs since CLAVATA1 did not directly interact with Rop1 (Wu and Yang, unpublished data). Furthermore, an untraditional GEF (SopE) without DH domain has been found in animals (Hardt et al., 1998). Thus, it is possible that RLK is responsible for the receiving of extra- or intra- cellular cues, which in turn activates a novel GEF-like protein, resulting in the activation of Rop. Moreover, non-receptor tyrosine kinase has been shown to stabilize the active Rho GTPases (Manser et al., 1993). Taken together, this novel STYLK may relay the extracellular signals received from the RLKs to the Rop signaling.

Based on the identity, this kinase is probably the animal tyrosine kinase Lck homolog in plants (Amrein et al., 1994). In animal, Lck is a tyrosine kinase that functions in the upstream of the small GTPases and acts on RasGAP or Rap GAP (Amrein et al., 1994). It is not known whether STYLKs interact with RopGAPs. Taken together, this kinase may activate both RopGAP and Rop, thus resulting in complicated regulation of Rop activation. The overexpression of this novel kinase did not have obvious effect on the pollen tube growth (unpublished observation), arguing that this hypothesis is possible. However, the overexpression of some other Ser/Thr kinases do not show up obvious phenotypes either while the dominant negative or constitutively active mutants of those kinases do have dramatic effects (Li et al., 2000). Therefore, constitutively active or/ dominant negative mutation at STYLK1 will be required to address the function of this kinase in the Rop signaling and pollen tube growth.
STYLK1 has high homology with Ser/Thr kinases such as Pak as well but does not contain CRIB motif that is responsible for the interaction with Cdc42/Rac/Rop. However, CRIB motif is unnecessary since this kinase does interact with active Rop1 even though the mechanism for the interaction of STYLK1 and Rop1 is unknown, suggesting that this kinase might be an effector. The overexpression did not show up obvious phenotype might simply indicate this kinase is not in the rate limiting step of the Rop signaling, the constitutively active or/and dominant negative mutation at STYLK1 should help to solve this puzzle. Further future work should direct to the knock-out of STYLK1.

Based on this dissertation research and previous knowledge of Rop1 signaling in pollen tubes, I propose a working model (Figure 6.1). In this model, signaling cues received by RLKs are transduced into the cell by STYLK1 to Rop1. Thus STYLK1 will activate Rop1 directly or indirectly. The activated Rop1 then activate RICs. Some RICs are real effectors of Rop1 while some others are the recruiters of Rop1 in the positive feedback loop (such as RIC4), others may be both effectors and recruiters of Rop1. RICs may activate the calcium signaling and actin reorganization. The localized calcium and actin dynamic then regulate the polarized exocytosis and control the tip growth in pollen tubes.
Figure 6.1. A working model for Rop1 signaling in the control of pollen tube growth. In this model RLK (receptor like kinase) and STYLK1 (Ser/Thr/ Tyr like kinase 1) are activators while RopGAP1 and AtGDI1 are negative regulators. RIC4 is a recruiter of Rop1 while some other RICs may be effectors or recruiters or both of Rop1. RIC4 might have effector function too. RIC1 apparently is not a recruiter of Rop1.

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Spatial Cues
(Tip growth)

RLK

STYLK

RopGAP

AtGDI1

GDP

Inactive

Tip Localized

Positive feedback loop

Localized Ca^{2+} influx and accumulation as well as actin

Polarized Exocytosis

Tip Growth

Directional Growth

Inactive

AtGDI1

Active

RICs

RIC4

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