Regulation of Membrane Trafficking, Cytoskeleton Dynamics, and Cell Polarity by ROP/RAC GTPases[1][W]

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Rho of plants (ROP) proteins, also known as RAC proteins, are Rho-related GTPases that function as molecular switches in a multitude of signaling cascades involved in the regulation of the actin and microtubule cytoskeleton, of vesicle trafficking, and of plant responses to hormones, stresses, or light (Yang, 2002; Berken, 2006; Nibau et al., 2006; Yang and Fu, 2007). Rho GTPases are Ras-related small guanine nucleotide-binding proteins (G-proteins) that bind GTP and GDP with high affinity and hydrolyze GTP inefficiently. Rho GTPases switch between GTP-on to GDP-off states by highly regulated GDP/GTP exchange and GTP hydrolysis (Bourne et al., 1991; Vetter and Wittinghofer, 2001). Only in the GTP-bound state can Rho GTPases interact with effectors to elicit downstream signaling. The GDP/GTP exchange is catalyzed by guanyl nucleotide exchange factors (GEFs), and GTP hydrolysis is enhanced by GTPase-activating proteins (GAPs). Like other members of the Ras superfamily of small G-proteins, Rho GTPases are soluble proteins that associate with and function at cell membranes by virtue of the posttranslational lipid modifications prenylation and *S*-acylation (Hancock et al., 1989; Michaelson et al., 2001). A third group of regulating proteins are Rho guanyl nucleotide dissociation inhibitors (RhoGDIs), which inhibit GDP/GTP exchange and facilitate the cycling of Rho GTPases on and off membranes (DerMardirossian and Bokoch, 2005). The ability of Rho GTPases to interact with membranes allows these proteins to regulate actin polymerization and vesicle trafficking at discrete sites of the plasma membrane and of internal membranes, which is essential for their role in the control of cell polarity (Ridley, 2006). As part of the *Plant Physiology* focus issue on membrane biology, this review focuses on subcellular targeting of plant ROP/RAC GTPases and on the role of these proteins in the regulation of membrane trafficking, cytoskeleton organization, and cell polarity. Other aspects of ROP/RAC biology, such as the role of these GTPases in hormonal or stress signaling, will only be summarized in brief. We refer interested readers to several excellent recent reviews on ROP/RAC GTPases that highlight these other topics (Molendijk et al., 2004; Xu and Scheres, 2005; Nibau et al., 2006; Yang and Fu, 2007; Berken and Wittinghofer, 2008; Kost, 2008). Throughout this review, we have opted to use the ROP nomenclature for the sake of clarity. However, we use the RAC terminology in instances in which there is no ROP nomenclature or when publications have used the term RAC rather than ROP. Finally, we apologize to those colleagues whose work we have not been able to cite due to lack of space.

ROP/RAC EVOLUTION

Based on cell biological studies in animals, the Rho superfamily was initially divided into three major subfamilies designated Rho, Rac, and Cdc42 (Ridley and Hall, 1992; Ridley et al., 1992; Hall, 1998). With the increased availability of sequence information, the Rho family has been expanded and is currently suggested to include eight to nine subfamilies (Boureux et al., 2007; Vega and Ridley, 2007). Two methods of ROP/RAC classification are described in the literature. One classification placed ROP/RAC GTPases as a branch in the Rac subfamily and divided them into two subgroups, designated type I and type II, according to the structure of the C-terminal hypervariable domain (Winge et al., 1997). The second classification method, which is based on nucleotide sequences, suggested that ROP/RAC GTPases diverged as a separate group prior to the separation between Rac and Cdc42 and can be divided into four subgroups, which were designated I, II, III, and IV. Subgroups I and II correspond to type II ROP/RAC GTPases, and subgroups III and IV correspond to type I ROP/RAC GTPases of the first classification method (Yang, 2002; Christensen et al., 2003; Vernoud et al., 2003). Recent sequence analysis of the Rho superfamily in different

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eurkaryotes (Boureux et al., 2007) may solve the existing discrepancy in the literature. This analysis suggests that Rac GTPases were the originating family of all Rho GTPases and that the Rho and Cdc42 families diverged from the Rac family later in evolution. The additional Rho subfamilies were created by diversification of the family in vertebrates, primarily in mammals (Boureux et al., 2007). Thus, both earlier analyses are correct. ROP/RAC GTPases are indeed more closely related to Rac, but they diverged as a separate Rac group prior to the divergence of Rac, Rho, and Cdc42. The unique features discovered in high-resolution three-dimensional structures of AtROP9/RAC7 (Sormo et al., 2006) and AtROP4/RAC5 (Thomas et al., 2007) support the notion that ROP/RAC GTPases form a unique subgroup of the Rho GTPases (Berken and Wittinghofer, 2008). In this review, the classification of ROP/RAC GTPases into type I and type II according to their hypervariable domain will be used to discuss differences in lipid modification and subcellular targeting existing between these two types.

STRUCTURE AND ACTIVITY

Similar to other Rho GTPases, ROP/RAC GTPases contain a G-domain, which is responsible for GTP binding/hydrolysis and for interaction with effector proteins, and a hypervariable domain, which determines subcellular targeting. ROP/RAC GTPases have a molecular mass of 21 to 24 kD and consist of around 200 amino acids. High-resolution three-dimensional structures of Arabidopsis (*Arabidopsis thaliana*) AtROP9/RAC7 (Sormo et al., 2006) and AtROP4/RAC5 (Thomas et al., 2007) show that these ROP/RAC GTPases contain a β-sheet core composed of six β-sheets surrounded by four α-helices. The β-sheets and α-helices are connected by five loops that contain five highly conserved G-box motifs (G1–G5) responsible for GTP/Mg^{2+} binding and GTP hydrolysis (Bourne et al., 1991; Vetter and Wittinghofer, 2001; Berken and Wittinghofer, 2008).

Like corresponding mutations in Ras (Lowy and Willumsen, 1993; Feig, 1999), replacing invariably conserved G1 Gly or G3 Gln residues abolishes the GTPase activity of ROP/RAC proteins and renders them constitutively active (CA; Lemichez et al., 2001; Klahre and Kost, 2006; Berken and Wittinghofer, 2008). By contrast, replacing equally highly conserved G1 Thr or G4 Asp reduces the affinity of ROP/RAC GTPases for guanine nucleotides, thereby stabilizing their interaction with GEFs (Lemichez et al., 2001; Berken et al., 2005; Berken and Wittinghofer, 2008). When expressed in living cells, nucleotide-free Ras and ROP/RAC mutants have dominant negative (DN) effects, presumably because they inactivate endogenous GEFs by forming nonproductive heterodimers with them. A detailed sequence comparison of dicot and monocot ROP/RAC GTPases has revealed the putative G-domain composition and showed that all four residues are highly conserved (Christensen et al., 2003). As will be detailed throughout this review, CA and DN mutants have been extensively utilized to study ROP/RAC functions (Supplemental Table S1).

In addition to the G-domain and hypervariable regions, all Rho proteins contain a helical domain called the insert region, labeled αI, that is suggested to interact with effectors and regulatory proteins. Three-dimensional crystal structures show that in AtROP4 and AtROP9, the insert domain is two and four amino acids shorter, respectively, compared with animal and fungal Rho GTPases (Sormo et al., 2006; Thomas et al., 2007).

As in all members of the Rho family, the hypervariable domain is located at the C-terminal end of ROP/RAC GTPases. In type I ROP/RAC GTPases, the hypervariable domain consists of a canonical CaaL box, which is prenylated primarily by geranylgeranyltransferase I (PGGT; Sorek et al., 2007), and a proximal polybasic domain. The hypervariable domain of type II ROP/RAC GTPases has a different structure and directs membrane attachment by a mechanism that likely involves *S*-acylation (Ivanchenko et al., 2000; Lavy et al., 2002; Lavy and Yalovsky, 2006).

SUBCELLULAR LOCALIZATION

ROP/RAC subcellular localization has been investigated by indirect immunofluorescence, GFP tagging, and cell fractionation/immunoblotting. Imaging experiments have shown that these GTPases are associated with the plasma membrane in a variety of cell types and display enhanced membrane association at growth sites in pollen tubes, root hairs, and leaf epidermal cells (Ivanchenko et al., 2000; Molendijk et al., 2001; Fu et al., 2002; Jones et al., 2002; Lavy et al., 2002, 2007; Bloch et al., 2005; Lavy and Yalovsky, 2006; Sorek et al., 2007). By immunoblotting, ROP/RAC GTPases were exclusively detected in the membrane fraction of extracts of vegetative cells (Sorek et al., 2007), whereas they were found in both the membrane and the cytoplasmic fraction of pollen tube extracts (Kost et al., 1999).

The subcellular localization of ROP/RAC GTPases is primarily determined by their C-terminal hypervariable domain. However, recent findings show that activation-dependent *S*-acylation of one or more G-domain Cys residues is associated with partitioning of ROP/RAC GTPases into nonionic detergent-resistant membranes (DRMs; Sorek et al., 2007; Fig. 1).

Prenylation and CaaX Processing of Type I ROP/RAC GTPases

The CaaL box Cys of type I ROP/RAC GTPases is prenylated in the cytoplasm primarily by PGGT (Caldelari et al., 2001; Sorek et al., 2007). Following prenylation, proteins undergo two additional post-translational modifications, collectively referred to as

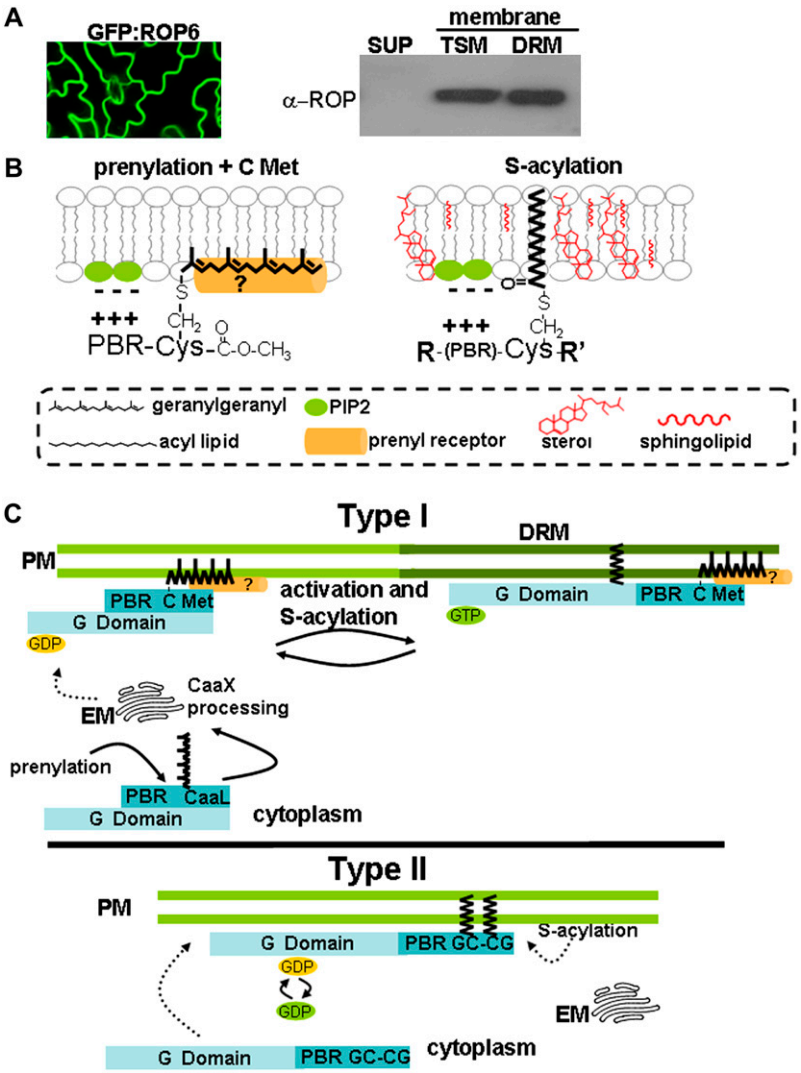

Figure 1. Posttranslational lipid modifications of type I and type II ROP/RAC GTPases. A, Left, GFP:AtROP6 is exclusively localized at the plasma membrane of leaf epidermal pavement cells. Right, Arabidopsis seedling protein extract separated by membrane flotation on a Suc density gradient and analyzed by immunoblotting using an anti-ROP/RAC polyclonal antibody. ROP/RAC GTPases are absent from the soluble fraction (SUP). In the insoluble membrane fraction, these proteins partition between nonionic detergent-soluble (TSM) and insoluble (DRM) membranes. B, Prenylation (left) and *S*-acylation (right) involve the formation of thioether and thioester bonds, respectively. CaaX box prenylation or GCCG box *S*-acylation is required for the membrane association of type I or type II ROP/RAC GTPase, respectively. The CaaX and GCCG boxes are located at the C termini of ROP/RAC GTPases, and the lipid modification of these domains is permanent. Following prenylation, the three C-terminal amino acids of the CaaX box of type I ROP/RAC GTPases are cleaved and the free carboxyl group of the isoprenyl Cys is methyl esterified (C-Met). Prenyl tails may directly insert into the membrane bilayer or bind to membrane-associated receptor proteins (Ashery et al., 2006; Belanis et al., 2008), which remain to be identified in plants. Proximal to the lipid modification domain, type I and type II RAC/ROP GTPases contain a polybasic region (PBR), which contributes to membrane association through direct binding to PtdIns 4,5-P_2. C, Type I ROP/RAC GTPases are prenylated in the cytoplasm before they are targeted to the ER for further CaaX box processing. Prenylated and carboxymethylated type I ROP/RAC GTPases may be transported from the ER to the plasma membrane along the secretory pathway or by another unknown mechanism. Upon activation, a G-domain Cys residue of at least some type I ROP/RAC GTPases is reversibly *S*-acylated, which results in transient partitioning of these proteins into sterol- and sphingolipid-rich membrane domains known as DRMs (darker green). By contrast, type II ROP/RAC GTPases are attached to the plasma membrane by virtue of stable *S*-acylation of two or more Cys residues in the C-terminal GCCG box. Stable *S*-acylation of this domain does not depend on the ROP/RAC activation status and presumably occurs directly at the plasma membrane.

CaaX processing. The first of these modifications involves proteolytic removal of the last three amino acids by either of two CaaX proteases called Ste24 and Rce1. In turn, the free carboxyl group of the isoprenyl Cys is methylated by isoprenyl carboxy methyltransferase (Fig. 1, B and C; Young et al., 2000). Homologs of all CaaX-processing enzymes have been identified and characterized in Arabidopsis (Rodriguez-Concepcion et al., 2000; Bracha et al., 2002; Narasimha Chary et al., 2002; Cadinanos et al., 2003). Similar to their animal

and yeast homologs, the Arabidopsis CaaX proteases and isoprenyl carboxy methyltransferases are likely localized at the endoplasmic reticulum (ER; Rodriguez-Concepcion et al., 2000; Bracha et al., 2002). This suggests that following prenylation in the cytoplasm, type I ROP/RAC GTPases are targeted to the ER (Fig. 1C). It is unknown whether transport from the ER to the plasma membrane occurs along the secretory pathway or by a different route.

Transient *S*-Acylation of Type I ROP/RAC GTPases

Membrane-associated type I GTPases partition between nonionic detergent-soluble (Triton X-100-soluble membrane [TSM]) and insoluble (DRM) fractions (Fig. 1A; Sorek et al., 2007). Constitutively active AtROP6^{CA} was exclusively localized in DRMs, and GDP/GTP exchanges induced dynamic partitioning of endogenous ROP/RAC proteins between DRMs and TSMs (Sorek et al., 2007). Analysis by gas chromatography-coupled mass spectrometry demonstrated that recombinant AtROP6 purified from TSMs was only geranylgeranylated (prenylated), while AtROP6 or AtROP6^{CA} purified from DRMs was geranylgeranylated and *S*-acylated by palmitic (C16) and stearic (C18) acids (Sorek et al., 2007). Transient *S*-acylation occurs at highly conserved Cys residues within the G-domain (Fig. 1C; Sorek et al., 2007) and may induce additional conformational changes in the activated GTP-bound protein. Interestingly, although the same Cys residues are present in animal and yeast Rho GTPases, *S*-acylation of these residues in nonplant proteins has not been demonstrated to date.

Type II ROP/RAC GTPases

The hypervariable domain of type II ROP/RAC GTPases consists of a unique sequence motif designated the GCCG box and a proximal polybasic domain. The GCCG box is composed of two Cys residues that undergo *S*-acylation (Fig. 1D; Lavy et al., 2002; Lavy and Yalovsky, 2006) and that are separated by five to six mostly aliphatic amino acids. The Cys residues are flanked by Gly residues. By contrast to the transient *S*-acylation of the G-domain described above, GCCG box *S*-acylation is stable (Lavy et al., 2002; Lavy and Yalovsky, 2006). Together with the lipid-modified GCCG box Cys residues, the aliphatic residues between them, the Gly residues flanking them, and the polybasic domain are required for membrane binding of type II ROP/RAC GTPases (Lavy and Yalovsky, 2006).

The Polybasic Domain, a Prenylation and Membrane-Binding Module

The polybasic domain has two essential functions: (1) it enhances PGGT-mediated prenylation by about one order of magnitude (James et al., 1995; Caldelari et al., 2001), and (2) it facilitates ROP/RAC membrane interaction (Del Pozo et al., 2002; Lavy and Yalovsky, 2006). It is now well established that polybasic domains in proteins function as interaction modules with phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P_3), which has not been detected in plant cells to date, and with phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2; Fig. 1B; Papayannopoulos et al., 2005; Heo et al., 2006; Kaadige and Ayer, 2006; Sun et al., 2007; Orlando et al., 2008). Importantly, it has been specifically demonstrated that the polybasic domains of Rho proteins and other small GTPases interact with both PtdIns 4,5-P_2 and PtdIns 3,4,5-P_3 (Heo et al., 2006). A polybasic region in N-WASP interacts with PtdIns 4,5-P_2 in a multivalent cooperative manner. This facilitates a highly sensitive switch-like mechanism that induces membrane recruitment of N-WASP specifically above a PtdIns 4,5-P_2 threshold level (Papayannopoulos et al., 2005). In yeast, the Cdc42 effector GIC2 interacts with PtdIns 4,5-P_2 in the membrane via a polybasic domain and with Cdc42 through a Cdc42/Rac-interactive binding (CRIB) domain. The interaction with PtdIns 4,5-P_2 is required for polar localization of GIC2 and for its function in polar cell growth (Orlando et al., 2008).

Removal of the polybasic domain of the Arabidopsis type II ROP/RAC AtROP8 abrogated its interaction with the membrane (Lavy and Yalovsky, 2006). Pollen tube ROP/RAC GTPases were shown to physically interact with a phosphatidylinositol monophosphate kinase (PtdIns P-K) activity in extracts of tobacco (*Nicotiana tabacum*) pollen tubes, and PtdIns 4,5-P_2, the product of PtdIns P-K activity, colocalizes with ROP/RAC GTPases at the apical plasma membrane of these cells (Kost et al., 1999). Based on these observations, it appears possible that ROP/RAC GTPases and PtdIns 4,5-P_2 maintain a positive feedback loop (Fig. 2; see also Fig. 4 below). Since polybasic domains function in a cooperative multivalent manner, PtdIns 4,5-P_2 may serve as a sensitive switch that above a threshold level triggers ROP/RAC accumulation. In turn, ROP/RAC accumulation would lead to the production of more PtdIns 4,5-P_2, promoting the recruitment of additional ROP/RAC molecules. This positive feedback loop may be tightly controlled by the ROP/RAC switch and is potentially further enhanced by the ability of PtdIns 4,5-P_2 to destabilize interactions between Rho GTPases and RhoGDIs. Work in animal cells has shown that through this mechanism, PtdIns 4,5-P_2 can promote Rho membrane association and subsequent activation (Fauré et al., 1999).

S-Acylation and RhoGDI

Under physiological conditions, prenylation facilitates the interaction between Rho proteins and RhoGDI (Di-Poi et al., 2001; DerMardirossian and Bokoch, 2005). In cocrystal structures, the geranylgeranyl moiety of Cdc42, Rac1, and Rac2 was shown to insert into a hydrophobic pocket formed by the immunoglobulin-

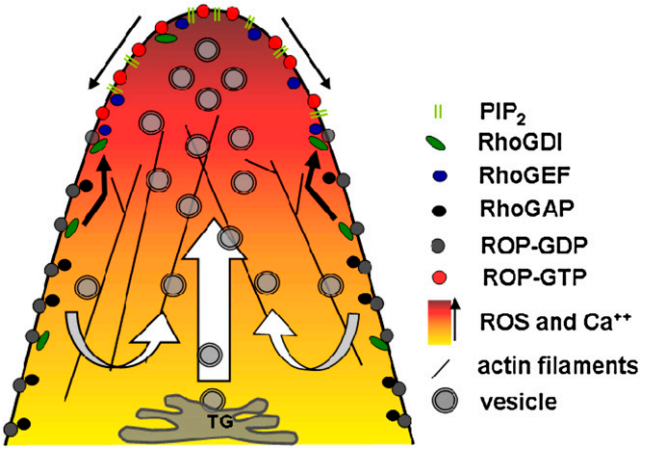

Figure 2. A model for ROP/RAC function during polar cell growth. Activated GTP-bound ROP/RAC GTPases accumulate at the plasma membrane at the apex of expanding cells based on lipid modification of the hypervariable domain, interactions of this domain with the membrane lipid PtdIns 4,5-P_2, RhoGDI-mediated ROP/RAC recycling, and, possibly, transport along membrane-trafficking pathways. ROP/RAC-GAPs associated with the plasma membrane at the flanks of the tip, together with spatially separated ROP/RAC-GEFs localized at the apex, contribute to the maintenance of highly focused apical ROP/RAC activity. This activity coordinates F-actin organization and membrane trafficking required for polarized cell expansion through the stimulation of multiple signaling pathways, including (1) the activation of PtdIns P-K, resulting in apical production of PtdIns 4,5-P_2, which is prevented from laterally spreading by PLC activity associated with the plasma membrane at the flanks of the tip and exerts multiple functions in the control of local ROP/RAC activation, F-actin organization, and membrane trafficking; (2) the stimulation of NADPH oxidase activity, which leads to the production of ROS, which contribute to the establishment of tip-focused Ca^{2+} gradients by opening Ca^{2+} channels; and (3) a number of additional ROP/RAC-dependent signaling mechanisms summarized in Figure 4. The polarization of ROP/RAC activity depends on the dynamic cycling of ROP/RAC GTPases between active and inactive states, which is based on the relatively inefficient GTP hydrolysis by these proteins, the spatial separation of activating GEF and inactivating GAP activities, RhoGDI-mediated recycling from sites of inactivation to the site of activation, and activation-dependent partitioning into discrete membrane microdomains.

like β sandwich of the RhoGDI (Hoffman et al., 2000; Scheffzek et al., 2000; Grizot et al., 2001). Given their structural conservation, plant RhoGDIs are predicted to function similar to their homologs in other organisms (Berken and Wittinghofer, 2008). Type II ROP/RAC GTPases are not prenylated in plants (Lavy et al., 2002), suggesting that they might be regulated by a RhoGDI-independent mechanism. Interestingly, in monocot grasses, type II ROP/RAC GTPases are more abundant than the type I proteins (Christensen et al., 2003). In mammalian cells, *S*-acylation of RhoA in the hypervariable domain inhibited its interaction with RhoGDI (Michaelson et al., 2001). It could be that transient G-domain *S*-acylation of activated type I ROP/RAC GTPases inhibits their accessibility for interaction with RhoGDI. *S*-Acylation may thus destabilize Rho interactions with RhoGDIs, similar to RhoGDI displacement factors such as PtdIns 4,5-P_2 (Fauré et al., 1999) and different proteins, including integrins (Del Pozo et al., 2002).

FUNCTIONAL IMPLICATIONS OF ACTIVATION-DEPENDENT *S*-ACYLATION OF ROP/RAC GTPASES

The findings on the activation-dependent transient *S*-acylation of AtROP6 and its consequential partitioning in DRMs have interesting regulatory implications. *S*-Acylation involves an unstable and reversible thioester bond, in contrast to prenylation, which is based on an irreversible thioether linkage (Fig. 1B). Due to its reversibility, *S*-acylation was suggested to play an important regulatory role in signaling processes (Smotrys and Linder, 2004). DRMs, often referred to as lipid rafts, are sterol- and sphingolipid-rich membrane microdomains that attract specific groups of proteins (Fig. 1B). Given their properties, lipid rafts were suggested to function as signaling hubs that can change their size and composition in response to external stimuli, favoring certain protein-protein interactions (Simons and Toomre, 2000). Characterization of DRMs in plants showed that, like their counterparts in animal cells and in yeast, they are enriched in sterols and sphingolipids. Furthermore, they contain characteristic glycosylphosphatidylinositol-anchored proteins and, importantly, a type I ROP/RAC protein (NtRAC5; Mongrand et al., 2004; Borner et al., 2005; Morel et al., 2006). In animal cells, *S*-acylated (palmitoylated) proteins partition into DRMs (Melkonian et al., 1999). Thus, transient *S*-acylation induces temporal partitioning of AtROP6 and likely other ROP/RAC GTPases to DRMs, where they potentially can interact with other proteins. Because constitutively active GTP-bound $Atrop6^{CA}$, which was always found to be both prenylated and *S*-acylated, localized exclusively in DRMs (Sorek et al., 2007), it is likely that type I ROP/RAC GTPases signal mostly from these membrane microdomains.

The lipid raft hypothesis is still bitterly debated (Munro, 2003). Yet, it is becoming accepted that DRMs are an inherent property of biological membranes (Hancock, 2006; Grennan, 2007). A unifying model for animal cells has been proposed that attempts to resolve the existing controversies about lipid rafts (Hancock, 2006). The model predicts that lipid rafts are short-lived entities that are stabilized by their protein constituents (i.e. *S*-acylated proteins attract sterols and sphingolipids, which in turn attract more proteins to form nanoclusters). According to this view, lipid rafts are short-lived microdomains that form and disintegrate. In line with this hypothesis, in yeast the levels and composition of sterols and sphingolipids are tightly linked (Pichler and Riezman, 2004), suggesting that increase in one component attracts the other. Thus, transient *S*-acylation of ROP/RAC GTPases may be responsible for temporally attracting certain proteins and molecules to discrete membrane domains.

Predictions based on modeling of the Ras-activated mitogen-activated protein kinase pathway in mammalian cells suggest that nanoclustering of Ras facilitates a mechanism that converts graded ligand inputs into fixed outputs. The model predicts that cells form Ras nanoclusters in direct proportion to the concentration of the input signal (e.g. epidermal growth factor), creating a high-fidelity signaling relay across the membrane. The concentration of the epidermal growth factor, which is analog like in its nature, is turned into a digital-like on/off reaction by the Ras switch. The signal is transmitted from Ras to the mitogen-activated protein kinase pathway, recreating an analog-like signal. The signal transmission is predicted to be fully dependent on Ras nanoclustering (Tian et al., 2007). Could a similar mechanism function in ROP/RAC signaling in plants? An analogous situation may be the auxin gradient-induced accumulation of ROP/RAC GTPases in trichoblasts at the future position of root hair formation that was detected using indirect immunofluorescence and GFP tagging (Carol et al., 2005; Fischer et al., 2006). In fact, auxin has been shown to activate ROP/RAC GTPases (Tao et al., 2002). It would be of interest to determine whether auxin-induced nanoclustering into DRMs is involved in the stimulation of ROP/RAC signaling by this hormone.

MOLECULAR MECHANISMS INVOLVED IN ROP/RAC-MEDIATED CELLULAR POLARIZATION

Mathematical models of Rho, Rac, and Cdc42 functions in cellular polarization predict that cycling between active and inactive states, together with fast diffusion to the cytosol of GDP-bound protein in complex with RhoGDI, are essential for obtaining robust spatial polarization rather than traveling waves (Maree et al., 2006; Jilkine et al., 2007). Models of Cdc42 function during bud formation in yeast predict that the energy obtained from GTP hydrolysis, along with RhoGDI-mediated relocation of GDP-bound Cdc42 to the cytoplasm, are required for polar bud growth and for the development of a single bud (Goryachev and Pokhilko, 2008). These models imply that discrete localization of polarizing factors, without cycling of Rho proteins between active and inactive states and relocation of the inactive form, is not sufficient to establish cell polarity. Mechanistically, the Rho switch mechanism that enables the binding and release of effectors, along with the spatial separation of the active and inactive states, form the basis for maintaining cell polarization.

Consistent with the theoretical models, numerous studies have demonstrated that the expression of constitutively active forms of both type I and type II ROP/RAC GTPases depolarizes cell expansion (Kost et al., 1999; Li et al., 1999; Fu et al., 2001, 2002; Molendijk et al., 2001; Jones et al., 2002; Chen et al., 2003; Bloch et al., 2005).

Figure 2 summarizes the current knowledge concerning factors involved in polarized ROP/RAC activation and downstream signaling, which is primarily based on studies in pollen tubes and root hairs. In pollen tubes, fluorescent ROP/RAC fusion proteins accumulate at the plasma membrane of the growing tip (Kost et al., 1999; Li et al., 1999; Klahre et al., 2006), where ROP/RAC activity was also detected using fluorescence resonance energy transfer-based analysis of effector binding (Hwang et al., 2005). RhoGAP was found to accumulate at the flanks of the tip (Klahre and Kost, 2006; Fig. 2). RhoGDI overexpression in tobacco pollen tubes effectively transferred the ROP/RAC GTPase NtRac5 from the plasma membrane to the cytoplasm and inhibited pollen tube growth. Furthermore, a mutant form of NtRAC5 specifically disrupted in its RhoGDI interaction was mislocalized to the flanks of the tip, where it appeared to remain essentially inactive (Klahre et al., 2006). These data suggest that RhoGDI-mediated recycling from the flanks of the pollen tube tip to the apex is responsible for ROP/RAC accumulation and activation at the apex of elongating pollen tubes.

Similar mechanisms appear to be involved in the polarization of ROP/RAC activity during root hair elongation. Indirect immunofluorescence and GFP tagging showed that ROP/RAC accumulation at a specific domain of the trichoblast plasma membrane, which is determined by an auxin gradient in the root epidermis, precedes root hair outgrowth (Molendijk et al., 2001; Carol et al., 2005; Fischer et al., 2006). Consistently, ROP GTPases regulate polar root hair growth (Molendijk et al., 2001; Jones et al., 2002; Bloch et al., 2005). Ectopic root hairs develop in the Arabidopsis RhoGDI mutant *scn1*, apparently as a consequence of enhanced and depolarized ROP/RAC accumulation at the trichoblast plasma membrane (Carol et al., 2005). This indicated that RhoGDI activity is essential for the polarization of ROP/RAC activity and cell expansion also during root hair development.

Consistent with the proposed RhoGDI function in the control of polarized ROP/RAC activation in pollen tubes, a minor proportion (less than 50%) of the ROP/RAC proteins in tobacco pollen tube protein extracts were detected in the insoluble fraction, whereas the rest were found in the soluble fraction (Kost et al., 1999). By contrast, in young seedling, leaves, and root protein extracts, endogenous ROPs were not identified in soluble fractions using protein immunoblots following centrifugal separation and membrane flotation-centrifugation assays (Sorek et al., 2007).

ROP/RAC targeting seems to depend particularly strongly on GDI function in tip-growing pollen tubes, whereas the GDI-bound soluble ROP/RAC fraction in other cell types appears to be below the detection limit of biochemical assays. Identification in these cell types of small fractions of GDI-bound soluble ROP/RAC GTPases undergoing rapid cycling between the cytoplasm and the plasma membrane may require sensitive fluorescence imaging techniques, such as spot fluorescence recovery after photobleaching, which can differentiate between lateral diffusion within mem-

branes and movement on/off membranes, or total internal reflection fluorescence, which enables monitoring of fluorescent structures in close proximity of the plasma membrane.

Plants contain a unique family of ROP/RAC-GEFs named PRONE (for plant-specific ROP nucleotide exchanger) after their catalytic domain (Berken et al., 2005; Gu et al., 2006). Identification of an interaction between the cytoplasmic domain of the pollen-specific receptor protein kinases LePRK1/LePRK2 and the PRONE family ROP/RAC-GEF kinase partner protein provided the first insight on how localized activation of ROP/RAC GTPases may be achieved (Kaothien et al., 2005; Shichrur and Yalovsky, 2006). PRONE ROP/RAC-GEFs appear to colocalize with active ROP/RAC GTPases to the plasma membrane at the pollen tube apex (Gu et al., 2006), which supports an important function of these proteins in localized ROP/RAC activation. PRK homologs may phosphorylate pollen-specific ROP/RAC-GEFs, causing their activation by relieving intramolecular autoinhibition (Zhang and McCormick, 2007). However, it remains to be demonstrated whether PRKs function as scaffolds that recruit both ROP/RAC-GEFs and ROP/RAC GTPases to specific membrane domains.

Given the theoretical models described above and available experimental data, the following model could be suggested (Fig. 2). ROP/RAC GTPases are recruited to highly specific membrane domains, for example by a morphogen (auxin) gradient, as in the case of root hairs (Fischer et al., 2006). Sensing of extracellular signals results in the activation of scaffold proteins that recruit and activate ROP/RAC-GEFs (Zhang and McCormick, 2007), facilitating ROP/RAC activation. ROP/RAC activation is transient and regulated by the ROP/RAC-GEFs, intrinsic GTPase activity, and ROP/RAC-GAPs. The half-life of ROP/RAC GTPase activity is about 10 min without a GAP (Lemichez et al., 2001; Molendijk et al., 2001) and 2 min in the presence of a GAP (Klahre and Kost, 2006). At least in growing pollen tubes, spatial separation of GAP activity to the flanks of the tip provides means to restrict the distribution of activated ROP/RAC GTPases to the apex. RhoGDIs can remove GDP-bound ROP/RAC GTPases from the membrane and function to facilitate highly localized ROP/RAC distribution (Carol et al., 2005; Klahre et al., 2006).

Through the regulation of the actin and microtubule cytoskeleton, and of membrane trafficking, activated ROP/RAC GTPases promote the establishment of robust cellular polarity.

CONTROL OF MEMBRANE TRAFFICKING BY ANIMAL AND YEAST RHO GTPASES

An important function of Rho GTPases in animal and yeast cells is the control of membrane trafficking. Yeast Rho GTPases accumulate at the plasma membrane at sites of directional cell expansion, where they promote localized secretion required for this process (Brennwald and Rossi, 2007). In animal cells, activated Rho GTPases are not only associated with the plasma membrane but also with intracellular compartments, including the Golgi and endosomal organelles. At these locations, Rho GTPases positively or negatively regulate specific membrane-trafficking events required for secretion, clathrin-dependent and -independent endocytosis, Golgi-to-ER transport, or recycling from endosomes back to the plasma membrane (Symons and Rusk, 2003; Ridley, 2006).

Although the molecular mechanisms by which activated Rho GTPases control membrane trafficking in animal and yeast cells are not fully understood, Rho-induced F-actin reorganization clearly plays a key role in these regulatory processes. Animal and yeast Rho GTPases promote F-actin nucleation via direct interactions with formins (Kovar, 2006) or with WASP/WAVE complex proteins that activate the Arp2/3 complex (Millard et al., 2004). In addition, Rho-dependent signaling pathways modulate the activity of key actin regulatory proteins such as ADF (for actin-depolymerizing factor)/cofilin (DesMarais et al., 2005). Activation of animal or yeast Rho GTPases is thought to result in the local formation of distinct F-actin structures. In turn, these F-actin structures can promote the formation of transport vesicles at donor membranes, stimulate or inhibit vesicle fusion with target membranes, or facilitate the directional movement of transport vesicles or larger membrane compartments through the cytoplasm (Bader et al., 2004; Ridley, 2006).

In yeast and animal cells, active Rho GTPases stimulate secretion not only via F-actin reorganization but also by directly interacting with and activating components of the exocyst. The exocyst is an octameric complex implicated in the tethering of post-Golgi vesicles, which promotes the fusion of these vesicles with the plasma membrane (TerBush et al., 1996; Brennwald and Rossi, 2007). Yeast Rho1, Rho3, and Cdc42 interact with Sec3 and Exo70 subunits of the exocyst complex (Guo et al., 2001; Novick and Guo, 2002; Roumanie et al., 2005). These interactions are thought to be required for the correct intracellular targeting of the exocyst complex and for its activation. Mammalian Sec3 homologs lack the Rho-binding domain present in yeast Sec3 (Guo et al., 2001), but the Rho GTPase TC10 directly interacts with an Exo70 homolog in mammalian cells and recruits this protein to lipid rafts in the plasma membrane. This in turn promotes delivery of the Glc transporter GLUT4 to the plasma membrane in response to insulin (Inoue et al., 2003).

Additional regulatory mechanisms by which animal Rho GTPases modulate membrane trafficking include the direct or indirect interaction with coat proteins (clathrin, coatmer/COP-1) involved in the formation and cargo loading of transport vesicles (Yang et al., 2001; Chen et al., 2005) and the recruitment of lipid kinases (PtdIns P-K) responsible for localized generation of the signaling lipid PtdIns 4,5-P_2 (Oude Weernink

et al., 2004). PtdIns 4,5-P_2 is a key regulator of membrane trafficking, which appears to have direct functions in the formation of endocytic transport vesicles (Cremona and De Camilli, 2001) as well as in the fusion of secretory vesicles with the plasma membrane (Bader et al., 2004). In addition, PtdIns 4,5-P_2 recruits Exo70 and Sec3 and thereby contributes to the correct intracellular targeting of the exocyst in yeast (He et al., 2007; Zhang et al., 2008). PtdIns 4,5-P_2 also stimulates Rho membrane association and activation, binds to and modulates the activity of a variety of actin regulatory proteins including ADF/cofilin (DesMarais et al., 2005), or may be hydrolyzed by phospholipase C (PLC) activity to inositol 3-phosphate (Ins 3-P), which opens Ca^{2+} channels to allow Ca^{2+} influx into the cytoplasm (Taylor, 2002). Elevated Ca^{2+} levels trigger the fusion of Golgi-derived vesicles with the plasma membrane in secretory animal cells (Bader et al., 2004).

ROP/RAC-REGULATED F-ACTIN STRUCTURES AND MEMBRANE TRAFFICKING ARE ESSENTIAL FOR POLAR CELL GROWTH IN PLANTS

The polarized growth of plant cells has long been thought to depend on internal turgor pressure built up by water accumulation in the large central vacuole and on microtubule-directed deposition of cell wall-reinforcing cellulose microfibrils. These cellulose microfibrils can restrict cell expansion in all directions other than the main growth axes. In recent years, it has become increasingly clear that F-actin structures and membrane trafficking controlled by ROP/RAC GTPases also play key roles in the directional expansion of plant cells (Smith and Oppenheimer, 2005; Hussey et al., 2006; Mathur, 2006).

Most plant cells undergo diffuse growth (i.e. they expand in all directions to some extent), although growth occurs mainly along one or more main axes. Diffuse cell growth has been shown to be associated with a network of actin cables and filaments extending throughout the cytoplasm, which displays a net alignment along growth axes, and with diffuse cortical F-actin structures underlying the plasma membrane at growth sites (Dong et al., 2001; Fu et al., 2005). By contrast, root hairs and pollen tubes are highly elongated uniaxial cells with species-specific length-diameter ratios of 100 to more than 1,000. Pollen tubes and root hairs expand exclusively at the apical end in a strictly polarized manner, based on a process known as tip growth. Longitudinally oriented actin cables in the shank that are apparently required for myosin-mediated organelle transport through the cytoplasm and fine F-actin structures at the tip are essential for the elongation of these cells (Kost, 2008). Apical F-actin structures in pollen tubes appear to include a subapical cortical F-actin ring or fringe (Kost et al., 1998; Lovy-Wheeler et al., 2005) and, possibly, fine filaments underlying the plasma membrane (Fu et al., 2001). Both of these F-actin structures were proposed to promote the transport of secretory vesicles to sites of their fusion with the plasma membrane (Fu et al., 2001; Cardenas et al., 2008), although the exact organization and function of apical F-actin in pollen tubes is still controversial.

A dense cortical F-actin network similar to the one underlying the plasma membrane in lamellipodia of motile animal cells has not been identified in expanding plant cells, indicating that membrane protrusion driven by actin polymerization does not play a role in the polarized growth of these cells. Rather, F-actin structures seem to promote membrane trafficking required for the local deposition of new cell wall material in the extracellular matrix at sites where plant cells expand (Wasteneys and Galway, 2003). A quantitative analysis of pollen tube growth has indicated that the delivery of sufficient material for cell wall construction to the apical growth site depends on the fusion of secretory vesicles with the plasma membrane at a much higher rate than is required for plasma membrane extension. This suggests an important role not only of secretion but also of endocytic membrane recycling in the polarized growth of plant cells (Derksen et al., 1995).

ROP/RAC GTPases clearly have important functions in the control of both diffuse cell expansion and tip growth. Overexpression of constitutively active ROP/RAC GTPases enhances and depolarizes diffuse cell growth (Molendijk et al., 2001; Bloch et al., 2005; Fu et al., 2005), whereas Arabidopsis mutants defective in the *SPIKE1* (*SPK1*) gene, which encodes a ROP/RAC-GEF required for ROP/RAC activation, show severe defects in diffuse cell expansion and remain highly stunted (Basu et al., 2008). In tip-growing cells, ROP/RAC GTPases accumulate at the plasma membrane specifically at the apex. Overexpression of wild-type or constitutively active ROP/RAC GTPases depolarizes the growth of these cells and can result in massive apical ballooning. By contrast, expression of dominant negative ROP/RAC GTPases strongly inhibits tip growth (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002). Effects of ROP/RAC overexpression on root hair growth are shown in Figure 3. Constitutively active GFP-AtROP11/RAC10^{CA} induced the depolarization of root hair growth (Fig. 3, C–E), while wild-type GFP-AtROP11/RAC10 caused root hair swelling without entirely abolishing polar cell expansion (Fig. 3B; Bloch et al., 2005).

Accumulating evidence summarized in the following sections strongly suggests that ROP/RAC GTPases control polarized cell growth by regulating membrane trafficking both via the control of actin organization and via actin-independent pathways. An important function of ROP/RAC-regulated F-actin structures in membrane trafficking has also been demonstrated during pathogen defense reactions. The prevention of the penetration of resistant barley (*Hordeum vulgare*) cells by the fungus powdery mildew was shown to involve ROP/RAC-dependent formation of actin filaments polarized toward the site of fungal attack. These

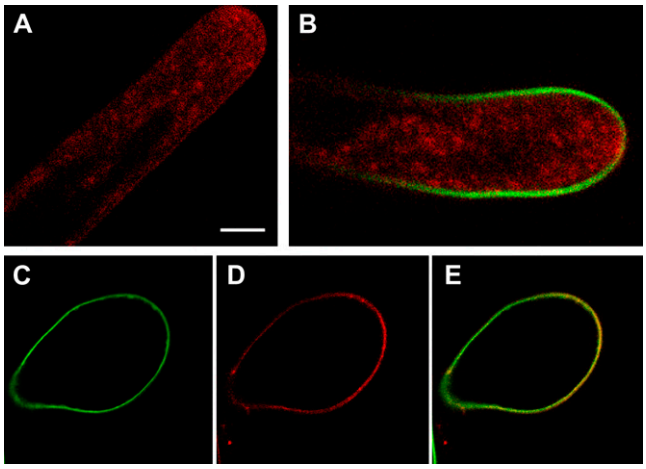

Figure 3. Internalization of FM4-64 is inhibited in swollen root hair of GFP-AtROP11/RAC10^{CA} plants. A, Extensive internalization of FM4-64 is detected within minutes after labeling of wild-type root hairs. B, FM4-64 is internalized into a partially swollen root hair of a transgenic plant expressing wild-type GFP-AtROP11/RAC10. C to E, FM4-64 was not internalized into swollen root hairs of transgenic plants expressing a constitutively active GFP-AtROP11/RAC10^{CA} even after 90 min of incubation at room temperature. C, GFP-AtROP11/RAC10^{CA}. D, FM4-64. E, GFP/FM4-64 overlay. Green indicates GFP and red indicates FM4-64. Adapted with permission from Bloch et al. (2005).

actin filaments are thought to promote membrane trafficking required for the deposition of additional cell wall material and of defense compounds at this site (Opalski et al., 2005).

DIRECT EVIDENCE FOR ROP/RAC-MEDIATED CONTROL OF MEMBRANE TRAFFICKING AND EXOCYST FUNCTION

Overexpression of constitutively active GFP-AtROP11/RAC10^{CA} in transgenic Arabidopsis plants was shown to block the uptake of the styryl dye FM4-64, an established tracer of endocytic membrane internalization, in root hairs and to interfere with the formation of brefeldin A (BFA) compartments in these and other cells (Bloch et al., 2005). BFA compartments are formed in plant cells treated with the Arf-GEF inhibitor BFA by the aggregation of aberrant endocytic endosomes and secretory (trans-Golgi network) organelles. These compartments have been shown to contain plasma membrane proteins and lipids that normally undergo endocytic recycling (Nebenführ et al., 2002; Geldner et al., 2003; Grebe et al., 2003). The effects of ROP/RAC overexpression on FM4-64 uptake by transgenic root hairs are shown in Figure 3. In nontransformed root hairs and in transgenic root hairs expressing GFP fused to wild-type AtROP11/RAC10, massive FM4-64 internalization was observed within minutes (Fig. 1, A and B). By contrast, FM4-64 internalization was not detectable even after prolonged incubation at room temperature in swollen root hairs expressing GFP fused to constitutively active AtROP11/RAC10^{CA} (Fig. 3, C–E; Bloch et al., 2005). Together, these observations strongly suggest a role of ROP/RAC GTPases in the control of endocytic membrane uptake. Considering the proposed function of lipid rafts in the regulation of endocytosis in animal and yeast cells (Hancock, 2006), it will be interesting to test whether the accumulation of activated ROP/RAC GTPases in DRMs has a function in the control of membrane internalization in plants.

Another direct link between ROP/RAC GTPases and the control of membrane trafficking was established by the observation that an Arabidopsis protein called ICR1 (for interactor of constitutively active ROP1) binds directly to AtSEC3A, a homolog of the yeast regulatory exocyst component Sec3 (Lavy et al., 2007). ICR1 is a member of a family of plant-specific coiled-coil scaffold proteins that interact with activated ROP/RAC GTPases (Lavy et al., 2007). In yeast, Sec3 is thought to function as a landmark exocyst subunit. Its recruitment to the plasma membrane by Cdc42 was proposed to mark the site of exocyst complex formation (Guo et al., 2001; Novick and Guo, 2002; Zhang et al., 2008). Genes encoding all eight exocyst subunits have been identified in Arabidopsis (Elias et al., 2003), and the existence of the exocyst complex was recently demonstrated (Hala et al., 2008). Interestingly, root hairs of maize (*Zea mays*) *sec3* mutants fail to elongate (Wen et al., 2005), pollen tube growth is abolished in Arabidopsis SEC8 and *sec6* mutants (Cole et al., 2005; Hala et al., 2008), and different forms of polar cell expansion are compromised in Arabidopsis *exo70a1* mutants (Synek et al., 2006). These observations demonstrate that the plant exocyst is required for polar cell growth, presumably because of its essential function in localized exocytosis. AtSEC3 lacks an N-terminal Rho interaction domain and does not bind directly to ROP/RAC GTPases (Lavy et al., 2007). However, AtSEC3 was shown to interact directly with ICR1, and ROP/RAC GTPases were demonstrated to recruit ICR1-AtSEC3 complexes to the plasma membrane (Lavy et al., 2007). *icr1* mutant plants display abnormally shaped leaf epidermal cells and compromised primary root development, a phenotype similar to that of some mutants defective in polar auxin transport (Lavy et al., 2007). It remains to be established whether polar auxin transport is indeed compromised in this mutant. However, it appears that the regulation of exocyst function and membrane trafficking by ROP/RAC GTPases through their interaction with ICR1 plays an important role in the control of polar cell expansion in plants (Lavy et al., 2007).

ROP/RAC GTPASES CONTROL CYTOSKELETAL STRUCTURES REQUIRED FOR POLARIZED CELL GROWTH BY REGULATING THE ARP2/3 COMPLEX AND RIC FAMILY EFFECTORS

Several ROP/RAC signaling pathways appear to regulate F-actin structures and to influence microtu-

bular organization during polar cell expansion. One of these pathways involves the DOCK180-like ROP/RAC-GEF SPK1 along with the WAVE and Arp2/3 complexes. Other pathways are based on CRIB domain-containing ROP/RAC effectors called RICs (for ROP-interacting CRIB) or on actin-binding proteins such as ADF/cofilin. Mutations in genes coding for all these regulatory factors have profound effects on directional cell expansion. Current knowledge concerning the functions of these factors is discussed in this and the following section.

Role of the WAVE and Arp2/3 Complexes in the ROP/RAC-Mediated Control of Cytoskeletal Organization and Cell Expansion

Trichomes are large cellular structures that protrude from the leaf epidermis. In Arabidopsis, they are single cells typically composed of a central stalk and three distal branches with pointed tips. Although the cell expansion mechanisms responsible for the development of the complex morphology of these cells are not fully understand, they are thought to depend in part on diffuse cell expansion and clearly require microtubules and the actin cytoskeleton (Mathur, 2005; Szymanski, 2005).

The *distorted* class of Arabidopsis mutants develop deformed trichomes with abnormal F-actin organization, swollen stalks, and short, mispositioned branches. Essentially identical cellular defects are caused by the treatment of developing trichomes with drugs that interfere with F-actin stability (Mathur et al., 1999; Szymanski et al., 1999). Interestingly, *distorted* mutants are defective in genes encoding homologs of components of either the WAVE or the Arp2/3 complex (Mathur, 2005; Szymanski, 2005). In animal cells, Rho activates the Arp2/3 complex through stimulation of the WAVE complex to induce F-actin nucleation. The observation that activated ROP/RAC GTPases directly interact with components of the regulatory WAVE complex suggested that also in plant cells Arp2/3 activity is modulated by Rho signaling (Basu et al., 2004; Uhrig et al., 2007).

Trichomes formed by mutants defective in the gene encoding the ROP/RAC-GEF SPK1 fail to branch (Qiu et al., 2002). Genetic and biochemical evidence indicates that SPK1-mediated ROP/RAC activation controls cell morphogenesis through stimulation of the WAVE and Arp2/3 complexes (Basu et al., 2008). Interestingly, SPK1 was also found to directly interact with WAVE complex proteins (Uhrig et al., 2007; Basu et al., 2008), suggesting that the WAVE complex may function as a scaffold that interacts with SPK1, ROP/RAC GTPases, and the Arp2/3 complex to coordinate local F-actin reorganization. Presumably, the WAVE complex is recruited to specific membrane domains by activated ROP/RAC GTPases. Immunofluorescence data show that in epidermal mutant *spk1* cells, the microtubular cytoskeleton is more strongly affected than F-actin organization (Qiu et al., 2002), suggesting that SPK1-controlled ROP/RAC GTPases also control microtubular structures in these cells, either directly by an unknown pathway or indirectly through WAVE-Arp2/3-mediated control of actin dynamics.

Analysis of *distorted* mutants has shown that, in addition to aberrant trichome morphogenesis, these plants display cell expansion and cell adhesion defects, which are particularly clearly detectable in the epidermis of rapidly growing organs such as etiolated hypocotyls and cotyledons (Mathur, 2005; Szymanski, 2005). The observed defects in cell adhesion indicate that ROP/RAC-controlled WAVE- and Arp2/3-dependent F-actin structures in epidermal cells may mediate membrane trafficking required for the secretion of cell wall material (Szymanski, 2005).

Interestingly, plants carrying mutant alleles of *DISTORTED* genes, which are ubiquitously expressed at low levels throughout plants (Mathur, 2005), display relatively mild phenotypes and a normal overall architecture, whereas cell expansion and organogenesis are much more strongly affected in *spk1* mutants. This suggests that ROP/RAC GTPases targeted by SPK1 GEF activity have important functions in the control of the diffuse expansion of a range of cell types but depend on the WAVE-Arp2/3 pathway to regulate actin organization and membrane trafficking, particularly strongly in trichomes and other epidermal cells.

Mutant *spk1* and *distorted* alleles are normally transmitted through the male gametophyte (pollen tube) during sexual reproduction and have only weak effects on root hair morphology (Qiu et al., 2002; Mathur, 2005; Szymanski, 2005), demonstrating that SKP1-dependent ROP/RAC activity or WAVE-Arp2/3 signaling are not essential for tip growth.

Role of RIC Family Effectors in the ROP/RAC-Mediated Control of Cytoskeletal Organization and Cell Expansion

RICs form a plant-specific family of ROP/RAC effectors that all contain a CRIB domain but share little homology outside of this domain with each other or with any other proteins (Wu et al., 2001). CRIB domains are also present in many effectors of animal and yeast Rho GTPases and confer specific binding to active GTP-bound Rho proteins (Pirone et al., 2001). Several Arabidopsis RICs have been reported to play key roles in the control of polar cell growth downstream of ROP/RAC activation.

Counteracting RIC1- and RIC4-dependent pathways have been proposed to underlie the ROP/RAC-dependent control of directional cell expansion in developing pavement cells in the epidermis of Arabidopsis leaves (Fu et al., 2005). AtROP2 and AtROP4 accumulate at the plasma membrane, preferentially in expanding lobes formed by these cells. Via RIC4 activation, these GTPases appear to promote the local formation of a cortical network of fine actin filaments beneath the plasma membrane in expanding lobes.

Active AtROP2 and AtROP4 also seem to bind and sequester RIC1, which can strongly associate with microtubules and is thought to promote the formation of highly ordered cortical microtubule arrays in non-expanding regions between lobes. In addition, RIC1 appears to have the ability to reduce AtROP2 and AtROP4 activity via an unknown negative feedback mechanism. Based on these observations, Fu and colleagues (2005) have proposed that a delicate balance between interacting RIC4 and RIC1 pathways, which establishes an alternating pattern of fine actin networks and highly ordered microtubule structures in the cell cortex, is responsible for the control of pavement cell morphogenesis by AtROP2 and AtROP4. Immunofluorescence studies (Qiu et al., 2002) have shown that alternating F-actin and microtubular structures are present in the cortex of cotyledon epidermal cells already at early developmental stages before lobes are formed. It will be interesting to determine the roles of SPK1, ROP/RAC activity, RIC proteins, and the WAVE-Arp2/3 complexes in the control of cytoskeletal organization at these early developmental stages.

Two different counteracting RIC-dependent pathways have also been proposed to participate in the control of tip growth by ROP/RAC GTPases (Gu et al., 2005). Similar to the situation in epidermal cells, RIC4 activated by AtROP1, which accumulates at the apical plasma membrane, was suggested to promote the formation of a network of fine actin filaments at the pollen tube tip. Tip growth of plant cells depends on a steep tip-focused Ca^{2+} gradient. As in secretory animal cells, elevated cytoplasmic Ca^{2+} levels in the low micromolar range (Pierson et al., 1996) are thought to promote secretion at the apex of tip-growing plant cells via F-actin regulation and/or via actin-independent pathways (Messerli et al., 2000). ROP/RAC-mediated activation of RIC3 appears to stimulate Ca^{2+} influx into the cytoplasm at the pollen tube tip, which induces depolymerization of the RIC4-induced F-actin network. In turn, the RIC4 pathway was found to down-regulate the RIC3 pathway in an F-actin-dependent manner. Consistent with the observation that the rate of cell expansion, as well as apical cytoplasmic Ca^{2+} levels, are often oscillating during tip growth, ROP/RAC-mediated control of this process may depend on a temporally coordinated balance between the antagonistic RIC4 and RIC3 pathways (Gu et al., 2005). Stretch-activated Ca^{2+} channels at the pollen tube apex, which allow Ca^{2+} influx into the cytoplasm at high growth rates, have also been proposed to play an important role in the control of oscillatory tip growth along with RAC/ROP-dependent RIC3 and RIC4 signaling pathways (Cardenas et al., 2008).

While RIC1, RIC4, and RIC3 undoubtedly play important roles as ROP/RAC effectors in the regulation of directional cell expansion, the molecular mechanisms they employ to control cytoskeletal organization and membrane trafficking remain to be identified.

FUNCTION OF OTHER ROP/RAC-CONTROLLED ACTIN REGULATORY FACTORS (ADF, FORMINS) IN THE CONTROL OF MEMBRANE TRAFFICKING AND POLAR CELL GROWTH

Like animal and yeast Rho GTPases, ROP/RAC GTPases can also modulate F-actin organization by regulating ADF/cofilin and, possibly, formin activity. The pollen-specific tobacco ADF homolog NtADF1 associates with F-actin structures in pollen tubes and contains a conserved regulatory phosphorylation site (Ser-6) near the N terminus (Chen et al., 2003). NtADF1 overexpression in pollen tubes depolymerizes fine actin filaments, inhibits cell expansion, and counteracts growth depolarization induced by ROP/RAC overexpression. Interestingly, ROP/RAC overexpression results in enhanced phosphorylation of NtADF1 at Ser-6, which reduces the ability of this protein to associate with F-actin, inhibit cell expansion, and block ROP/RAC-induced growth depolarization (Chen et al., 2002, 2003). These observations suggest that, similar to the situation in animal cells, ROP/RAC activation can promote F-actin assembly not only via the stimulation of the Arp2/3 complex (see above) but also by inducing ADF phosphorylation and inactivation. Homologs of LIM kinases, which phosphorylate ADF in response to Rho activation in animal cells, are not found in plants. Instead, plant ADF was shown to be phosphorylated by calmodulin-like protein kinases (Allwood et al., 2001).

As discussed above, animal Rho GTPases promote F-actin nucleation alternatively through the Arp2/3 complex or through formins. Because Arp2/3 functions appear to be largely restricted to trichomes and other epidermal cells in plants, it is tempting to speculate that ROP/RAC-mediated modulation of formin activity may play an important role in the control of F-actin organization during the expansion of other cells in these organisms. Members of a structurally diverse family of Arabidopsis proteins containing an FH2 domain, which is responsible for the F-actin-nucleating activity of animal and yeast formins, have been shown to display such activity in vitro and/or to promote F-actin formation in vivo (Staiger and Blanchoin, 2006). Interestingly, one of these Arabidopsis formins (AtFH5) is associated with the expanding cell plate, a membranous compartment mediating the construction of a new cell wall between daughter cells during cytokinesis (Ingouff et al., 2005). AtFH5 is essential for the completion of cytokinesis, possibly because it plays a role in the massive vesicle trafficking to and from the cell plate that is required for the normal function of this structure (Jurgens, 2005). A more extensive functional characterization of plant formins, which all seem to be missing domains homologous to those mediating direct binding of animal or yeast formins to Rho GTPases, is required to determine whether any of these proteins act downstream of ROP/RAC activation in the control of actin organization and membrane trafficking.

ROS PRODUCTION BY ROP/RAC-DEPENDENT NADPH OXIDASE ACTIVITY PROMOTES Ca^{2+} INFLUX INTO THE CYTOPLASM REQUIRED FOR TIP GROWTH

ROP/RAC GTPases have been reported to directly bind to and activate NADPH oxidase subunits (Wong et al., 2007). In root hairs (Foreman et al., 2003) and in pollen tubes (Potocky et al., 2007), NADPH oxidase activity responsible for the apical accumulation of reactive oxygen species (ROS) was shown to be essential for cell elongation. Through the stimulation of plasma membrane Ca^{2+} channels, ROS are thought to be essential for the establishment of a tip-focused Ca^{2+} gradient required for root hair growth (see above; Foreman et al., 2003). Interestingly, ROS levels at the root hair tip were recently shown to fluctuate periodically in coordination with oscillations in extracellular pH and in cell elongation rate (Monshausen et al., 2007).

In addition to presumably regulating actin organization and membrane trafficking, elevated Ca^{2+} levels at the root hair apex were recently demonstrated to stimulate NADPH oxidase activity. This creates a positive feedback loop that is likely to contribute to the maintenance of the polarity of root hair growth (Takeda et al., 2008). Root epidermal cells of Arabidopsis *scn1* mutants missing a RhoGDI isoform are unable to polarize ROP/RAC activity and consequently fail to form normal root hairs (see above). ROS production is also enhanced and depolarized in these cells, suggesting that NADPH oxidase activity required for root hair formation is under the control of ROP/RAC GTPases (Carol et al., 2005).

ROP/RAC ACTIVITY PROMOTES APICAL ACCUMULATION OF SIGNALING LIPIDS IN TIP-GROWING CELLS

PtdIns 4,5-P_2

As discussed above, like animal Rho GTPases, pollen tube ROP/RAC GTPases physically interact with PtdIns P-K activity, which generates the signaling lipid PtdIns 4,5-P_2 (Kost et al., 1999). ROP/RAC GTPases and PtdIns 4,5-P_2 both accumulate to high levels at the apex of pollen tubes (Kost et al., 1999; Dowd et al., 2006) and root hairs (van Leeuwen et al., 2007; Stenzel et al., 2008). Sequestering PtdIns 4,5-P_2 by high-level overexpression of GFP fused to the PH domain of rat PLCδ1 inhibits pollen tube elongation (Kost et al., 1999). These observations suggest that PtdIns 4,5-P_2 may act as a ROP/RAC effector in tip-growing cells, which promotes vesicle fusion with the apical plasma membrane directly, by recruiting exocyst components, or via its ability to modulate the activity of actin regulatory proteins. Recent evidence indicates that, besides fusion of secretory vesicles, fission of endocytic vesicles may also occur at the apical plasma membrane of pollen tubes (Moscatelli et al., 2007; Zonia and Munnik, 2008), another process known to be modulated by PtdIns 4,5-P_2. In addition to regulating membrane trafficking as a ROP/RAC effector, PtdIns 4,5-P_2 potentially participates in ROP/RAC activation as part of positive feedback loops that help to polarize ROP/RAC signaling and cell expansion at the apex of tip-growing cells (Kost, 2008).

PtdIns 4,5-P_2 hydrolysis by PLC activity associated with the pollen tube plasma membrane at the flanks of the tip appears to be required to restrict the distribution of this lipid to the apex (Dowd et al., 2006; Helling et al., 2006). Diacyl glycerol, the lipid product of PLC-mediated PtdIns 4,5-P_2 hydrolysis, also accumulates at the pollen tube apex, apparently depending on an endocytic recycling mechanism that transports this lipid to the apex from the site of its generation at the flanks of the tip (Helling et al., 2006). The activation of protein kinase C by diacyl glycerol is a well-characterized and important signaling pathway in animal cells (Yang and Kazanietz, 2003), but homologs of this kinase do not seem to exist in plants (Meijer and Munnik, 2003). The characterization of possible functions of diacyl glycerol in the ROP/RAC-dependent control of membrane trafficking in pollen tubes requires the identification of factors whose activity is modulated by this lipid.

Ins 3-P

Interestingly, PLC-mediated PtdIns 4,5-P_2 hydrolysis also generates Ins 3-P, a soluble molecule well known for its ability to induce Ca^{2+} influx into the cytoplasm of pollen tubes (Franklin-Tong et al., 1996; Monteiro et al., 2005) and other cells (Taylor, 2002). It will be interesting to investigate the possibility that PLC-mediated Ins 3-P production links ROP/RAC-stimulated PtdIns 4,5-P_2 synthesis to the establishment of the tip-focused Ca^{2+} gradient, which appears to have important functions in the control of membrane trafficking in tip-growing cells (see above).

MUTUAL DEPENDENCE OF ROP/RAC SIGNALING AND MEMBRANE TRAFFICKING?

While most of the evidence summarized above links ROP/RAC signaling to the control of membrane trafficking, it appears likely that membrane trafficking in turn also affects ROP/RAC signaling. As discussed above, like other Rho proteins, type I ROP/RAC GTPases presumably undergo postprenylation CaaX processing at the ER before they are transported to the plasma membrane, possibly along the secretory pathway. The promotion of secretion by ROP/RAC GTPases, along with the transport of these proteins on the surface of secretory vesicles to sites of their activation at the plasma membrane, potentially creates a positive feedback mechanism that contributes to the maintenance of cellular polarization. As exemplified by the proposed transport of diacyl glycerol from the flanks of

the pollen tube tip to the apex, endocytic membrane uptake or recycling mechanisms may regulate the intracellular distribution of membrane-associated factors involved in ROP/RAC signaling, including ROP/RAC-activating transmembrane receptors or ROP/RAC-GEFs. Again, the control of such mechanisms by ROP/RAC signaling would create an excellent opportunity for positive or negative feedback regulation that could play a very important role in the control of cell polarization. Consistent with the proposed interdependence of membrane trafficking and Rho signaling, blocking secretion and/or endocytic recycling by BFA treatment prevents the polarized accumulation of type I ROP/RAC activity at the plasma membrane of root epidermal cells, which is required for root hair outgrowth (Molendijk et al., 2001). However, BFA did not induce the accumulation of AtROP11/RAC10 in BFA bodies or prevented its accumulation at the plasma membrane (Bloch et al., 2005). This indicated that the intracellular targeting of this protein, and possibly other type II ROP/RAC GTPases, does not depend on BFA-sensitive membrane trafficking.

Another implication of the stimulation of membrane trafficking by activated ROP/RAC GTPases is that the membrane domains with which these proteins are associated are presumably undergoing constant remodeling. At the tip of rapidly elongating pollen tubes, where membrane trafficking is particularly dynamic, GAP- and GDI-dependent ROP/RAC recycling from the flanks to the apex was proposed to be required to compensate for the constant lateral displacement of ROP/RAC activity caused by the massive fusion of secretory vesicles with the plasma membrane (Kost, 2008). It appears likely that, consistent with mathematical models of Rho-mediated cellular polarization (see above), the polarization of ROP/RAC signaling and membrane trafficking in cells undergoing diffuse growth is maintained by dynamic feedback regulation rather than by the stable association of polarity determinants with growth sites.

CONCLUSION AND OUTLOOK

Recent years have seen much progress in our understanding of the regulation of ROP/RAC function and of the downstream signaling network stimulated by their activity. Although Rho regulation and down-

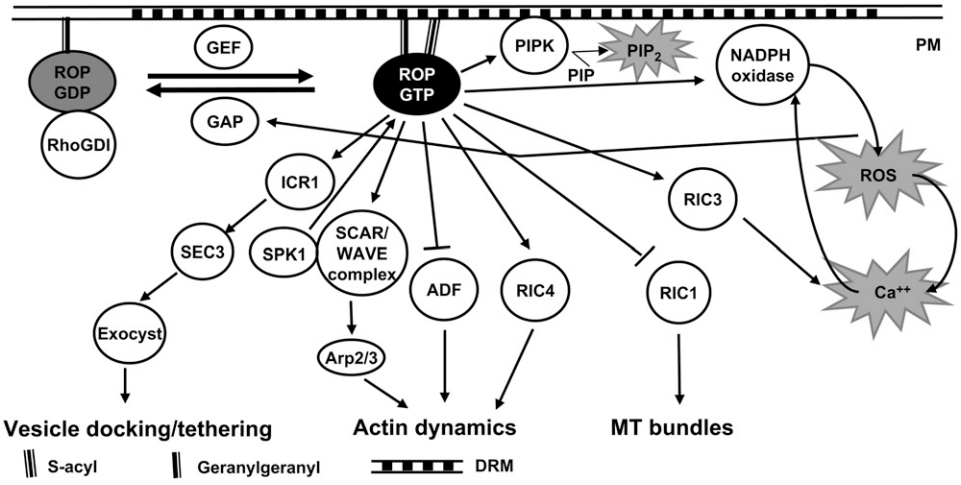

Figure 4. ROP/RAC-dependent signaling network. ROP/RAC signaling occurs at discrete domains of the plasma membrane (PM). ROP/RAC GTPases regulate actin dynamics through different pathways. WAVE and Arp2/3 complexes induce F-actin nucleation, possibly downstream of local ROP activation by the DOCK-180 ROP-GEF SPK1. ROP/RAC activity suppresses ADF/cofilin-induced actin depolymerization and stimulates RIC4-dependent actin polymerization. ROP/RAC GTPases also bind to and sequester RIC1, which locally prevents this protein from promoting the formation of cortical microtubular structures. The ROP/RAC-interacting scaffold protein ICR1 binds to the exocyst subunit SEC3, directly linking ROP activation to the control of secretion. Observed changes in cell morphology induced by ICR1 overexpression suggest that this protein may also contribute to the regulation of cytoskeletal organization. Activation of NADPH oxidases by ROP/RAC GTPases results in ROS production, which induces Ca^{2+} influx into the cytoplasm by opening Ca^{2+} channels. In turn, Ca^{2+} binding enhances NADPH oxidase activity, which generates a positive feedback loop. ROP/RAC activation also increases cytoplasmic Ca^{2+} levels through RIC3 interaction. Elevated cytoplasmic Ca^{2+} levels are thought to depolymerize F-actin structures and to promote the fusion of secretory vesicles with the plasma membrane. ROS production was also shown to stimulate the expression of a ROP/RAC-GAP during plant responses to oxygen deprivation (Baxter-Burrell et al., 2002). In addition, ROP/RAC GTPases physically interact with PtdIns P-K (PIPK) activity, which generates PtdIns 4,5-P_2 (PIP_2). This signaling lipid has multiple functions, including the promotion of vesicle fusion and fission, the regulation of actin-binding proteins, and the recruitment of exocyst components. Through destabilization of the ROP/RAC interaction with GDI and by directly binding to the polybasic region of ROP/RAC GTPases, PtdIns 4,5-P_2 also promotes ROP/RAC membrane association and activation, which creates another positive feedback loop. Stars denote PtdIns 4,5-P_2, ROS, and Ca^{2+}, which are involved in many signaling cascades, including ROP/RAC-independent pathways. Unspecific stimulation of such pathways is presumably prevented by the spatial and temporal restriction of ROP/RAC signaling.

stream signaling have remained highly conserved during evolution, plants have developed a range of unique mechanisms involved in these processes, including (1) ROP/RAC activation by plant-specific GEFs, (2) ROP/RAC-dependent signaling by plant-specific RIC and ICR1 effectors, and (3) PLC-mediated spatial restriction of PtdIns 4,5-P_2 distribution. Despite these variations in the underlying signaling mechanism, a key function of all Rho proteins, including ROP/RAC GTPases, appears to be the control of membrane trafficking, either through F-actin reorganization or actin-independent pathways.

The systems biology of ROP/RAC signaling appears to be highly complex. Direct evidence has been generated for an important function of ROP/RAC GTPases in the control of F-actin reorganization, membrane trafficking, and polar cell growth. A variety of factors have been identified that link ROP/RAC activation to the regulation of these processes in different cell types. Some of these factors, including Ca^{2+}, PtdIns 4,5-P_2, and ROS, are involved in a multitude of regulatory pathways, many of which are presumably ROP/RAC independent (Fig. 4). Spatial and/or temporal restriction of ROP/RAC activation and downstream signaling, therefore, is likely to be required to prevent nonspecific stimulation of independent pathways. To locally contain ROP/RAC signaling, plant cells appear to employ a complex network of interacting regulatory mechanisms. ROP/RAC targeting and local activation are tightly controlled by prenylation and *S*-acylation of the hypervariable domain, transient raft association depending on G-domain *S*-acylation, direct binding to PtdIns 4,5-P_2, GEF-dependent activation, GAP/GDI-mediated recycling, and, possibly, membrane trafficking. Additional mechanisms contributing to the spatial and temporal restriction of ROP/RAC signaling, at least in tip-growing cells, include (1) the highly specific intracellular targeting of regulatory factors such as GEFs and GAPs by unknown processes, (2) the maintenance by PLC activity of membrane domains enriched in PtdIns 4,5-P_2, which potentially acts both upstream and downstream of ROP/RAC activation, and (3) the coordinated oscillation of the activity of ROP/RAC GTPases (Hwang et al., 2005) and of ROP/RAC-dependent signaling factors (see above).

A key challenge of ongoing and future research is to understand how cells undergoing directional expansion integrate the many regulatory mechanisms and pathways involved in localized ROP/RAC signaling to coordinate F-actin-dependent membrane trafficking underlying this process. It will be essential to investigate in detail the intracellular distribution ROP/RAC GTPases expressed in expanding cells and to further explore the possibility that some of them may be associated with endomembrane compartments. An important part of this investigation will be the further analysis of lipid modifications of different ROP/RAC GTPases and of the effects of these modifications on RhoGDI interaction as well as on intracellular targeting. GEFs and GAPs controlling ROP/RAC activity during specific cellular processes need to be identified, and their intracellular targeting needs to be studied. In addition, it will be important to further characterize (1) the exact cellular functions of ROP/RAC effectors, (2) the membrane trafficking processes involved in polar plant cell expansion, and (3) the role of different F-actin structures in these processes.

Experiments based on analysis of the effects of overexpressing constitutively active or dominant negative ROP/RAC variants were instrumental in the acquisition of our current knowledge concerning the role of ROP/RAC signaling in the control of membrane trafficking and polar cell growth. Analysis of the effects of knocking out or knocking down the expression of single ROP/RAC GTPases, or of combinations of these proteins, in plant cells undergoing directional expansion will be important to confirm and extend this knowledge. Together with the experimental approaches summarized above, mathematical modeling will be required to achieve an integrated understanding of the complex regulatory mechanisms and cellular processes underlying ROP/RAC-controlled membrane trafficking during polar cell growth.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Function of ROP/RAC GTPases and their regulating proteins.

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