Plant Communications
Review article

CellPress
Partner Journal

Signaling network controlling ROP-mediated tip growth in *Arabidopsis* and beyond

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https://doi.org/10.1016/j.xplc.2022.100451

ABSTRACT

Cell polarity operates across a broad range of spatial and temporal scales and is essential for specific biological functions of polarized cells. Tip growth is a special type of polarization in which a single and unique polarization site is established and maintained, as for the growth of root hairs and pollen tubes in plants. Extensive studies in past decades have demonstrated that the spatiotemporal localization and activity of Rho of Plants (ROPs), the only class of Rho GTPases in plants, are critical for tip growth. ROPs are switched on or off by different factors to initiate dynamic intracellular activities, leading to tip growth. Recent studies have also uncovered several feedback modules for ROP signaling. In this review, we summarize recent progress on ROP signaling in tip growth, focusing on molecular mechanisms that underlie the dynamic distribution and activity of ROPs in *Arabidopsis*. We also highlight feedback modules that control ROP-mediated tip growth and provide a perspective for building a complex ROP signaling network. Finally, we provide an evolutionary perspective for ROP-mediated tip growth in *Physcomitrella patens* and during plant–rhizobia interaction.

Key words: Rho GTPases, feedback, polarization, root hair, pollen tube

Li E., Zhang Y.-L., Qin Z., Xu M., Qiao Q., Li S., Li S.-W., and Zhang Y. (2023). Signaling network controlling ROP-mediated tip growth in *Arabidopsis* and beyond. Plant Comm. **4**, 100451.

INTRODUCTION

Most eukaryotic cells are polarized, and polarization is essential for the function of polarized cells. Cell polarization is a consequence of the asymmetric distribution of proteins and lipids, as well as cell wall components in plants (Qin and Dong, 2015; Muroyama and Bergmann, 2019). Tip growth is a special type of cell polarization process in which a single and unique growth axis is established and maintained, leading to a tubular or cylindrical cell shape. Examples of tip growth include budding and shmoo formation in yeast, growth of fungal hyphae and moss protonema cells, and growth of pollen tubes and root hairs in plants (Qin and Dong, 2015).

Extensive studies in plants have shown that the small GTPase ROPs play an instructive role in cell polarization. As key molecular switches, ROPs switch between GDP-bound inactive forms and GTP-bound active forms through guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs convert inactive ROPs into active ROPs, whereas GAPs activate the GTPase activity of ROPs to cause their inactivation (Wu et al., 2000; Berken et al., 2005). The third class of ROP regulators, guanine nucleotide dissociation inhibitors (GDIs), extract ROPs from the plasma membrane (PM) and sequester and protect inactive ROPs in the cytoplasm (Zhang and McCormick, 2010; Feng et al., 2016). Active ROPs mediate the dynamics of actin microfilaments (MFs) and microtubules (MTs), the concentration gradient of calcium (Ca^{2+}), the production of reactive oxygen species (ROS), and vesicle transport through effector proteins, thereby regulating cell polarization.

Here, we summarize the critical role of ROPs in tip-growing plant cells, i.e., root hairs and pollen tubes, focusing on molecular mechanisms that mediate the dynamic and asymmetric PM distribution of active ROPs. We also review recent findings indicating that feedback modules are a general theme in ROP-mediated tip growth.

Published by the Plant Communications Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and CEMPS, CAS.

ROPs REGULATE POLLEN TUBE GROWTH

Pollen tubes deliver sperm cells to the embryo sacs through a long and complex journey, thus playing a key role in the fertilization of flowering plants (Higashiyama and Yang, 2017). Pollen germination and pollen tube growth are stages of polarity establishment and maintenance, respectively. Upon pollen hydration and germination *in vitro*, the level of active ROPs increases rapidly (Chen et al., 2013), implying a role for ROPs in polarity establishment during pollen germination, a presumption that has yet to be demonstrated genetically.

In contrast to the process of polarity establishment, extensive studies have demonstrated a crucial role for ROPs in polarity maintenance during pollen tube growth. Among 11 ROP isoforms encoded in the *Arabidopsis* genome, four, *ROP1*, *ROP3*, *ROP5*, and *ROP9*, are expressed in pollen (Li et al., 1998, 1999, 2013; Zheng and Yang, 2000). Overexpressof the dominant negative (DN) form of *ROP1* ($ROP1^{DN}$) or downregulation of *ROP1* inhibited pollen tube growth, whereas overexpression of *ROP1* or constitutively active (CA) *ROP1* ($ROP1^{CA}$) induced isotropic growth of pollen tubes (Li et al., 1999; Zheng and Yang, 2000). Manipulating the level or activity of *ROP3* and *ROP5* showed a similar, albeit weaker, effect. $ROP3^{CA}$ overexpression induced tube bulging (Li et al., 2013). Overexpression of *ROP5* caused pollen tube widening in a dose-dependent manner, and overexpression of $ROP5^{CA}$ induced isotropic growth of pollen tubes similar to that caused by overexpression of $ROP1^{CA}$ (Feng et al., 2016). In contrast to the three type I ROPs that are associated with the apical PM of growing pollen tubes, ROP9 is distributed at the subapical PM of growing pollen tubes, and its overexpression resulted in a dosage-dependent pollen tube depolarization (Li et al., 2013).

Distinct distribution or organization of intracellular activities is critical for pollen tube growth. Growing pollen tubes contain a tip-ward Ca^{2+} gradient with maximal concentrations close to the apical PM and high levels of ROS also at the apical region (Li et al., 1999, 2018a; Breygina and Klimenko, 2020). The dynamic distribution of actin MFs in pollen tubes has been demonstrated in recent years. Growing pollen tubes contain patches of short and fine actin MFs at the apical clear zone, an actin fringe at the subapical domain, and longitudinal actin cables in the shank area (Gu et al., 2003; Li et al., 2018a). A recent model suggests that actin MFs within both the apical and subapical regions of pollen tubes are generated from the PM at the tip, and they are arrayed into a structure defined as the "apical actin structure" (Qu et al., 2017; Xu and Huang, 2020). Exocytic vesicles, as marked by members of RabA4, accumulate at the apical clear zone in an inverted cone shape for the delivery of wall- and membrane-building materials (Szumlanski and Nielsen, 2009; Li et al., 2018a). All these intracellular activities were reported to be regulated by ROP signaling. An injection of anti-ROP1 antibody caused the disappearance of the Ca^{2+} gradient, suggesting that the maintenance of the tip-ward Ca^{2+} gradient depends on ROPs (Lin and Yang, 1997). Overexpression of $ROP1^{DN}$ disrupted the formation of short actin bundles at the apex and induced transverse actin bands behind the apical region (Fu et al., 2001; Gu et al., 2003). By contrast, overexpression of *ROP1* caused an extensive longitudinal actin MF penetrating to the apex (Fu et al., 2001; Gu et al., 2003), suggesting that the distribution of actin MFs relies on ROP activity. Alteration of ROP activities also interferes with exocytic trafficking in pollen tubes. Overexpression of $ROP1^{CA}$ caused a more compact distribution of secretory vesicles at the apex, whereas overexpression of $ROP1^{DN}$ reduced the accumulation of secretory vesicles, indicating a positive role for ROPs in exocytosis (Lee et al., 2008; Lee and Yang, 2008).

A few effectors through which ROPs mediate intracellular activities in pollen tubes have been uncovered. ROP-interactive CRIB motif-containing proteins (RICs) play pivotal roles in relaying ROP signaling into a Ca^{2+} gradient and dynamic assembly of actin MFs (Wu et al., 2001). RICs interact specifically with active ROP GTPases, indicating that they are ROP effectors. Among the 11 members of the RIC family, RIC1 was activated by ROP6 to control the organization of MTs by promoting the MT-severing activity of katanin (KTN1), which contributes to the formation of the jigsaw-puzzle shape of leaf pavement cells (Fu et al., 2009; Lin et al., 2013). In addition to its role in cell polarization, RIC1 may be responsive to developmental and environmental cues such that it mediates the reorganization of MTs induced by salt stresses (Li et al., 2017) and positively regulates auxin responses but negatively regulates abscisic acid (ABA) responses during root development (Choi et al., 2013). Recent studies have demonstrated that RIC1 directly binds actin MFs and severs actin MFs in the presence of Ca^{2+} at the apical region, whereas it caps the barbed ends of actin MFs to prevent their growth in the cytoplasm of the shank (Figure 1) (Zhou et al., 2015). *RIC1* overexpression reduces apical actin MFs and tube growth, whereas its loss-of-function produced extended actin MFs at the apex and enhanced pollen tube growth (Zhou et al., 2015). Two other RICs, RIC3 and RIC4, relay ROP signaling directly or indirectly by mediating actin MF dynamics, Ca^{2+} signaling, and exocytotic trafficking (Figure 1). RIC3 regulates the influx of Ca^{2+} across the apical PM, whereas RIC4 promotes F-actin assembly, two processes that interact to maintain pollen tube growth (Gu et al., 2005). Indeed, *RIC3* overexpression resulted in the loss of short actin bundles at the apex and the protrusion of longitudinal actin cables to the apex, whereas *RIC4* overexpression converted the dynamic F-actin into a dense F-actin network (Gu et al., 2005). In addition, the assembly or stabilization of actin MFs mediated by RIC4 is critical for the accumulation of secretory vesicles in the apex, whereas the disassembly or destabilization of actin MFs mediated by RIC3 is required for vesicle docking or fusion (Lee et al., 2008; Lee and Yang, 2008).

Interactors of constitutive active ROP 1/ROP interactive partner 1 (ICR1/RIP1) belong to a five-member family in *Arabidopsis* (Lavy et al., 2007; Li et al., 2021). ICR1/RIP1 also interacts with active ROPs (Lavy et al., 2007). It promotes the accumulation of exocytic vesicles in the apical region of growing pollen tubes, likely through its interaction with the exocyst complex (Lavy et al., 2007) (Figure 1). Overexpression of *ICR1*/*RIP1* resulted in depolarized growth of pollen tubes, similar to that caused by *ROP1* overexpression (Li et al., 2008). ROPs may regulate ROS production via PM-associated NADPH oxidases. In *Arabidopsis*, *RBOHH* and *RBOHJ* are two NADPH oxidase-coding genes that are highly expressed in pollen tubes and whose functional loss severely compromised tube growth (Kaya et al., 2014). Although the direct interaction between ROPs and

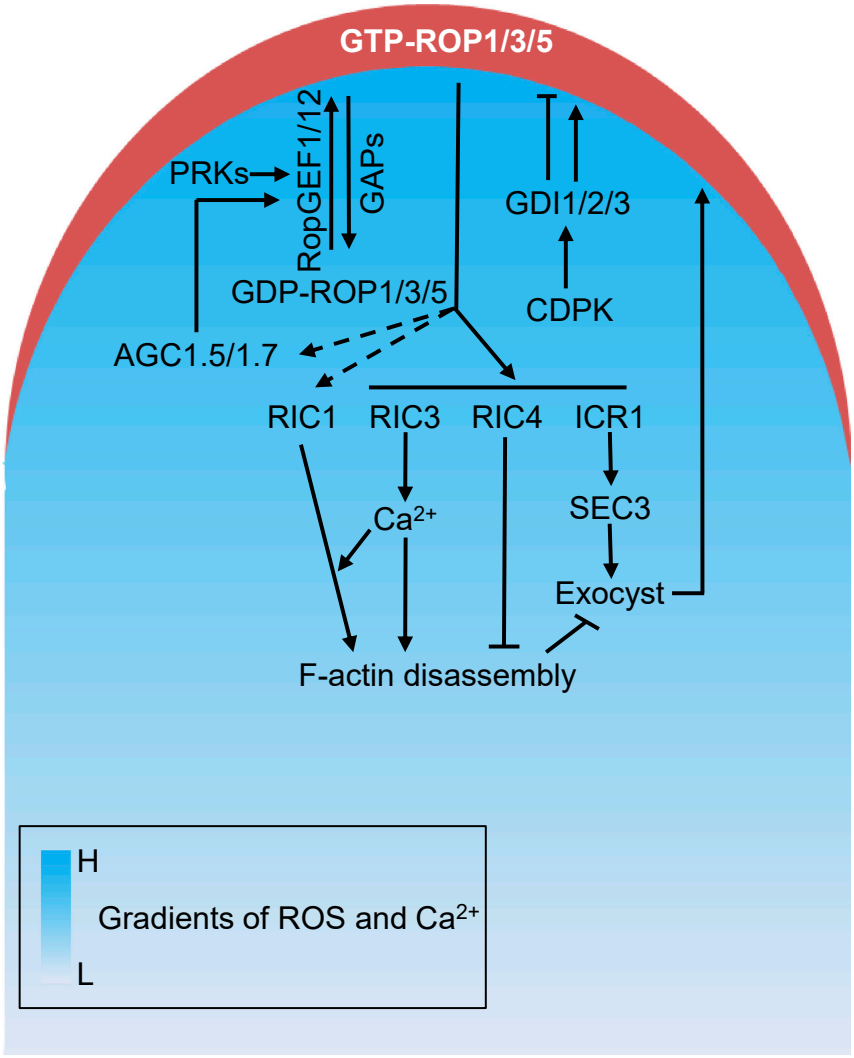

Figure 1. ROP signaling network during tip growth of pollen tubes.
During tip growth of pollen tubes, RopGEFs convert GDP-bound inactive ROPs into GTP-bound active ROPs at the apical PM (shown in red), whereas RopGAPs convert active ROPs into inactive ROPs. RopGEFs are recruited to the PM through their interaction with PRKs and are phosphorylated by AGC1.5 kinases. The localization and stability of ROPs are also mediated by GDIs. Different effector proteins, such as RIC1, RIC3, RIC4, and ICR1, initiate intracellular activities upon interaction with active ROPs, and intracellular activities in turn affect the dynamic targeting or activity of ROPs. Solid lines indicate confirmed interactions, and dashed lines indicate implied interactions.

pollen-expressed *RBOHH* or *RBOHJ* needs to be experimentally verified, ROPs have been reported to interact with other NADPH oxidases during pathogen-induced ROS production (Hu et al., 2020), suggesting a similar mechanism for ROP-mediated ROS production during pollen tube growth.

ROPs REGULATE ROOT HAIR GROWTH

Root hairs are cylindrical extensions from root epidermal cells that play crucial roles in water uptake, nutrient assimilation, and environmental sensing and are essential for the evolutionary success of vascular plants (Grierson et al., 2014). Transcriptional cascades determine whether or not a root epidermal cell becomes a hair-forming cell, i.e., a trichoblast or an atrichoblast (Grierson et al., 2014; Vissenberg et al., 2020). Root hair growth includes two stages: the establishment of a root hair initiation domain (RHID), in which certain factors distribute asymmetrically at the PM of the RHID without causing morphological changes, and the maintenance of tip growth, in which the RHID initiates a bulge that rapidly expands to form a root hair.

The *Arabidopsis* genome encodes 11 ROP isoforms, which are classified into two subfamilies based on their C-terminal sequences. Type I ROPs have a C-terminal CaaX motif for prenylation, whereas type II ROPs contain C-terminal domains with several basic amino acid residues for phospholipid binding and CG box motifs for S-acylation (Bloch and Yalovsky, 2013). Among them, three type I ROPs (ROP2, ROP4, and ROP6) and two type II ROPs (ROP10 and ROP11) (Winge et al., 2000) have been reported to mediate root hair growth. *Arabidopsis* ROP2 associates with the RHID during root hair initiation and throughout root hair growth (Jones et al., 2002). Functional loss of *ROP2* or the expression of $ROP2^{DN}$ resulted in short root hairs (Jones et al., 2002). Interestingly, the expression of $ROP2^{DN}$ also caused a reduction in RHIDs (Jones et al., 2002). By contrast, the expression of *ROP2* or $ROP2^{CA}$ induced additional and misplaced hairs, as well as longer root hairs or even balloon-shaped root hairs (Jones et al., 2002), suggesting that ROP2 mediates the polarity establishment and maintenance of root hairs. Similar to ROP2, *Arabidopsis* ROP4 and ROP6 are also localized at RHIDs, and the *rop2;rop4;RNAirop6* triple mutant grew root hairs shorter than those of *rop2* (Molendijk et al., 2001; Gendre et al., 2019), further supporting a redundant role of the three ROP isoforms in root hair growth. ROP10 localizes at the PM of the root hair shank, and the expression of $ROP10^{DN}$ induced shorter, wavy root hairs (Hirano et al., 2018), whereas overexpression of $ROP11^{CA}$ resulted in isotropic root hair growth (Bloch et al., 2005), suggesting a key role for ROP10 and ROP11 in polarity maintenance during root hair growth.

Similar to pollen tubes, growing root hairs exhibit a defined distribution or organization of specific intracellular activities. Growing root hairs contain fine actin meshes at the apex and actin bundles aligned longitudinally behind the apex (Jones et al., 2002; Bloch et al., 2005). A tip-focused Ca^{2+} gradient and ROS with peak concentrations at the apical region are associated with root hair growth (Foreman et al., 2003; Jones et al., 2007; Takeda et al., 2008). In *Arabidopsis* root hairs, RabA4b-positive secretory

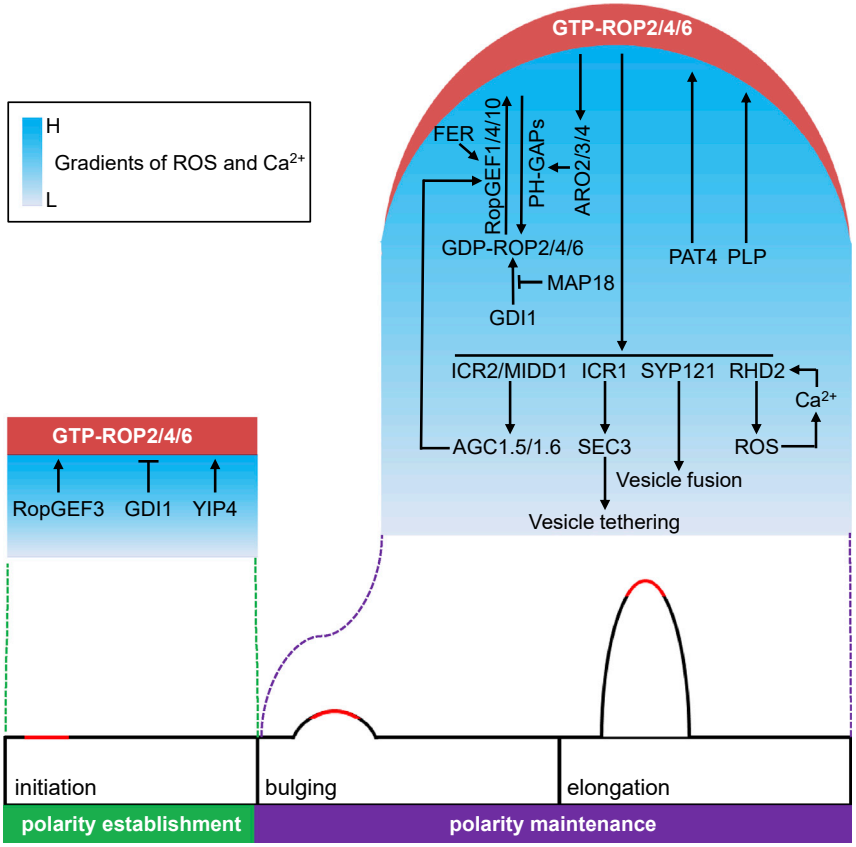

Figure 2. ROP signaling network during the initiation and elongation of root hairs.
Root hair growth includes both polarity establishment and maintenance, i.e., hair initiation and hair elongation, as shown in the cartoon. During root hair initiation, RopGEF3 is critical for the enrichment of ROPs at the RHIDs (in red); YIP4 facilitates and GDI1 inhibits the restricted accumulation of ROPs. During root hair elongation, the conversion between GDP-bound inactive ROPs and GTP-bound active ROPs is mediated by several RopGEFs and PH-GAPs. ROP-AROs-PH-GAP forms a negative feedback module, whereas ROPs-ICR2/MIDD1-AGC1.5-RopGEF forms a positive feedback module to maintain the dynamic active ROP domains at the PM. ROP signaling is also influenced by post-translational modifications of ROPs by competitive binding with either RhoGDI1 or MAP18 and by interaction with FERONIA-RopGEFs.

vesicles form an inverted cone-shaped pattern, and FM4-64, a lipophilic dye that enters cells through endocytosis, labels a similar area, indicative of an active endocytic domain (Bolte et al., 2004; Preuss et al., 2004; Zhang et al., 2015). The other cytoskeletal components, MTs, play a critical role in root hair growth. MTs are organized into bundles along the longitudinal shank area but are hardly detected in the apical domain of growing root hairs (Molendijk et al., 2001). Studies in the past decade have indicated a direct regulation of these intracellular activities in root hairs by ROPs. In root hairs, overexpression of *ROP2*^{CA} or *ROP11*^{CA} resulted in extensive and randomly oriented actin bundles, whereas overexpression of *ROP2*^{DN} caused actin bundles to penetrate to the apex and a loss of fine actin meshes in the apex (Jones et al., 2002; Bloch et al., 2005). Overexpression of *ROP6*^{CA} induced more randomly oriented and shorter MTs in the shanks of root hairs (Molendijk et al., 2001). The tip-focused Ca^{2+} gradient and ROS production also depend on ROP signaling, as either enhancement of ROP activities by expression of *ROP2*^{CA} or reduction in ROP activities by expression of *ROP2*^{DN} interfered with the tip-focused Ca^{2+} gradient and ROS production (Jones et al., 2007).

Effectors through which ROPs mediate various intracellular activities in root hairs have been reported. Similar to the situation in pollen tubes, NADPH oxidases encoded by *Arabidopsis RBOHC/ROOT HAIR DEFECTIVE 2* (*RHD2*) play a crucial role in ROS production during root hair growth (Figure 2) (Foreman et al., 2003). Loss of *RBOHC* function resulted in reduced ROS accumulation and root hair growth and abolished the effect of *ROP2*^{CA} overexpression on root hair growth (Foreman et al., 2003; Jones et al., 2007; Takeda et al., 2008), indicating an epistatic interaction between ROPs and RBOHC. Also, similar to that in pollen tubes, overexpression of *ICR1/RIP1* resulted in swollen root hairs (Lavy et al., 2007) resembling those caused by overexpression of ROPs. In addition to the ICR1/RIP1-Exocyst pathway for vesicle tethering, ROPs were recently reported to regulate vesicle fusion by promoting PM-targeting of SYP121, a Qa-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), as well as the assembly of the SNARE complex (Figure 2). Functional loss of *SYP121* resulted in reduced root hair growth, whereas overexpression of *SYP121* resulted in enhanced root hair growth (Cui et al., 2022). Furthermore, overexpression of *SYP121* partially rescued the defects in root hair growth caused by *ROP2* loss of function, demonstrating their genetic interaction (Cui et al., 2022). A few other protein factors are likely to be downstream effectors of ROPs for the dynamic assembly of actin MFs or MTs based on their root hair defects, as well as interactors in other cellular systems; these include Kinesin-13A, ARMADILLO-REPEAT KINESIN1/MORPHOGENESIS OF ROOT HAIR2 (ARK1/MRH2), actin depolymerizing factors, and so on (Ruzicka et al., 2007; Yang et al., 2007; Oda and Fukuda, 2013; Eng and Wasteneys, 2014). However, it is unclear whether they physically interact with active ROPs, and their genetic interactions with ROPs remain to be investigated.

UPSTREAM REGULATORS OF ROPs IN POLLEN TUBES AND ROOT HAIRS

The *Arabidopsis* genome encodes 14 plant-specific RopGEFs, all of which contain a plant-specific ROP nucleotide exchanger (PRONE) domain that catalyzes GDP-GTP exchange, in addition to hypervariable N- or C-terminal sequences (Berken et al., 2005). The role of RopGEFs was first established in pollen tubes. Overexpression of multiple RopGEFs in pollen tubes compromised tube growth, resulting in wavy to bulging growth (Gu et al.,

2006; Zhang and McCormick, 2007). Interestingly, overexpression of PRONE domains alone resulted in the most severe polarity defects, i.e., isotropic growth of pollen tubes (Gu et al., 2006; Zhang and McCormick, 2007), suggesting that the hypervariable sequences inhibit the catalytic activity of the PRONEs. In contrast to gain-of-function studies in pollen tubes, loss-of-function studies have mainly been used to uncover the role of RopGEFs in root hairs. Functional loss of *Arabidopsis RopGEF4* and *RopGEF10* resulted in a reduced ROP-GTP level and shorter root hairs, and the *ropgef1;ropgef4;ropgef10* triple mutant showed a further reduction in root hair elongation (Huang et al., 2013; Li et al., 2021), demonstrating the role of RopGEFs in ROP signaling during root hair growth (Figure 2). Root hair development was used as an experimental system to demonstrate that RopGEFs are critical not only for polarity maintenance but also for polarity establishment. *Arabidopsis* RopGEF3 accumulates asymmetrically in trichoblast cells before the formation of the RHID (Denninger et al., 2019). Functional loss of *RopGEF3* abolished the accumulation of ROP2 in the RHID and resulted in delayed root hair initiation (Denninger et al., 2019). Interestingly, ectopic expression of *RopGEF3* in atrichoblasts resulted in local accumulation of ROP2 (Figure 2) (Denninger et al., 2019), suggesting that RopGEF3 is sufficient for polar accumulation of ROP2 and the establishment of polarity. Multiple RopGEFs show either pollen-specific or pollen-enriched expression patterns, and it remains unclear whether functional loss of certain RopGEFs affects pollen germination, a polarity establishment process, owing to their presumed functional redundancy.

The *Arabidopsis* genome encodes ten GAPs for ROPs, six of which contain a Cdc42/Rac-interactive binding (CRIB) motif and four of which contain a Pleckstrin Homology (PH) domain (Wu et al., 2000; Hwang et al., 2008; Li et al., 2018b). In tobacco pollen tubes, a CRIB-GAP interacts with active ROPs through its CRIB domain at the apical PM, stimulates the GTPase activity of ROPs, and thereby restricts active ROPs from spreading toward the flanks to maintain tip growth (Klahre and Kost, 2006). Interference with endogenous CRIB-GAP activity induces tube ballooning, whereas overexpression of CRIB-GAP inhibits pollen germination and tube growth owing to a narrow apical ROP domain (Klahre and Kost, 2006). The functions of two *Arabidopsis* PH-type RopGAPs, REN1 (ROP1 enhancer 1) and REN4, have recently been revealed. REN1 is localized at exocytic vesicles (Hwang et al., 2008). Functional loss of *REN1* induced ballooning of pollen tubes, correlating with an expansion of active ROPs at the PM (Hwang et al., 2008). REN4 is distributed at the lateral and apical PM of growing pollen tubes, as well as in the vegetative nucleus and cytosol. Further studies demonstrated that REN4 is periodically localized to the apical PM, and its apical distribution is associated with a reduction in pollen tube growth, whereas its exclusion from the apical PM precedes rapid tube growth (Li et al., 2018b). Functional loss of *REN4* resulted in wider and wavier tubes, resembling the phenotype induced by weak *ROP1* overexpression (Li et al., 2018b). These results indicate that GAPs promote the dynamic removal of ROPs from the subapical PM during tip growth.

The *Arabidopsis* genome encodes three RhoGDIs, among which *GDI1/SUPERCENTIPEDE1* (*SCN1*) is constitutively expressed, whereas *GDI2* and *GDI3* are specifically expressed in pollen (Bischoff et al., 2000; Feng et al., 2016). Loss of *GDI1/SCN1* function resulted in ectopic RHIDs and an expansion of ROP distribution at the PM in a single trichoblast (Carol et al., 2005), suggesting that GDI1/SCN1 is responsible for the restricted distribution of ROPs during root hair initiation and growth. In comparison, functional loss of all three *Arabidopsis GDI*s caused an expansion of ROPs at the apex, leading to shorter and wider pollen tubes (Feng et al., 2016). The distinct consequences of *RhoGDI* loss of function in two types of tip-growing cells imply a cell-type-specific regulation of ROPs by GDIs.

REGULATORS OF THE REGULATORS

Tip growth is a self-organizing process. However, it can be influenced by extracellular signals, such as female guidance cues for pollen tube growth or rhizobia for root hair growth. A common theme in the tip growth of plant cells is the interaction between receptor-like kinases (RLKs) and RopGEFs. *Arabidopsis* FERONIA (FER), one of the *Catharanthus roseus* RLK1-like subfamily kinases (CrRLK1Ls), interacts with root hair-functional RopGEF4/10, and *FER* loss of function resulted in defective root hair growth (Figure 2) (Duan et al., 2010; Huang et al., 2013). In addition, ectopic expression of *ROP2* partially restored root hair growth of a weak *fer* allele (Duan et al., 2010), indicating their genetic epistasis. *Arabidopsis* Pollen Receptor Kinase 2 (PRK2) interacts with pollen tube-functional RopGEF12 to release the autoinhibition of its catalytic domain by its C terminus, leading to activation of ROP GTPases in pollen tubes (Figure 1) (Zhang and McCormick, 2007). Indeed, overexpression of a C-terminally truncated but not full-length *RopGEF12* substantially compromised pollen tube polarity (Zhang and McCormick, 2007). A few other PRKs have been reported to induce directional growth of pollen tubes through ROP signaling upon the sensing of female guidance cues, and these PRKs do interact with RopGEFs (Takeuchi and Higashiyama, 2016). Buddha's Paper Seal 1/2 (BUPS1/2), two CrRLK1Ls recently reported to be critical for pollen tube reception, were found to form a tripartite complex with RopGEFs and ROPs (Zhu et al., 2018), hinting at a similar signaling pathway from cell surface RLKs to intracellular activities.

Although these RLKs interact with RopGEFs, it is unclear whether they regulate RopGEFs by phosphorylation. In fact, ectopic expression of both wild-type and a kinase-dead version of PRK2 resulted in depolarized growth of pollen tubes (Zhao et al., 2013), despite the fact that PRK2 shows *in vitro* phosphorylation activity toward RopGEFs (Chang et al., 2013). Similar to ROPs, RopGEFs are distributed both at the apical PM of growing pollen tubes and in the cytosol. The apical PM association of RopGEFs is crucial for their activity in ROP signaling (Li et al., 2018a, 2021). Recently, a subfamily of tip-growing cell-specific cytoplasmic kinases was demonstrated to regulate ROP signaling through phosphorylation of RopGEFs. AGC1.5 and AGC1.7, two members of the AGCVIII subfamily kinases, phosphorylate RopGEF1/12 and restrict their localization to the apical PM of growing pollen tubes (Figure 1) (Li et al., 2018a). Functional loss of *AGC1.5* and *AGC1.7* compromised the maintenance of pollen tube growth, which was consistent with the random distribution of RopGEFs and mistargeted ROP-GTP (Zhang et al., 2009; Li et al., 2018a).

Phosphorylation may also influence the activity of GDIs. A study in *Petunia inflata* indicated that PiCDPK1, a pollen-specific

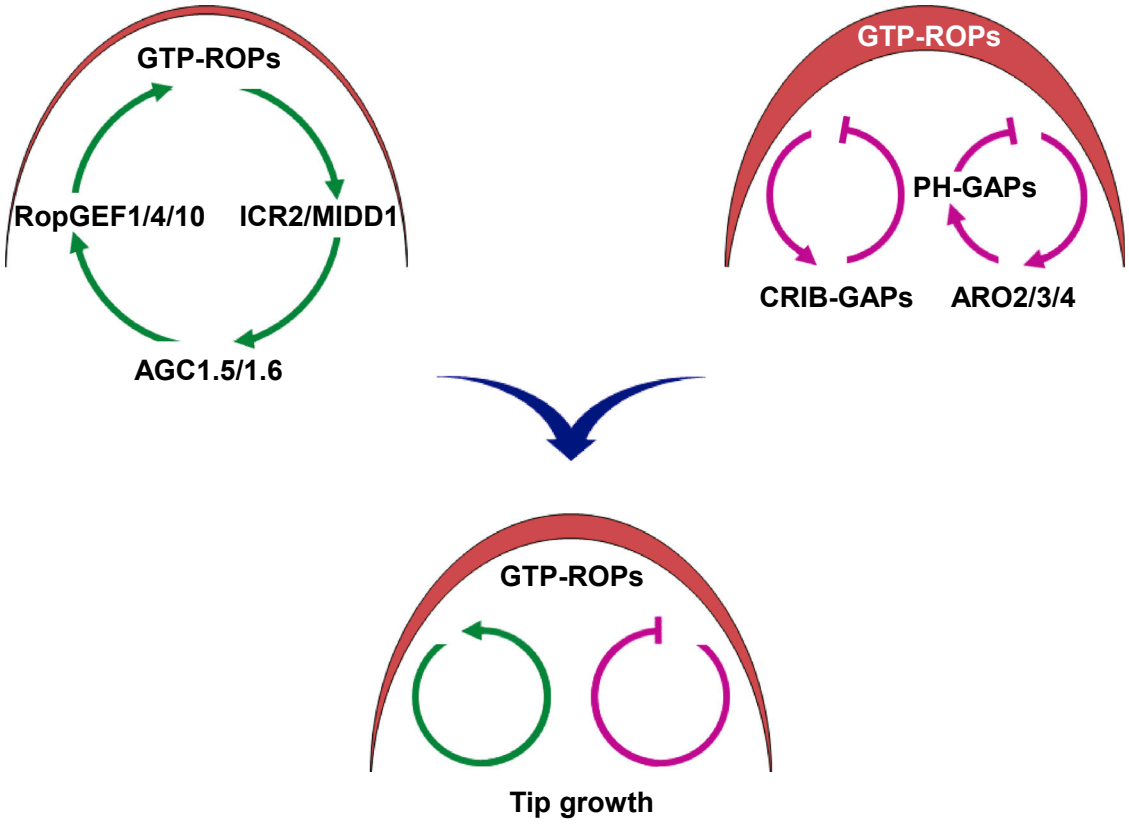

Figure 3. Feedback modules in ROP-mediated tip growth.
Positive feedback on an activator contributes to restricted ROP activity at the apex of tip-growing cells. Active ROPs, through their effector proteins ICR2 and MIDD1, recruit AGC1.5 subfamily kinases and promote their kinase activity toward RopGEFs. Phosphorylated RopGEFs are restricted at the apical PM to activate ROPs and maintain their spatiotemporal distribution. Inhibitors with positive feedbacks prevent the expansion of ROP activity through the interaction between active ROPs and their inhibitors, either directly or indirectly through a mediator. ROP activities are tightly controlled by positive and negative feedbacks to maintain the dynamic restriction of apical ROPs, thereby enabling continuous tip growth.

calcium-dependent protein kinase, interacted with and phosphorylated PiGDI1 (Figure 1) (Scheible et al., 2022). Overexpression of *PiCDPK1* induced extremely short pollen tubes with almost spherical tips, resembling those caused by *ROP* overexpression (Yoon et al., 2006; Scheible et al., 2022). Co-expression of *PiGDI1* suppressed the depolarized pollen tube growth caused by excess *PiCDPK1*, suggesting that PiCDPK1 negatively regulates GDI1 by phosphorylation (Scheible et al., 2022). Although a similar mechanism has not been reported in *Arabidopsis*, CPK3, a calcium-dependent protein kinase, regulates the development of leaf pavement cells by phosphorylating GDI1 in *Arabidopsis* (Wu et al., 2013). As important calcium sensors in plant cells, CPKs and CDPKs may regulate polarity maintenance in root hairs and pollen tubes by a similar phosphorylation mechanism (Qian and Xiang, 2019; Menesi et al., 2021).

Competitive protein–protein interaction is another paradigm in the regulation of ROP regulators. During root hair polar growth, MAP18 (MT-associated protein 18), also known as PCaP2 (PM–associated Ca^{2+} binding protein 2), interfered with the interaction between GDI1/SCN1 and ROP2 (Figure 2) (Kang et al., 2017). MAP18 was first identified as an MT-associated protein but has versatile functions in mediating actin MF dynamics and lipid binding (Wang et al., 2007; Zhu et al., 2013; Kato et al., 2019). During root hair tip growth, MAP18 interacts genetically and physically with ROP2 to mediate ROP signaling (Kang et al., 2017). Functional loss of *MAP18* compromised ROP2 distribution at the PM in root hairs and caused shorter root hairs, whereas *MAP18* overexpression resulted in multiple tips or branching hairs (Kang et al., 2017), resembling the phenotype of *scn1/gdi1* (Carol et al., 2005). Indeed, MAP18 inhibits the interaction between GDI1/SCN1 and inactive $ROP2^{DN}$ but not $ROP2^{CA}$ (Kang et al., 2017), suggesting that MAP18 competes with GDI1 for interaction with ROP2 to promote ROP2 activation and signaling during root hair growth.

LIPIDATION AND VESICULAR TRAFFICKING REGULATE ROP SIGNALING DURING TIP GROWTH

ROPs are post-translationally modified by prenylation and S-acylation, which are critical for their subcellular targeting and activities during tip growth (Figure 2). Type I ROPs contain a C-terminal CaaX motif for prenylation and internal Cys residues for S-acylation, whereas type II ROPs are S-acylated at the C-terminal hypervariable region (Bloch and Yalovsky, 2013; Feiguelman and Fu, 2018). Functional loss of *PLURIPETALA* (*PLP*), which encodes the shared α subunit of two

heterodimeric protein isoprenyltransferases in *Arabidopsis*, significantly reduced the accumulation of ROP2 at the apical PM of elongating root hairs (Chai et al., 2016). Consistent with this finding, *plp* mutants showed much shorter and fewer root hairs than the wild type (Chai et al., 2016). Protein S-acylation is a reversible post-translational modification that attaches palmitate or a saturated lipid group to the sulfhydryl group of a Cys, thus affecting protein stability, subcellular localization, and activity (Magee and Seabra, 2005; Hemsley and Grierson, 2008; Greaves and Chamberlain, 2011; Running, 2014). It was demonstrated that 2-bromopalmitate (2-BP), a specific inhibitor of protein S-acylation, reduced root hair growth and caused a shift of ROP2 from the PM to the cytoplasm (Zhang et al., 2015). Indeed, *Arabidopsis* protein *S*-acyl transferase 4 (PAT4) interacts with ROP2 genetically (Wan et al., 2017). *PAT4* loss-of-function caused a reduced PM association of ROP2 in root hairs and shorter root hairs (Wan et al., 2017).

Recent studies have also indicated that vesicular trafficking regulates ROP signaling. Two *trans*-Golgi network-localized proteins, YPT-INTERACTING PROTEIN (YIP) 4a and 4b, mediate secretory trafficking of proteins and wall components. Their functional loss compromised root hair growth and reduced the accumulation of ROPs at the RHID (Gendre et al., 2019). The multiple and ectopic hair phenotype associated with overexpression of $ROP2^{CA}$ was suppressed in *yib4a;yib4b*, confirming their genetic epistasis (Gendre et al., 2019). Indeed, although ROPs without lipid modifications are soluble proteins, genetic interference with vesicular trafficking by expression of a DN form of ADP-ribosylation factor 1 (ARF1) (Xu and Scheres, 2005), expression of a DN form of Sar1, or functional loss of AP-1 caused the accumulation of ROPs at different endomembrane compartments (Ge et al., 2020), supporting a role for vesicular trafficking in ROP targeting.

FEEDBACK MODULES IN ROP-MEDIATED TIP GROWTH

Establishment and maintenance of cell polarity relies on local and dynamic accumulation of polarity regulators (McCaffrey and Macara, 2012; Muroyama and Bergmann, 2019). Feedback modules are necessary for the robustness yet flexibility of cell polarization (Chau et al., 2012; Wu and Lew, 2013). Several feedback modules have been identified in ROP-mediated tip growth in plant cells; these include inhibitors with positive feedbacks, global inhibitors, and positive feedbacks on an activator (Figure 3) (Hwang et al., 2010; Chau et al., 2012; Kulich et al., 2020; Li et al., 2021).

Two negative feedback modules consistent with the ‘‘inhibitor with positive feedback’’ pattern have been demonstrated in ROP-mediated tip growth (Figure 3). The presence of an active ROP-binding CRIB domain (Wu et al., 2000) in a few RopGAPs hinted at positive feedback on the inhibitor. Indeed, in tobacco pollen tubes, a CRIB-GAP was shown to associate with the apical PM, likely through binding with active ROPs (Klahre and Kost, 2006). By such an interaction, the CRIB-GAP restricts active ROPs from spreading toward the shank PM during pollen tube growth (Klahre and Kost, 2006; Hwang et al., 2010). Thus, active ROPs recruit their inhibitors, i.e., CRIB-GAPs, by direct interaction through the CRIB domain to attenuate their own activities. A similar module was recently reported in root hairs. Active ROPs interact with ARMADILLO REPEAT ONLY (ARO) proteins, which in turn interact with the PH-GAP REN1 and are critical for the subcellular localization of REN1 (Kulich et al., 2020). Functional loss of several *ARO*s resulted in the spreading of ROP2 to the entire PM of root hairs (Kulich et al., 2020), indicating a defect in the restriction of dynamic ROP localization. In this case, active ROPs recruit their inhibitors, i.e., PH-GAPs, by indirect interaction through AROs to attenuate their own activities.

Both RhoGDIs and RopGAPs have been proposed to confer a global inhibition to restrict the distribution or attenuate the spread of active ROPs during pollen tube growth (Hwang et al., 2010). Overexpression of *RopGAPs* or *RhoGDIs* suppresses the expansion of active ROP domains induced by overexpression of *ROPs*, indicating that RhoGDIs and RopGAPs limit the lateral propagation of apical ROP domains (Klahre and Kost, 2006; Hwang et al., 2010). Indeed, functional loss of all three RhoGDIs in *Arabidopsis* caused wider pollen tubes and enlarged the distribution of active ROPs at the apex (Feng et al., 2016).

A recent study uncovered a positive module for ROP-mediated root hair growth (Figure 3). *Arabidopsis* AGC1.5 subfamily kinases are critical for tip growth; functional loss of AGC1.5 subfamily kinases caused either meandering pollen tubes or reduced elongation of root hairs (Zhang et al., 2009; Li et al., 2018a, 2021), indicating compromised positive feedback for maintenance of active ROP domains during tip growth. Indeed, AGC1.5 subfamily kinases interact with both ROP activators and ROP effectors (Li et al., 2021). In root hairs, active ROPs recruit the ROP effectors ICR2 and MIDD1/ICR5, both from the ICR/RIP family (Li et al., 2008, 2021), to the apical ROP domain, whereas ICR2 and MIDD1 not only induce the re-localization of AGC1.5 kinases to the apical PM but also promote their kinase activities toward RopGEFs (Li et al., 2021). Phosphorylation of RopGEFs by AGC1.5 subfamily kinases enabled their dynamic and restricted localization at the apical PM and ensured polarity maintenance during root hair growth (Li et al., 2021). Thus, through oligomerization and phosphorylation, active ROPs are able to maintain their dynamic localization at the apical PM of tip-growing cells in plants (Li et al., 2021).

EVOLUTIONARY PERSPECTIVES

Considerable progress has recently been made in understanding ROP-mediated polar growth in *Physcomitrella patens*. Similar to their roles in pollen tubes and root hairs, ROPs play a key role in the polar growth of *P. patens* by affecting cell adhesion, cell wall deposition, and actin dynamics (Burkart et al., 2015). During polar growth, PpROPs form a steep gradient at the growing apex of the cell, and their polar localization predicts future growth sites (Cheng et al., 2020). Mutants of all four *PpROP* genes comprise a disordered clump of spherical cells that are unable to form gametophores (Cheng et al., 2020). Similar regulators, including RopGEFs, RopGDIs, and RopGAPs, have been functionally characterized in *P. patens* (Eklund et al., 2010). Silencing *PpRopGEFs* or *PpRopGDIs* results in smaller plants with globular cells that resemble *PpROP*-RNAi cells (Bascom et al., 2019). By contrast, loss of PpRopGAP activity

either impairs protoplast regeneration or is lethal, similar to overexpression of *PpROPs* (Burkart et al., 2015; Bascom et al., 2019). All these results suggest that ROP signaling in cell polarization is evolutionarily conserved.

ROP-mediated cell polarization is also critical for plant–rhizobia interaction. In legumes, rhizobia attach to the tips of root hairs and redirect root hair growth to entrap themselves within an infection chamber (Esseling et al., 2003; Murray, 2011; Fournier et al., 2015). The infection thread (IT), a specialized transcellular compartment, extends from the infection chamber down through the root hair to the root cortex, where a nodule primordium is produced (Fournier et al., 2008, 2015; Liu et al., 2020). The IT is the key structure for rhizobial entry to the nodule primordium, a cell polarization process mediated by ROPs (Liu et al., 2020). Knocking down *MtROP8* caused larger numbers of infection events in response to *Sinorhizobium meliloti* inoculation, whereas reducing *MtROP9* expression promoted mycorrhizal and early hyphal root colonization but reduced rhizobial infection compared with the wild type (Kiirika et al., 2012; Wang et al., 2014). Similarly, LjROP6 is critical for rhizobial infection and nodule formation in *Lotus japonicas* (Ke et al., 2012; Wang et al., 2015; Liu et al., 2020). Upstream regulators of ROPs, such as membrane receptors and GEFs (Liu et al., 2020), have also been functionally characterized in several legume species, further demonstrating that ROP signaling in cell polarization is a universal theme during plant evolution.

CONCLUDING REMARKS

The crucial role of ROPs in tip growth, a special type of cell polarization, has been unequivocally established. ROPs are also in a good position to relay extracellular cues into intracellular activities, central to cell survival and plant fitness in a changing environment. The upstream regulators and downstream activities of ROP signaling are gradually being revealed. Future efforts should aim to connect individual dots and pathways into a finer network, focusing in particular on feedback modules and the biological consequences of molecular interactions. Although tip growth is a special type of cell polarization, mechanisms characterized by studying tip-growing cells will facilitate our understanding of cell morphogenesis in general.

FUNDING

This work is funded by the National Natural Science Foundation of China (32100295 to E.L.) and the Natural Science Foundation of Shandong Province (ZR2021QC001 to E.L.).

AUTHOR CONTRIBUTIONS

E.L., Y.-L.Z., S.L., and Y.Z. initiated this review and drafted the manuscript; E.L. and Y.-L.Z. wrote the manuscript with assistance from Z.Q., M.X., Q.Q., and S.-W.L.; E.L. and S.L. prepared the figures. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors declare no conflict of interest.

Received: June 23, 2022
Revised: August 24, 2022
Accepted: September 12, 2022
Published: September 15, 2022

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