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Review

ROPs in the spotlight of plant signal transduction

A. Berken

Max Planck Institute of Molecular Physiology, Department of Structural Biology, Otto-Hahn-Strasse 11,
44227 Dortmund (Germany), Fax: +49 231 1332699, e-mail: antje.berken@mpi-dortmund.mpg.de

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Abstract. Small guanine nucleotide binding proteins of the Rho family called ROP play a crucial role as regulators of signal transduction in plants. They participate in pathways that influence growth and development, and the adaptation of plants to various environmental situations. As members of the Ras superfamily, ROPs function as molecular switches cycling between a GDP-bound 'off' and a GTP-bound 'on' state in a strictly regulated manner. Latest research provided fascinating new insights into ROP regulation by novel guanine nucleotide exchange factors, unconventional GTPase activating proteins, and guanine nucleotide dissociation inhibitors, which apparently organize localized ROP activation. Important progress has also been made concerning signaling components upstream and downstream of the ROP cycle involving receptor-like serine/threonine kinases and effectors that can manipulate cytoskeletal dynamics, intracellular calcium levels, H_2O_2 production and further cellular targets. This review outlines the fast developing knowledge on ROP GTPases highlighting their specific features, regulation and roles in a cellular signaling context.

Keywords. Plant, ROP, Rac, GTPase, small G protein, molecular switch, signal transduction.

Introduction

Signal transduction in eukaryotic organisms including plants is often regulated by small guanine nucleotide binding proteins (G proteins) [1, 2]. Their regulatory role is based on their function as molecular switches that are turned on in response to upstream signals whereupon reaction cascades are induced to generate a cellular response. The small G proteins, also known as small GTPases, constitute a large superfamily termed after its founding member Ras [1, 3]. Proteins in the five subfamilies Ras, Rho, Arf, Ran and Rab all share a common structural design and molecular mechanism [4]. The conserved structural element is referred to as G domain that fulfills the basic functions of the proteins: It binds the guanine nucleotides GDP and GTP with high affinities and slowly hydrolyzes GTP. The nature of the bound nucleotide ultimately determines the activation status of the molecules. Activation occurs by exchanging bound GDP for GTP, and subsequent hydrolysis of GTP returns the molecular switch back to the inactive state [1, 3, 4]. GTP hydrolysis induces a conformational change in the structure of the proteins affecting mainly the two so-called switch regions I and II in the G domain, which contact the γ-phosphate in the GTP-bound form [4]. This structural change goes along with strongly altered affinities for regulatory proteins and effector molecules. The latter relay signals to downstream targets as they bind specifically to the GTP-bound conformation involving the switch regions [4, 5]. That way, the Arf, Rab and Ran proteins control vesicular or nuclear trafficking processes in the cell, while Ras and Rho proteins function as checkpoints in signaling networks [1, 3]. The Rho family can be further grouped into distinct classes with specific signaling functions [6, 7]. These include the best characterized members Rho, Rac and Cdc42 in mammals, which regulate cytoskeletal reorganization, cell cycle progression, morphogenesis, motility and gene expression [7]. Plants apparently lack true Ras proteins and the Rho family is represented by a unique class termed ROP for 'Rho proteins of plants' [2, 8, 9]. As the sole *bona fide* signaling small GTPases, the ROPs are believed to play a leading part as multitasking regulators of plant signal transduction.

ROP GTPases – Lessons from sequence and structure prediction

The ROP story began in 1993 when a plant homologue of Rho-type small G proteins was discovered in the garden pea [10]. Since then, representatives of the ROP family have been identified in mosses, conifers and both monocot and dicot flowering plant species [11, 12], suggesting a ubiquitous appearance in embryophytes. ROP proteins are quite similar to animal and fungal Rho family members [2, 8, 11, 12], and the highest conservation is found in the five sequence motifs, G1–G5, which are responsible for GDP/GTP and Mg^{2+} binding and GTPase hydrolytic activity [13]. Based on a slightly higher overall similarity to Rac [2, 8], ROPs have often been designated as plant RAC proteins, and to date the ROP/RAC nomenclature is still a matter of debate. In this review, the term 'ROP' will mainly be used for reasons of simplicity and to reduce confusion with the animal Rac proteins.

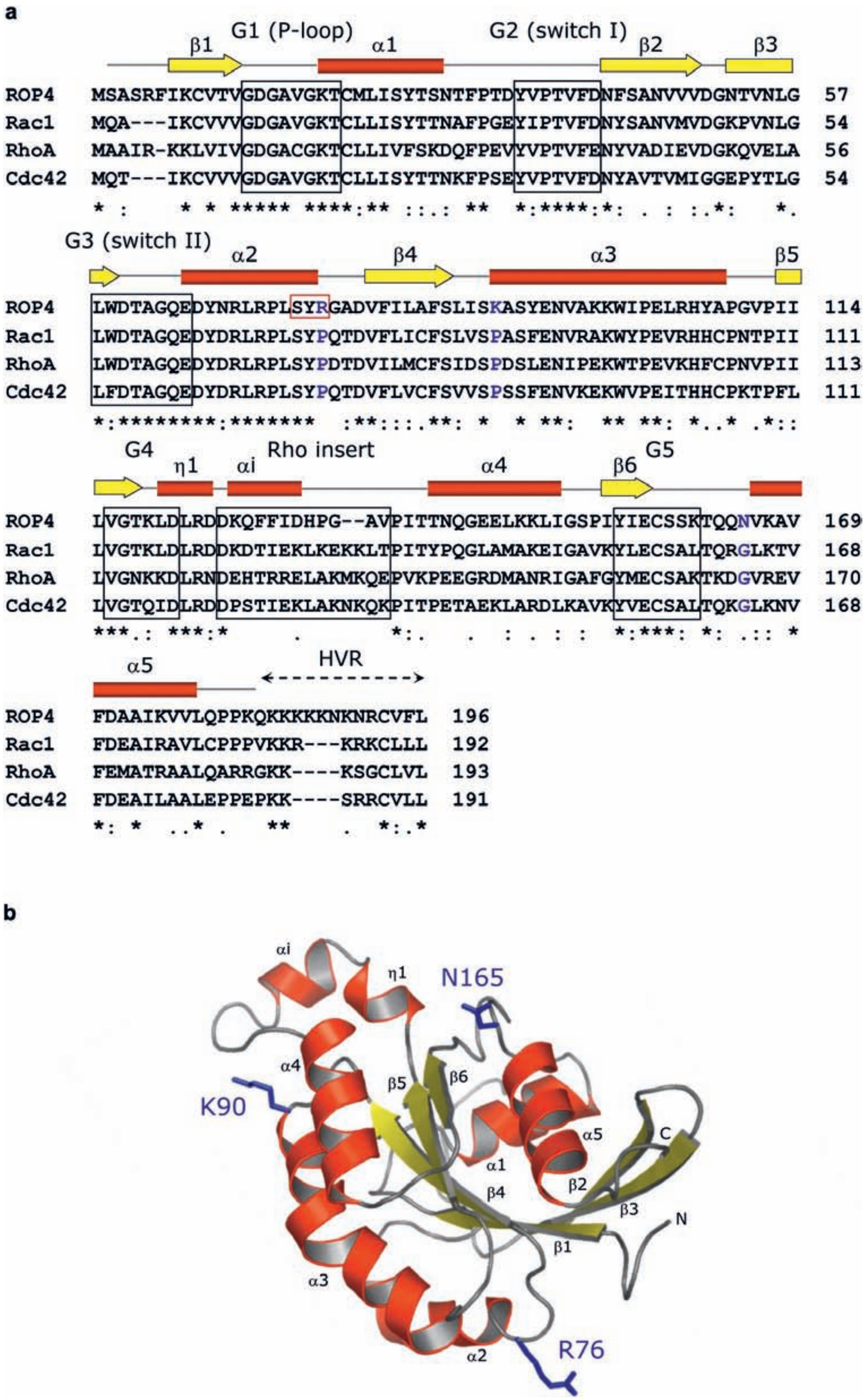
When comparing sequences, ROPs are clearly distinct from Rac as well as Rho and Cdc42, and make up a separate subgroup in phylogenetic trees of the Rho family [2, 14, 15]. Particular differences in ROP *versus* other Rho family sequences may attract special attention with respect to features that are specific for the plant proteins, as exemplified with *Arabidopsis thaliana* (At) ROP4, human RhoA, Rac1 and Cdc42 (Fig. 1a). A conserved arginine present in ROP proteins [11, 12] (R76 in AtROP4) downstream of switch II gives rise to a possible recognition site (SYR) for serine/threonine kinases that is never found in non-plant Rho GTPases [2]. In this context, it has been suggested earlier that ROPs associate with receptor-like kinases in high molecular weight complexes at the plasma membrane (PM) [16, 17]. Pronounced sequence variations also lie in the C-termini and a shortened Rho insert region. The latter makes Rho-type GTPases unique within the Ras superfamily and was discussed to be involved in certain effector interactions or effector activation [18, 19]. To date, only few homologues of animal and fungal Rho effectors have been identified in plants, raising the speculation that the characteristic and shortened insert region in ROP may contribute to define targets in plant-specific signaling pathways.

Unique features of ROP may also be seen from three-dimensional structure models. Structure prediction for AtROP4 reveals the conservation of the G domain [4] composed of a central six-stranded β-sheet ($\beta1$–$\beta6$) surrounded by five α-helices ($\alpha1$–$\alpha5$), the helix αi of the Rho insert and a short helical structure ($\eta1$) upstream of the insert (Fig. 1b). Similar models have been computed for two ROP proteins from *Medicago sativa* in which a shortened helix αi is present in MsRac4, while the corresponding region in MsRac1 shows no helix at all [20]. In the AtROP4 model certain amino acids that fundamentally differ between plant and non-plant Rho GTPases are orientated towards the surface of the molecule, making them possible candidates to add to ROP-specific interactions. These include R76 following $\alpha2$, a conserved lysine (K90 in AtROP4) at the beginning of $\alpha3$, which is replaced by an invariant proline in the animal and fungal Rho GTPases, and a surface exposed asparagine (N165 in AtROP4) instead of a glycine upstream of helix $\alpha5$. The role of these residues in ROPs, however, remains to be established.

Further implications may come from comparing ROP sequences within the plant kingdom. ROPs are encoded by a multigene family in most plant species characterized to date including eleven members in *Arabidopsis* [8, 11], and seven and nine sequences in rice and maize, respectively [12]. The ROP proteins display a high degree of sequence conservation and some family members are particularly alike, suggesting gene duplications late in evolution [11]. Still, there is diversity in the ROP family. ROPs from angiosperms were divided into four phylogenetic groups (I–IV) [2, 8, 12], and the evolution of different ROPs may be an adaptation to the lack of Ras, Rho, Rac and Cdc42 proteins in plants [8, 11]. However, the functional significance to this division into four classes remains to be established. On the other hand, two superordinate ROP groups have been described before, which can be distinguished by their posttranslational lipid modifications [11, 21, 22]. These are defined by the most variable region at the C-terminus of the ROP proteins, also known as hypervariable region (HVR) [21–23]. Such lipid modifications in the HVR are necessary to attach small GTPases to the membrane, where they function in signal transduction [24]. Depending on the HVR sequence, ROPs likely get prenylated and/or palmitoylated on cysteine residues and localize to the membrane [21–23]. Interestingly, a tobacco ROP (NtRAC5) was found in membrane microdomains described as lipid rafts [25], which are believed to function as signaling platforms capable of facilitating efficient and specific signal transduction [26]. The establishment of ROPs in discrete signaling complexes of lipid rafts likely contributes to their regulation of a variety of vital processes in the cell.

Polar growth – ROPs lead the way

Localization studies, gene knockouts and silencing of ROPs, as well as transgenic manipulations of the protein level and activation status with overexpressed (OX) wild-type (wt) ROPs and constitutively active (CA) or dominant negative (DN) mutants [8] have contributed



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ROP4   MSASRFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGNTVNLG  57
Rac1   MQA---IKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLG  54
RhoA   MAAIR-KKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELA  56
Cdc42  MQT---IKCVVVGDGAVGKTCLLISYTTNKFPSEYVPTVFDNYAVTVMIGGEPYTLG  54
       * :    * * ***** ****:** ::.: **  *:****:*: . : :.*:   *.

ROP4   LWDTAGQEDYNRLRPLSYRGADVFILAFSLISKASYENVAKKWIPELRHYAPGVPII  114
Rac1   LWDTAGQEDYDRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPEVRHHCPNTPII  111
RhoA   LWDTAGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPII  113
Cdc42  LFDTAGQEDYDRLRPLSYPQTDVFLVCFSVVSPSSFENVKEKWVPEITHHCPKTPFL  111
       *:********:*******  :**:::.**: *  * **:  ** **: *..* .*::

ROP4   LVGTKLDLRDDKQFFIDHPG--AVPITTNQGEELKKLIGSPIYIECSSKTQQNVKAV  169
Rac1   LVGTKLDLRDDKDTIEKLKEKKLTPITYPQGLAMAKEIGAVKYLECSALTQRGLKTV  168
RhoA   LVGNKKDLRNDEHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREV  170
Cdc42  LVGTQIDLRDDPSTIEKLAKNKQKPITPETAEKLARDLKAVKYVECSALTQKGLKNV  168
       ***.: ***:*     .       *:.   .  : . : :  *:***: *: .:: *

ROP4   FDAAIKVVLQPPKQKKKKKNKNRCVFL  196
Rac1   FDEAIRAVLCPPPVKKR---KRKCLLL  192
RhoA   FEMATRAALQARRGKK----KSGCLVL  193
Cdc42  FDEAILAALEPPEPKK----SRRCVLL  191
       *: *  ..* .   **    .  *:.*
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Figure 1. Sequence and structural features of ROP. (*a*) Alignment (http://www.ebi.ac.uk/clustalw) of *Arabidopsis thaliana* ROP4 (Q38937), human (h) Cdc42 (NP_001034891), hRac1 (NP_008839) and hRhoA (NP_001655) representing the conserved elements G1–G5 of the G domain [13], the Rho insert and the hypervariable region (HVR) [2, 15]. Red box: putative phosphorylation site; Consensus line: * identical residues; **:** conserved substitutions; **.** semi conserved substitutions. Secondary structure elements and blue residues refer to the ROP4 model depicted in (*b*). Modeling of ROP4 (http://salilab.org/modeller/modeller.html) is based on the structure of hRac1 (PDB: 1mh1) aligning residues 2–181 of hRac1 and 2–182 of ROP4. (N: N-terminus, C: C-terminus).

significantly to uncover their involvement in polar cell expansion (Fig. 2a). Polar growth applies to tip-growing cells represented by pollen tubes and root hairs, but also to regions in diffuse growing epidermal cells and probably trichomes [27–30]. ROPs were localized at the PM in the apical region of pollen tubes where growth takes place [31], and negative manipulations of the pollen-specific ROPs AtROP1 and AtROP5 inhibited tube growth [32–34]. In contrast, OX wt ROPs or CA mutants were ectopically localized at the PM and caused growth depolarization, implying that the establishment of polarity depends on the localized activation of ROP in the tip region [33, 34]. Interestingly, impaired pollen tube growth in response to altered copy numbers of *AtROP9* (*AtRAC7*) was implicated in reduced seed set of transgenic tobacco plants, suggesting the importance of ROP GTPases in sexual reproduction [35]. However, tube growth is not only spatially controlled by activated ROPs but, as shown recently, also temporally regulated by oscillating ROP activity [36]. Similarly to pollen tubes, ROPs seem to regulate polar tip growth of root hairs [37–39]. ROPs localize to the future site of swelling formation, which precedes the outgrowth of hairs from trichoblasts, indicating an additional role of ROPs in early processes of root hair development [37, 38]. Equivalent mechanisms likely apply for the polarization of diffuse growth of unspecialized leaf epidermal cells (pavement cells), which contributes to their morphogenesis [27, 28]. In wt *Arabidopsis* leaves, differentiated pavement cells form pronounced lobes and indentations, resulting in a jigsaw-puzzle-shaped morphology, which was impaired by *CA*- or *DN-Atrop2* expression [40]. Lobe formation occurs in several stages starting from pentagonal or hexagonal initial cells that begin to build multiple outgrowths [30]. The DN form of AtROP2 affected only the early stages of cell expansion, and AtROP2 tagged with the green fluorescent protein (GFP) preferentially localized to sites of lobe initiation [40], similar to what is described for future root hair sites on trichoblasts [27]. Later in pavement cell development, GFP-tagged AtROP2 is concentrated at the tip of newly formed lobe primordia, indicating that their further polarized expansion proceeds by mechanisms comparable to tip growth [40]. Taken together, localized ROP signaling seems to provide a common mechanism for the regulation of polar growth.

ROPs are probably linked to various aspects of polar growth by their influence on cytoskeletal elements, calcium (Ca^{2+}) fluxes and reactive oxygen species (ROS) (Fig. 2a) [8, 41–43]. Accumulating data reveal that ROP signaling specifically promotes the assembly of dynamic, fine filamentous (F)-actin at growth sites in pollen tubes, root hairs, and pavement cells [38, 40, 44–47], and this is believed to spatially fine-tune the delivery of Golgi-derived vesicles with cell material [28]. Cell expansion is usually antagonized by bundled cortical microtubules (MTs) that co-align with cellulose fibrils, which ultimately prevent outgrowth [48]. In this context, ground-breaking work in pavement cells proposed that AtROP2 and AtROP4 not only promote actin assembly but at the same time inhibit MT bundling in the outgrowing lobes, while bundling is locally induced by ROP in the indentations [47]. ROPs presumably also influence the intracellular Ca^{2+} level at the tip of growing pollen tubes [33, 46] and root hairs [37], which may regulate vesicle fusion with the apical PM region [49, 50]. Stimulation of actin assembly and Ca^{2+} accumulation at the tip were shown to be separate functions of AtROP1 in pollen tubes [46]. Yet, actin and Ca^{2+} counteract each other as F-actin can negatively regulate the influx of extracellular Ca^{2+} [51], which in turn can promote actin disassembly at high intracellular concentrations [46]. There is also increasing evidence that polar growth is affected by the ROP-dependent formation of ROS [52–54], which are believed to activate Ca^{2+} channels [53], or may lead to cell expansion, independently of Ca^{2+}, due to wall loosening by non-enzymatic cleavage of cell wall polysaccharides [43]. In mammals, ROS are generated through the action of the NADPH oxidase complex, also called respiratory burst oxidase (RBO) [55]. However, plants lack homologues of two complex subunits, $p47^{phox}$ and the mammalian Rac effector $p67^{phox}$ [2, 55], and thus the important ROP-ROS connection is still an unsolved question (see below).

The input of ROPs for the outcome of plant-microbe interactions

Another physiological role of ROPs is emerging for the regulation of plant responses to microbes (Fig. 2b). Plant-microbe interactions can be beneficial, as in symbiotic *Rhizobium*-legume and mycorrhizal-root associations, but in other cases, microbes act as pathogens that either cause diseases in susceptible species or provoke a resistance response that is associated with successful defense reactions [56, 57]. First hints for a possible role of ROPs in symbiotic interactions come from transcriptional analyses in which Rho-like GTPases were found to be differentially expressed in ectomycorrhizal root tissue formed between *Betula pendula* and *Paxillus involutus* [58], and in *Sinorhizobium meliloti* infected roots of *M. sativa* [59].

More data clearly demonstrate the involvement of ROPs in plant responses to pathogenic microbes [15, 41, 42]. CA and DN mutants of the rice ROP protein OsRAC1 altered the resistance (R)-gene-mediated defense responses of rice against blight disease-causing *Xanthomonas oryzae* and the blast fungus *Magnaporthe grisea* [60]. OsRAC1-dependent resistance is likely accomplished through the production of H_2O_2 and other ROS, resulting in an oxidative burst to cause cell death, synthesis of

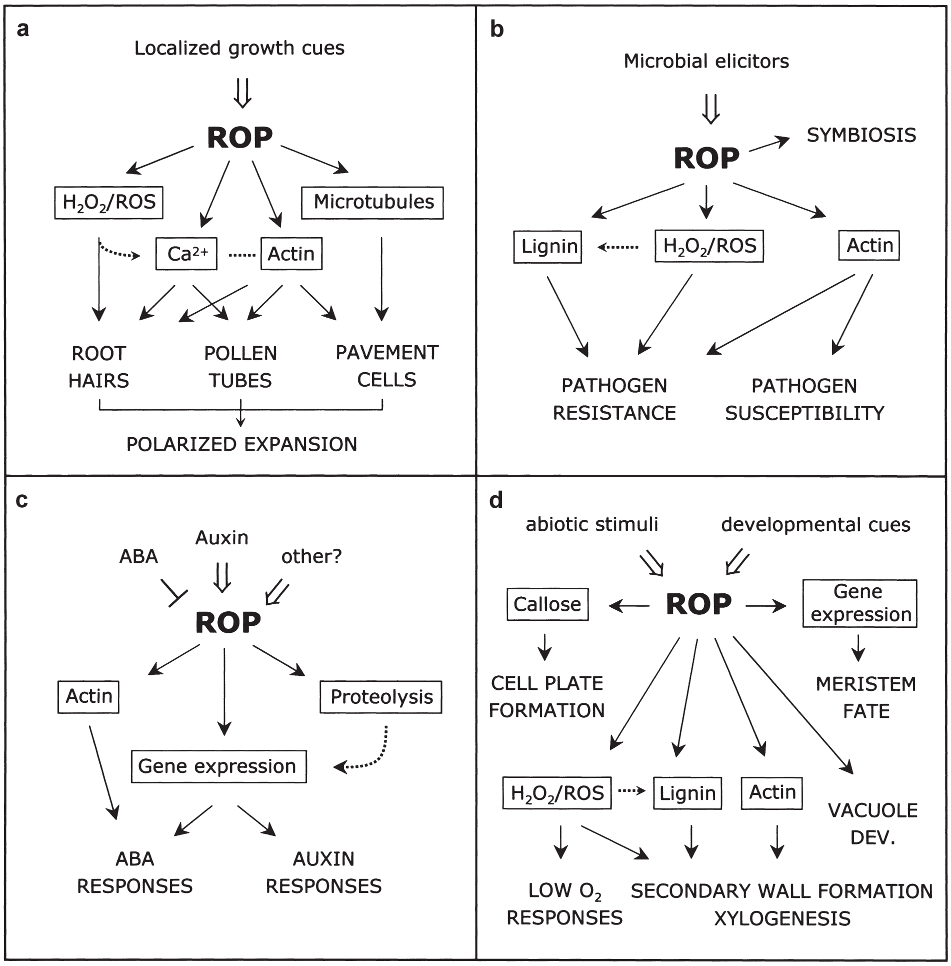

Figure 2. Simplified flow charts for ROP-dependent processes in plants. ROP signaling in response to diverse upstream cues involves a set of downstream tools (boxed) to implement aspects of polar growth (*a*), plant-microbe interactions (*b*), phytohormone responses (*c*), and other processes in development (DEV.) and stress adaptation (*d*). Cross-relations occur as indicated by dotted lines.

phytoalexins and expression changes in defense-related genes [60–62]. At the same time, OsRAC1 apparently prevents ROS removal as it was shown to down-regulate the ROS scavenger metallothionein [63]. Studies in other systems further substantiated the importance of ROPs in elicitor-induced defense responses associated with H_2O_2/ROS and cell death [64–67].

Still, the ROP-dependent defense machinery relies not only on ROS (Fig. 2b). Lignin represents an undegradable mechanical barrier to pathogens, and OsRAC1 was found to regulate the biosynthesis of lignin precursors (monolignols) that can be polymerized with H_2O_2 by the peroxidase activity in the cell wall [68]. Further, the actin cytoskeleton seems to play a crucial role in resistance mechanisms by mediating the polarized deposition of defense-related compounds at microbial penetration sites [69, 70]. In this context, recent work discovered that a barley ROP protein (RACB) positively regulates the polarization of actin filaments to physically block tissue penetration by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) attacking mutant barley cells that lack the susceptibility factor MLO [71]. Positive manipulation of ROP signaling can, on the other hand, also enhance the susceptibility of plants to pathogens, and this effect appears to be specific for distinct ROP proteins as seen for the barley/*Bgh* [23, 72, 73] and the rice/*M. grisea* [60, 74] systems. Hence, isoform specific ROP activation may contribute to the final outcome of a pathogen attack. The underlying mechanisms in susceptibility pathways perhaps engage actin-driven polar membrane growth resulting in PM invaginations as observed during the establishment of fungal haustoria in barley [73]. These invaginations are reminiscent of endocytosis, which occurs in root hairs and was recently linked to ROP activity [39].

Phytohormonal cues – Regulatory clues

Several observations suggest that ROP GTPases play a role in phytohormone signaling (Fig. 2c), and thus help to coordinate several aspects of plant growth, development and physiology [8, 42]. Initial studies using transgenically expressed *CA*- and *DN-rop* constructs in *Arabidopsis* revealed pleiotropic developmental phenotypes and altered responses to exogenous application of abscisic acid (ABA), auxin and brassinolides [75]. However, a direct involvement of ROP in phytohormone-induced pathways remained ambiguous considering that the observed readouts might also result from complex cross-talks with other pathways, possibly involving G protein-coupled receptors (GPCRs) and heterotrimeric G proteins, which have also been implicated in phytohormone signaling [76]. The participation of ROP in ABA signaling was further substantiated as ABA treatment caused inactivation of AtROP3 (AtRAC1) and down-regulation of AtROP10 promoter activity in *Arabidopsis* [77, 78]. Additional forward and reverse genetic approaches demonstrated that individual ROPs, and specifically AtROP10, likely act as negative regulators of multiple ABA responses including stomatal closure, the control of seed dormancy and germination, and the suppression of root growth and gene expression [73, 77–79]. In the assumed pathways, the negative regulation of ROP activity by ABA is an essential early signaling event to induce ABA responses, while ABA suppression of ROP gene transcription could be crucial for sustained ABA action [78]. However, to date it is still unclear how ROPs interfere with ABA responses. During stomatal closure, ABA treatment induced a breakdown of F-actin in guard cells in *Arabidopsis* and barley, and this effect was blocked by CA forms of ROP probably through their function in actin reorganization [73, 77]. Moreover, various negative effects of AtROP10 seem to be accomplished by a specific and differential modulation of the ABA sensitivity of certain genes encoding regulatory proteins like mitogen-activated protein kinases (MAPKs) and transcription factors known to function in ABA responses [79].

In contrast to ABA signaling, ROPs might work as positive regulators in auxin pathways [80, 81]. ROP activation was demonstrated in auxin-exposed tobacco seedlings, and the OX tobacco ROP NtRAC1 or its CA mutant activated auxin responsive promoters and induced certain phenotypes similar to auxin-overproducing mutants [80]. Opposing effects were observed when ROP signaling was inhibited by *DN-Ntrac1* expression or RNA interference. Since auxin can activate MAPKs it was suggested that NtRAC1 may activate MAPK cascades to regulate auxin-dependent gene expression [80]. Moreover, subsequent work of the same group provided evidence that ROPs mediate the auxin-induced ubiquitin/26S proteasome-dependent degradation of transcriptional regulators of the auxin/indol acetic acid (AUX/IAA) protein family that otherwise act as repressor for auxin-inducible gene expression [81]. Plant growth and development, and many physiological processes strongly depend on the selective removal of short-lived proteins involving ubiquitin and the 26S proteasome [82], and it will be highly interesting to unravel if ROP GTPases use proteolysis as a more general tool to regulate different signaling pathways.

Other jobs for ROPs

ROPs are believed to regulate further processes during plant development and stress responses (Fig. 2d) [2, 8, 14, 42]. ROP signaling was implicated in vacuole development, callose synthesis for cell plate formation, and the establishment of secondary cell walls in differentiating cotton fibers, which likely relies on H_2O_2 to promote cellulose synthesis [2, 8, 41]. Secondary wall formation arises as well during xylogenesis, and xylem-specific expression patterns in *Arabidopsis* indicate a role for AtROP7 in the development of xylem vessels [83]. The underlying machinery may comprise actin reorganization for vesicle-dependent deposition of wall components. Noticeably, the actin-disrupting drug cytochalasin D was associated with thinner cell walls in interfascicular fiber cells of *Arabidopsis* [84]. ROP-dependent synthesis of lignin and H_2O_2 are possibly also engaged in xylogenesis to strengthen secondary walls, and to induce cell death that accompanies final trachea formation [68, 85].

ROPs may also influence the fate of meristematic tissue. A ROP protein was found as part of the active *Arabidopsis* CAVATA1 complex, which controls gene expression during stem cell differentiation in the shoot meristem, possibly involving a MAPK cascade [16]. Additional roles for ROP GTPases might be expected in plants during abiotic stress situations as seen for oxygen deprivation [86]. In *Arabidopsis* seedlings, lack of oxygen was proposed to generate the transient activation of ROP signaling leading to the production of H_2O_2, which induces the expression of alcohol dehydrogenase to increase ethanolic fermentation. At the same time, H_2O_2 stimulates the up-regulation of the ROP deactivator RopGAP4 (see below) to restrict the formation of ROS. Future studies will uncover if this exciting kind of negative feedback regulation also applies to other ROP-controlled processes.

Regulation of the ROP switch – ON and OFF it goes!

The emergence of ROP GTPases as key players in a multitude of cellular processes has fostered a great effort to unravel their regulation in the cell. However, this chapter of the ROP story is ambiguous because the analogous mechanisms from animals and fungi cannot simply be translated into the plant system. The biochemistry of ROPs is similar to other Rho GTPases in terms of slow intrinsic GTP hydrolysis rates of 2×10^{-4}–6×10^{-4} s^{-1} ([37], and unpublished observations), high-affinity GDP/GTP binding reflected in equilibrium dissociation constants (*K*d) in the low nanomolar range (2 nM for *N*-methylanthraniloyl-GDP and AtROP4) (unpublished observations), and slow intrinsic nucleotide exchange with dissociation rate constants (K_{obs}) from 0.78×10^{-4} s^{-1} to 6.8×10^{-4} s^{-1} [87, 88]. Consequently, a fast GDP/GTP cycling of the ROPs requires the catalysis of two classes of regulatory proteins, GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which both differ significantly from their non-plant counterparts.

RopGAPs – The tic with the CRIB

GAPs usually interact specifically with the activated, GTP-bound conformation of small G proteins engaging the switch regions, and promote GTP hydrolysis by up to five orders of magnitude *in vitro* [89]. The reaction mechanism frequently involves an arginine residue that resides in the catalytically active GAP domain of the proteins. This so-called arginine finger is inserted into the active site of the G protein to stabilize the transition state during GTP hydrolysis [89]. Plant RopGAPs from different species including *Arabidopsis* [90] and lotus [91] have the GAP domain in common with their non-plant homologues, and this domain contains an invariant arginine that aligns with the arginine finger in RhoGAPs [90]. In addition to the GAP domain, most RopGAPs exhibit an upstream proline-rich region (PRR) and a 16-amino acid Cdc42/Rac-interactive binding (CRIB) motif in the designated CRIB domain near the N-terminus (Fig. 3) [90]. The PRR contains a core consensus motif PXXP (P: proline; X: any amino acid) known as binding motif for Src homology (SH)-3 domains that arrange processes such as increasing local concentration of proteins, altering their subcellular location and mediating the assembly of large multi-protein complexes [92]. However, the function of this region in RopGAPs is still unknown. Noticeably, a CRIB motif as in RopGAPs has never been found in other Rho family GAPs, but it is frequently present in Cdc42

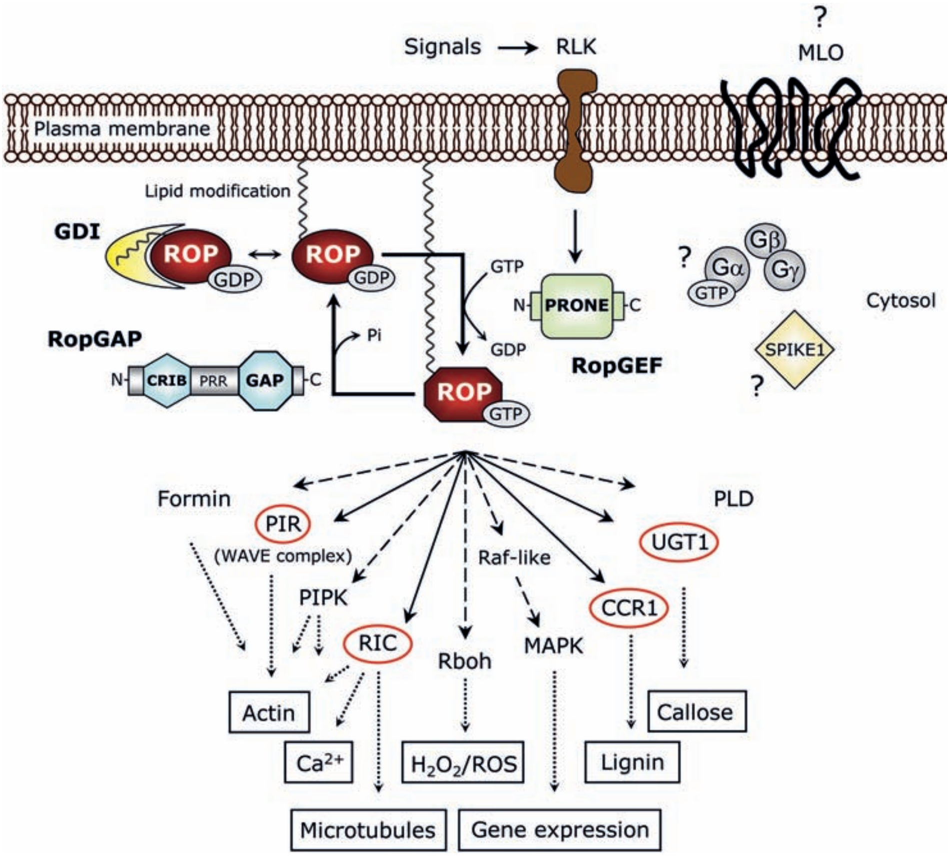

Figure 3. Model for ROP-regulated pathways in plants. ROP is anchored in the membrane via lipid modifications, or resides in the cytosol bound to GDI. Cycling between the GDP- and GTP-bound conformations is catalyzed by RopGAP and RopGEF, shown schematically by their domain structure composition. (Lipid moieties are the same in the GDP- and GTP-bound form of ROP; different length was chosen for the more concise illustration of the regulatory cycle involving GEF and GAP). ROPs relay signals from receptor-like kinases (RLK) via RopGEF to effector molecules (red circles) that influence a variety of downstream elements in the cell (boxed). Links to hypothetical or indirect ROP targets are indicated by dashed arrows. Question marks denote the yet-undefined role of MLO, the subunits $G\alpha$, $G\beta$ and $G\gamma$ of heterotrimeric G proteins, and SPIKE1 in ROP-dependent pathways. CCR: Cinnamoyl-CoA reductase; MAPK: mitogen-activated protein kinase; PIPK: phosphatidylinositol monophosphate kinase; PIR: p53-inducible RNA; PLD: phospholipase D; Rboh: respiratory burst oxidase homologue; RIC: ROP-interactive CRIB motif-containing protein; UGT: UDP-glucose transferase.

and Rac effectors [93], where it serves as a binding site for the GTP-bound small G proteins. This interaction involves the switch regions, so that CRIB containing effectors usually compete with GAPs for Rho binding and thus can inhibit RhoGAP activity [89]. By contrast, the CRIB motif in RopGAPs is essential for RopGAP activity as it presumably mediates high-affinity binding to ROPs [90]. Still, it remains unclear why and how plants use the CRIB/GAP constellation to down-regulate ROPs. In semiquantitative interaction assays using the β-galactosidase reporter of the yeast two-hybrid system, the isolated CRIB domain of RopGAP2 differentially interacted with distinct ROPs from *Arabidopsis*, while the GAP domain showed less specific binding (unpublished observations). In view of the diversity of ROP proteins within a given plant species, the CRIB domain of RopGAPs may contribute to define the specificity for distinct ROP substrates.

RopGEFs – PRONE to switch

Compared to the sparsely understood inactivation reaction, ROP activation had long been a complete mystery. No RopGEFs had been identified when plant genomes were screened for homologues of 'common' RhoGEFs like the diffuse B cell lymphoma (Dbl)-type GEFs with conserved Dbl-homology (DH) and pleckstrin-homology (PH) domains [2, 8, 9, 14]. Another protein family termed CZH (CDM-Zizimin-homology) has been introduced to function as RhoGEFs in eukaryotic organisms, and it is still controversial if certain family members, *i.e.* the human Dock180 protein, require a co-factor named ELMO (engulfment and cell motility) for nucleotide exchange activity [94]. *Arabidopsis* and rice hold a single CZH protein termed SPIKE1 [94, 95], and the *Arabidopsis* genome reveals at least six predicted proteins with ELMO-like domains [96]. The *Arabidopsis spike1* mutant displayed conditional seedling lethality, distorted trichomes, altered leaf shape and defects in pavement cell morphology [95]. The latter resemble defects induced by transgenic mutants of ROP [40], and a pathway from SPIKE1 via ROP to the actin cytoskeleton has been proposed to operate during epidermal cell lobe formation [96]. Nevertheless, neither the function of SPIKE1 as a GEF, nor the relevance of ELMO-like proteins in plants have yet been documented in print, except for a conference abstract in which recombinant SPIKE1 was reported to have GEF activity [97].

On the other hand, plants possess a unique family of RhoGEFs whose members have clearly been demonstrated to specifically activate ROP small G proteins *in vitro* and *in vivo*, and thus were designated RopGEFs [87, 88]. RopGEFs are abundant proteins in plants, and are differentially expressed in diverse tissues [87, 88, 98]. Their primary structure is characterized by a highly conserved catalytic domain designated PRONE (plant-specific ROP nucleotide exchanger) (Fig. 3) [87], which was stored in the database as domain of unknown function (DUF) 315 [87, 88]. PRONE was found to stimulate nucleotide dissociation from ROP with catalytic properties comparable to Dbl-type GEFs [87], but the catalytic mechanism is still unknown. GEF catalysis is believed to follow a push-and-pull mechanism affecting the switch regions of small G proteins [4], and it will be highly interesting to ascertain if this mechanism also applies to the PRONE-type GEFs. The three-dimensional structure of PRONE in a complex with ROP will be required to address this question, and this may also help to determine the features that make ROP the exclusive substrate for PRONE [87].

PRONE is flanked by the variable N- and C-termini of the RopGEFs which may be important for the regulation of the proteins. An autoinhibitory mechanism involving the N- and C-terminal regions was proposed for the regulation of RopGEF1 from *Arabidopsis*, and an intramolecular interaction between the C-terminus and the catalytic domain may block GEF activity [88]. The activating factors for RopGEFs are so far unknown, and more data are required to unravel the regulation of these molecules in more detail. Most interestingly, the tomato RopGEF kinase partner protein (KPP) was found associated with the membrane and interacted with the pollen-specific receptor-like kinases (RLKs) LePRK1 and LePRK2 (see below) [98], implying that RopGEF activity may be regulated by RLKs. Activity of RopGEF1 was correlated with polarized growth of pollen tubes [88], and further lines of evidence *in planta* support the notion that RopGEFs activate the ROP switch *in vivo*. Pollen-expressed RopGEFs were localized at the tip of pollen tubes, and overexpression phenotypes demonstrated aberrant actin distribution and growth depolarization as seen with OX wt or CA forms of ROP [88, 98]. Contrariwise, the effects of OX RopGEF1 could be reverted by co-expressing RopGAP1 [88]. A pathway can be envisioned in which localized cues at pollen tube tips trigger RopGEF-dependent activation of ROP via RLKs at the PM to induce cellular actin rearrangements for polar growth [99].

GDIs – More than just cytosolic sequestration

A third class of regulatory molecules for Rho GTPases covers the guanine nucleotide dissociation inhibitors (GDIs), which affect the activation status and subcellular localization of Rho proteins [100]. GDIs usually form high-affinity complexes with lipid-modified small G proteins shielding the lipid moiety from the solvent. This sequesters the GTPases in the cytosol (Fig. 3) and negatively regulates their activation at the membrane [100]. Plant GDIs from *Arabidopsis* and tobacco were described by their similarity to animal RhoGDIs and were shown to interact with distinct ROPs in yeast two-hybrid tests and *in vitro* [101, 102]. Their role for the regulation of ROP recruitment to the PM is assumed because overexpression of the *Arabidopsis* RhoGDI1 removed GFP-tagged ROPs from the PM in tobacco protoplasts and pollen tubes [8, 80]. Further experimental data suggest the importance of GDIs for the negative regulation of ROP signaling in plants. RhoGDI1, like RopGAP1, interfered with the NtRAC1-dependent activation of an auxin-responsive promoter [80], and suppressed depolarized growth and the reorganization of the actin cytoskeleton induced by OX AtROP1 in pollen tubes [44]. A model for polar pollen tube growth has been proposed in which GDIs are involved in restricting the ROP signaling pathway to the tip [8]. Compelling evidence for such a spatial regulation of ROP-mediated cell growth by plant RhoGDIs came recently from the *Arabidopsis* RhoGDI1 mutant *supercentipede1* (*scn1*), which exhibited multiple sites of root hair growth (bulges) on single trichoblasts and multiple growing axes from single bulges [54]. Supernumerary hair initiation sites in mutant plants were found to be associated with the ectopic and sustained accumulation of AtROP2 and the formation of ROS, implying that AtRhoGDI1 controls the pattern of root hair growth by regulating ROP activity and the ROP-dependent production of ROS at growth sites. Similar mechanisms may be expected for polar growth processes in pollen tubes and pavement cells giving RhoGDIs a key role in organizing spatially restricted cell growth in plants.

The ups and downs of ROP signaling

Although our understanding of the switch reaction of ROP GTPases and their impact on pivotal processes in plants is rapidly proceeding, the upstream events that elicit ROP-dependent reaction cascades, as well as the downstream effectors that mediate the biological function are just beginning to be uncovered. Consulting animal and fungal systems as templates has often been unsatisfactory as plants frequently bank on different concepts for the perception and transmission of signals to generate a cellular response.

How to launch ROP-dependent pathways in plants?

Plants seemingly lack receptor tyrosine kinases that can induce Rho-mediated pathways in animals in response to ligand binding [6], and much attention has been drawn to receptor-like kinases as the prominent cell surface receptors for extracellular signals in plants. Like ROPs, RLKs participate in plant development, disease resistance and hormone perception [103]. They are characterized by an extracellular domain often comprising multiple leucine-rich repeats (LRRs), a transmembrane (TM) region and a cytosolic kinase domain with serine/threonine-type specificity. A role for RLKs in ROP signaling can be assumed from the CLAVATA1 complex enclosing a ROP protein and the LRR-type RLK CLAVATA1 [16]. Likewise, the pollen-specific tomato RLKs LePRK1 and LePRK2 were found in a PM complex with ROP [17]. The link between the PRKs and ROP was established when the tomato RopGEF KPP was characterized as binding partner of the cytosolic domains of LePRK1 and LePRK2, which possibly involves the variable C-terminus of KPP [98]. This finding argues for a pathway in which PRKs relay pollen or pistil signals via RopGEF to ROP to affect pollen germination or pollen tube growth [99]. Phosphorylation might be an important instrument in the pathway because LePRK2 and KPP were found to be phosphorylated in pollen [98, 104]. Whether phosphate moieties influence the catalytic activity of RopGEFs and/or affect the interaction between pathway components still needs to be examined. A direct phosphorylation of ROPs by RLKs can be imagined too, keeping in mind the putative phosphorylation site in ROPs. Beyond PRK and probably CAVATA1 signaling, the RLK-RopGEF-ROP route may be more generally relevant in plants (Fig. 3). ROPs may interact with an as-yet-undefined PM-associated auxin reception mechanism [81], and a LRR-type RLK termed RPK1 has recently been shown to be involved in early ABA perception in *Arabidopsis* [105]. It is tempting to speculate that aspects of phytohormone signaling involve RLKs and ROPs via RopGEFs.

Yet, RLKs are probably not the only upstream players to feed into ROP pathways (Fig. 3). Heterotrimeric G proteins have been linked with OsRAC1 in disease resistance mechanisms of rice [106, 107]. Such heterotrimeric G proteins transduce signals from GPCRs in the PM to the cell interior when ligand binding induces their dissociation into the Gα and G$\beta\gamma$ subunits, which can subsequently signal to Rho GTPases involving GEFs and protein kinases [6]. Few putative GPCRs with seven TM spans (7TMS) have so far been described in plants, and two heterotrimeric G proteins exist in *Arabidopsis* and rice, respectively [76]. These are assembled by varying the single Gα and Gβ subunits with two available Gγ subunits. Mutation of the gene for Gα in rice was found to be accompanied by reduced resistance to blast fungus infection and suppressed H_2O_2 production [106]. The mutation was complemented by the CA form of OsRAC1, supporting the idea that OsRAC1 operates downstream of Gα. However, evidence for the input of a GPCR in this pathway is missing, and the connection between Gα and OsRAC1 remains to be elucidated. In barley, the susceptibility factor MLO for *Bgh* infection is a 7TMS protein reminiscent of a GPCR, and as RACB and MLO are required for fungal entry, it was speculated that they might be functionally linked [72]. Still, MLO-related defense modulation to *Bgh* seems to work without heterotrimeric G proteins, and MLO has recently been predicted to act downstream of RACB [73], suggesting a more complex regulatory network.

ROP effector molecules – Who is on duty?

ROP signaling is frequently implemented by cytoskeletal rearrangements, Ca^{2+} fluxes, ROS production, altered gene expression and other instruments in the cell that are ultimately affected by ROP effectors (Fig. 3). Actin polymerization has been the focus of many studies, and it is now emerging that distantly related equivalents of the pentameric animal WAVE (Wiskott-Aldrich syndrome protein family Verprolin-homologous protein) complex activate the actin nucleating Arp2/3 complex in plants [28, 29, 108]. The plant homologue of the regulatory WAVE complex subunit SRA-1/PIR121 (specifically Rac1-associated/p53-inducible RNA), termed PIR in *Arabidopsis*, likely works as an effector of AtROP2 that interacts with the GTPase with strong specificity for the GTP-bound form [109]. In analogy to the animal system, it may be assumed that binding of ROP to PIR induces the WAVE subunit-dependent actin polymerization in plants.

Plants also contain homologues of diaphanous-related formins that operate as Rho effectors in animals and yeast to induce actin polymerization upon binding of Rho to its GTPase binding domain (GBD) [110]. Overexpression of the *Arabidopsis* formin AFH1 was shown to stimulate actin cable formation in pollen tubes and resulted in depolarized phenotypes similar to those induced by OX wt or CA forms of ROP [111]. This might indicate that ROPs and formins function in the same pathway. Nonetheless, a GBD has never been identified in plant formins [110], and to date there is no evidence for a direct link to ROP. Still, one may guess that formins are subject to indirect ROP regulation possibly involving a larger protein complex. GBDs in animal Cdc42 and Rac effectors frequently contain a CRIB motif [93]. Plants possess a unique family of small polypeptides with the CRIB motif, known as ROP-interactive CRIB motif-containing proteins (RIC1–RIC11 in *Arabidopsis*) [112]. The RICs have been established as putative ROP effectors through their CRIB motif-dependent interaction with activated ROPs. It was recently demonstrated *in planta* that RIC4 is a target of AtROP1 and promotes the assembly of fine F-actin at pollen tube tips as a necessary element for polar expansion [46]. In leaf pavement cells, activated AtROP2 was in charge and interacted at the lobe tips with RIC4, which in turn stimulated fine cortical F-actin required for lobe outgrowth [47]. Interestingly, the RICs not only affect actin assembly in these scenarios but influence other targets, resulting in a coordinated regulation of polar growth. AtROP1 was shown to engage RIC3 in pollen tubes to stimulate influx and accumulation of tip-focused Ca^{2+} [46]. In pavement cells, RIC1 was found co-localized with cortical MTs and likely promoted their organization into bundled structures [47]. Activated AtROP2 suppressed the function of RIC1, and it was proposed that AtROP1 in its activated form sequesters RIC1 away from the MTs, and thus prevents bundle formation. Except the CRIB motif, the RICs display no other domains with an assigned function, and the molecular mechanisms of RIC-mediated actin assembly, Ca^{2+} accumulation and MT organization so far remain elusive. It was proposed that RICs act as adaptors that link ROPs to the actual signal relaying molecules [112], raising the need to identify binding partners of RICs.

Other pathways connecting ROPs with actin and Ca^{2+} signaling in pollen tubes may involve phosphatidylinositol monophosphate kinase (PIPK) which, in analogy to animal systems, has been suggested to be a ROP effector in plants [2, 8, 14]. GTP-dependent binding of ROP to PIPK has never been shown, but AtROP7 (AtRAC2) was found to be physically associated with tobacco pollen tube PIPK activity, which likely involves additional factors [34]. PIPK synthesizes phosphatidylinositol 4, 5-bisphosphate (PI 4, 5-P_2), which is known to regulate actin organization by interacting with actin binding proteins [113]. It serves as substrate for phospholipase C (PLC) for the synthesis of inositol 1, 4, 5-triphosphate (IP_3), which may affect Ca^{2+} levels during pollen tube elongation [34]. Phospholipase D (PLD) has also been linked to ROP GTPases [114]. PLDs are widely distributed in all organisms including plants [115]. They generate phosphatidic acid, which is generally believed to have an important signaling role. There is much evidence that RhoA, Rac1 and Cdc42 activate human PLD1 in the presence of GTPγS [115], and likewise a *Brassica campestris* ROP was found to affect the activity of a rat PLD [114]. However, the connection between ROP and the plant PLDs needs to be substantiated in future studies.

A great deal work has addressed the important route from ROPs to ROS. *Arabidopsis* contains several homologues (AtRboh) of the mammalian NADPH oxidase subunit gp91^{phox}, which catalyzes oxygen reduction to initiate H_2O_2 and ROS formation [15, 55]. Based on knockout mutants, the isoforms AtRbohD, F and C have clearly been correlated with the formation of ROS in response to pathogen infection and in growing root hairs, respectively [52, 116]. Tobacco NtRbohD and NtRAC5 were both found in lipid rafts upon elicitation of cells with cryptogein [25], indicating that the proteins are functionally linked. In addition, ROP-dependent ADH induction via ROS in *Arabidopsis* was found to be sensitive to a NADPH oxidase inhibitor [86], and the analysis of ROS formation in single and double knockouts of AtRhoGDI1 and AtRbohC [54] also implied an essential function of ROPs in the regulation of plant Rboh proteins. ROPs could either directly activate these enzymes [54] or activation may require a mediator.

Other ROP effectors target developing or mature cell walls as seen for the cell plate-associated putative callose synthase complex subunit UDP-glucose transferase (UGT1) that displayed specificity for activated AtROP1 [117], or the OsRAC1 effector cinnamoyl-CoA reductase (OsCCR1), an enzyme involved in the generation of monolignols for lignin biosynthesis [68]. ROPs have also been implicated in gene expression, and certain pathways may involve a MAPK cascade. OsRAC1 has been shown to regulate the activation of OsMAPK6 during pathogen defense, and both proteins seem to be part of the same protein complex when the GTPase is activated [107]. However, no direct interaction is evident. OsRAC1 also controls H_2O_2 production [60, 61], which can induce MAPKs in rice [118]. This argues for a model in which a MAPK cascade is activated via the ROP-dependent formation of H_2O_2 as second messenger [15]. However, ROPs might as well be linked to MAPKs involving so far-uncharacterized Raf-like kinases that can associate with ROP in the yeast two-hybrid system [14]. It may be expected from more yeast two-hybrid screens and analyses of ROP-containing protein complexes that additional effectors are discovered which will further contribute to our understanding of the ROP-signaling network in plants.

Concluding remarks

A wealth of knowledge on ROP signaling has been gathered since the discovery of this unique class of Rho GTPases, and their vital function in the life cycle of plants is now becoming evident. Numerous molecules that operate around the ROP switch have been identified in the past, but the breakdown of complete reaction cascades and their interactions is still not finished at all. The list of participants supplied in this review is likely to expand rapidly with the help of genetics, biochemistry and proteomic approaches. Particularly, the identification of RopGEFs and their relation to RLKs opens new roads to unravel the early events of signal transduction for polar growth and a variety of other cellular processes. The RICs appear to be key molecules with importance for succeeding signaling steps, and they seem to provide a means of cross-talk between antagonizing and probably synergistic pathways. Still, we do not yet understand their mode of action, and the identification of their binding partners appears to be a promising strategy to further unravel ROP signaling pathways in plants.

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