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REVIEW

A RHOse by any other name: a comparative analysis of animal and plant Rho GTPases

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Rho GTPases are molecular switches that act as key regulators of a many cellular processes, including cell movement, morphogenesis, host defense, cell division and gene expression. Rho GTPases are found in all eukaryotic kingdoms. Plants lack clear homologs to conventional Rho GTPases found in yeast and animals; instead, they have over time developed a unique subfamily, ROPs, also known as RAC. The origin of ROP-like proteins appears to precede the appearance of land plants. This review aims to discuss the evolution of ROP/RAC and to compare plant ROP and animal Rho GTPases, focusing on similarities and differences in regulation of the GTPases and their downstream effectors.

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Introduction

The Ras superfamily of small GTPases constitutes a large class of monomeric GTP-binding proteins with a molecular weight of 20–40 kDa found in all eukaryotes [1]. The basic property of these proteins is their ability to bind and hydrolyze GTP. Thus, the GTPases can exist in two states: they are active when GTP is bound and inactive when GDP is bound. The ability to cycle between active and inactive states makes these proteins an ideal "molecular switch" for the transmission of discrete "on-off" signals in the cell [2].

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Abbreviations: CRIB (Cdc42/Rac-interactive binding); DH (Dbl homology); FH (formin homology); GAP (GTPase-activating protein); GDI (GDP dissociation inhibitor); GEF (GDP exchange factor); PH (Pleckstrin homology); PRONE (plant-specific Rop nucleotide exchanger); Rboh (Respiratory burst oxidase homolog); RIC (ROP-interactive CRIB-containing protein); RLK (Receptor-like kinase); ROP (Rho-related GTPase from plants); ROS (reactive oxygen species)

The Ras superfamily is classified into five families: Rab, Arf/Sar, Ran, Ras and Rho. Rab and Arf/Sar family GTPases function in intracellular vesicle trafficking; Ran is involved in nuclear transport, whereas the Ras and Rho family GTPases regulate signal transduction from the plasma membrane. Curiously, Ras GTPase homologs have not been found in viridiplantae (land plants and green algae). Research on Rho GTPases has mostly been focused on members of the Rac, Cdc42 and Rho subfamilies [3]. The Rho GTPase family in plants is unusual; no clear homologs to the Rac, Rho or Cdc42 subfamilies have been reported. Instead, plants contain a unique subfamily of Rho GTPases, called ROPs (Rho-related GTPase from plants) [4-7]. ROPs are sometimes referred to as RACs, because the primary amino acid sequences are most homologous to animal RACs. Being the only small GTPase family dedicated to signal transduction in plants, ROPs have been shown to be key regulators of a number of cellular processes. Some of the mechanisms by which ROPs receive or transmit signals are conserved between animals and plants, whereas others are plant specific. In this review, we examine the evolution of Rho GTPases in eukaryotic organisms and compare plant ROPs with Rho family GTPases from other organisms, looking at similarities and differences in regulation

of GTPase activity and downstream pathways.

Rho subfamilies

In mammals, the Rho GTPase family consists of 22 members which can be divided into eight subgroups: Cdc42, Rac, Rho, Rnd, RhoD, RhoH, RhoBTB and Miro [8]. In plants, however, only two subgroups appear to exist: Miro and ROP/RAC. With a size of about 70 kDa, the Miro (mitochondrial rho) GTPases are not really small GTPases; these proteins contain two domains with similarity to small GTPases, as well as two Ca^{2+}-binding EF hands [9]. The N-terminal GTPase domain shows significant similarity towards Rho GTPases. In yeast, the Miro homolog Gem1p is localized to the outer mitochondrial membrane [10]. Yeast cells lacking Gem1p contain globular, collapsed or grape-like mitochondria, indicating a role for Gem1p in regulating mitochondrial morphology. The *Arabidopsis* genome contains three genes encoding putative Miro homologs (At3g05310, At3g63150 and At5g27540). No results have yet been published on plant Miro homologs.

In contrast, the other subgroup of plant Rho-like GTPases, ROP/RAC, has been subject to intense research since their discovery more than ten years ago [11-13]. The ROP family in *Arabidopsis* comprises 11 members [7]. Plant ROP GTPases carry a number of conserved amino acid substitutions compared with animal Rho GTPases that identify them as a unique subgroup. These substitutions are found in the GTP binding motifs, as well as in several of the exposed loops. The difference is particularly evident in the insert region, an exposed loop corresponding to residues 124-135 in human Rac1, where ROPs contain a two- to four-amino acid deletion compared to animal Rho GTPases. Figure 1 summarizes mechanisms for regulation of ROP activity and downstream effectors.

Proteins regulating Rho GTPase activity

The intrinsic GTPase activity of Rho GTPases hydrolyzes GTP to GDP, leading to conformational changes that abolish interactions with downstream effectors. The switch back to the GTP-bound state occurs when GDP is released and replaced by GTP.

The cycling of Rho GTPases between GTP- and GDP-bound states is regulated by three protein classes: GTPase-activating proteins (GAPs), GDP dissociation inhibitors (GDIs) and GDP exchange factors (GEFs).

RhoGAP

GAPs increase the intrinsic GTPase activity by stabilizing a transition state in which a hydrolytic water molecule is positioned proximal to the terminal phosphate group of GTP [14]. Essential for the GAP activity is a conserved arginine residue (the "arginine finger") that is inserted

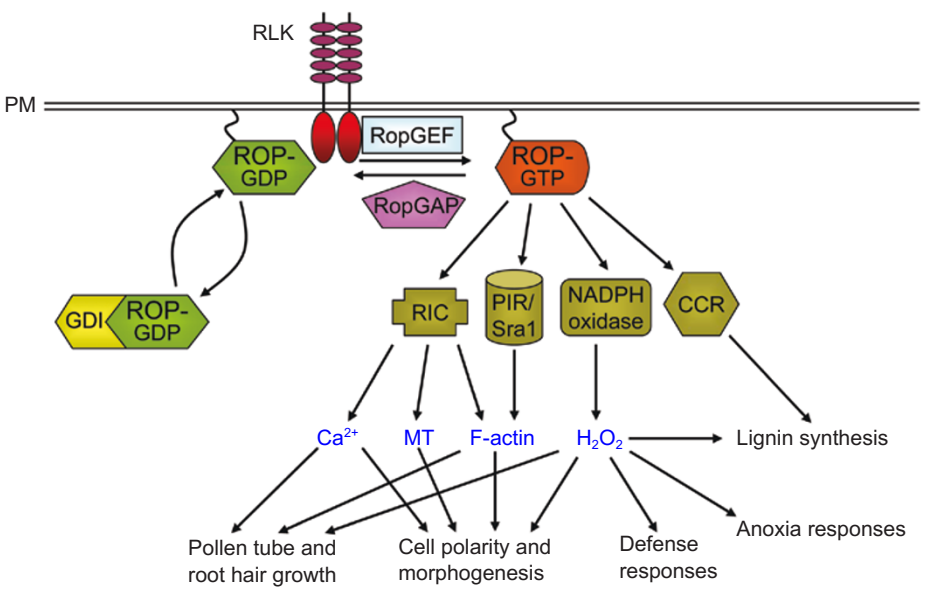

Figure 1 Overview of ROP signaling pathways. Extracellular signals activate a receptor-like kinase at the cell surface. RLK-mediated activation of RopGEF induces the exchange of ROP-bound GDP with GTP. GTP-bound, active ROP interact with a number of effectors to regulate a wide variety of cellular processes. ROP signaling is attenuated by RhoGAPs, whereas RhoGDIs may be important for both spatial and temporal regulation of ROP activity.

into the GTPase active site. The best characterized among RhoGAP-like protein families in plants is the RopGAP family, consisting of six members in *Arabidopsis* [15,16]. RopGAPs have an unusual domain organization, with a Cdc42/Rac-interactive binding (CRIB) motif adjacent to the RhoGAP domain. In animals, the CRIB motif has been found exclusively in Rac and Cdc42 effectors. *In vitro* assays have shown that RopGAP1 enhances the GTPase activity of ROP1; the CRIB motif is necessary both for this activity and for effective binding to ROP [16]. The CRIB motif of the RopGAPs may stabilize the transition state, thereby facilitating the GAP activity. Another possibility is that the CRIB motif could mediate crosstalk between different ROPs; binding of one ROP to the RopGAP CRIB could activate the RopGAP, leading to inactivation of another ROP.

One RopGAP has been shown to take part in a ROP rheostat regulating the response to oxygen deprivation in *Arabidopsis* [17]. Under anoxic conditions, an unidentified ROP induces production of hydrogen peroxide [H_2O_2] through the activation of NAPDH oxidase (discussed below). H_2O_2 triggers the expression of alcohol dehydrogenase (ADH), an enzyme involved in ethanolic fermentation. A negative feedback loop to ROP is provided by the H_2O_2-induced expression of RopGAP4, which terminate ROP signaling, thereby preventing damaging production of reactive oxygen species (ROS).

In addition to the RopGAP family, the *Arabidopsis* genome encodes two uncharacterized protein families containing a conserved RhoGAP domain. A small family of putative RhoGAPs, consisting of three members in *Arabidopsis*, contains an N-terminal Pleckstrin homology [PH] domain, followed by a RhoGAP domain (Vernoud V, Hwang J-U, Yang Z, unpublished data). PH domains are 100-120 amino acid protein modules with the ability to bind phosphoinositides, and are found in a diverse set of proteins, amongst them RhoGEFs and RhoGAPs [18]. The domain organization indicates that lipid signaling may be important for regulation of the activity and/or localization of these RhoGAPs. The GAP activity has still not been shown biochemically for members of this family. A third putative RhoGAP family, represented by a single gene (At5g61530) in *Arabidopsis*, does not contain any known protein motifs besides the GAP domain.

RhoGDI

RhoGDIs regulate Rho GTPases a) by inhibiting the dissociation of bound nucleotide from their binding GTPase partners; b) by inhibiting GTP hydrolysis and interaction with GAPs, GEFs and effectors; and c) by extracting Rho GTPases from the plasma membrane, thereby maintaining a cytoplasmic pool of GTPases [19, 20]. The *Arabidopsis* genome encodes three RhoGDI homologs with high similarity to mammalian RhoGDI, except for the N-terminal 50 amino acids. Interaction between ROP and RhoGDI has been shown in *Arabidopsis* and tobacco [21, 22]. The residues Asp45 and Asp185 of human RhoGDI-1, which has been shown to be necessary for inhibition of GDP dissociation from Cdc42 [23] are conserved in all three *Arabidopsis* RhoGDIs. Recently, the *Arabidopsis* RhoGDI1 has been found to be involved in root hair development, probably through regulation of ROP2 localization [24]. The root hair cells of *supercentipede1* (*scn1*) plants, in which RhoGDI1 function is disrupted, develop multiple growth sites along the outer face. Wild type root hair cells produce only one growth site per cell. Studies of ROP2 localization showed that whereas ROP2 becomes restricted to the root hair growth site in wild type plants, it is ectopically localized at the cell surface of *scn1* root hair cells.

RhoGEF

RhoGEFs facilitate the exchange of GDP with GTP, thereby switching the GTPase to its active state. Two classes of RhoGEFs have been identified in animals. Members of the Dbl family of RhoGEFs all contain a Dbl homology [DH] domain followed by a PH domain [25]. Surprisingly, no DH domains have yet been reported in plants. The other RhoGEF family is characterized by two conserved domains called Dock homology region (DHR) 1 and 2 [25]. The first member of this family that was characterized as a RhoGEF was Dock180, also termed Dock1 [26]. *Arabidopsis* contains a single gene encoding a Dock-like protein called SPIKE1 (SPK1) [27]. *spk1* mutant plants show defects in actin filament and microtubule organization and are seedling-lethal. However, GEF activity towards ROP has still not been shown for SPIKE1. Homologs of the Dock-associated protein ELMO are also present in plants.

Recently, a novel, plant-specific family of RhoGEFs termed RopGEF has been identified through yeast two-hybrid screens using dominant negative Rop mutants as bait [28, 29]. This family consists of 14 members in *Arabidopsis* that share a large central domain (315 amino acids), named PRONE (plant-specific Rop nucleotide exchanger) or DUF315, necessary for RopGEF activity. The plant specificity of the RopGEFs is supported by the inability of RopGEF1 to increase nucleotide exchange in human Rac1. In contrast, the DH-PH region of the human RhoGEF Tiam1 showed GEF activity towards *Arabidopsis* ROP4, although at a lower level compared with RopGEF1 [28]. Overexpression of RopGEF1 in pollen tubes resulted in depolarization of pollen tube growth, similar to the phenotype observed when a constitutively active (CA) ROP1 mutant is expressed [29]. Expression of a dominant

negative (DN) ROP1 mutant suppressed the RopGEF1 overexpression phenotype, further supporting a role for ROPGEF1 as an upstream activator of ROP1.

Signaling to RopGEFs – a role for receptor-like kinases

How are extracellular signals passed through the plasma membrane to the Rho GTPases? In animal cells, transmembrane receptor tyrosine kinases (RTKs) constitute a large and diverse family of cell surface receptors that play important roles in fundamental cell processes [30]. A number of RhoGEFs (and, to a lesser extent, RhoGAPs) are activated by RTKs, leading to activation or inactivation of downstream Rho GTPases [31, 32]. However, receptor tyrosine kinases appear to be absent in plants. Instead, a large family of receptor-like kinases (RLKs) with diverse extracellular domains and a cytoplasmic serine-threonine kinase domain has been found in plants; members of this family are involved in a plethora of signaling processes, such as hormonal responses, pathogen recognition, meristem development and self-incompatibility [33, 34].

Interestingly, a RopGEF was isolated in a yeast two-hybrid screen with the pollen-specific tomato RLKs LePRK1 and LePRK2 [35]. The RopGEF, termed KPP (kinase partner protein), interacted with the cytoplasmic domain of LePRK1 and LePRK2 and was phosphorylated *in vivo*, suggesting that KPP may be a substrate of LePRK1/LePRK2. Overexpression of KPP in pollen led to depolarized growth of the pollen tube, resulting in balloon-shaped pollen tube tips.

ROP has also been implicated in signaling downstream of the RLK CLAVATA1 (CLV1). CLV1 is part of a protein complex regulating the balance between cell differentiation and cell division in aerial meristems; *clv1* mutant plants show ectopic accumulation of stem cells at shoot and flower meristems [36]. An unidentified ROP GTPase was immunologically detected in the 450 kDa active CLV1 complex, together with the protein phosphatase KAPP . Still, one or more components of the complex were not identified. By analogy to the PRK-KPP interaction, it is not unlikely that a RopGEF might link ROP to the CLV1 complex.

Plant ROP vs. animal Rho effectors and downstream processes

The actin cytoskeleton

In both animals and plants, Rho GTPases play pivotal roles in regulation of the actin cytoskeleton [38-40]. In mammalian cells, cell shape, polarity and movement are governed by the coordinated activity of different Rho GTPases. Expression of constitutively active and dominant negative mutants of Cdc42, Rac and Rho induce different structures when expressed in tissue culture [41-43]. Activation of Cdc42 leads to the generation of actin-rich finger-like protrusions called filopodia. Active Rac induce actin-rich surface protrusions called lamellipodia. Rho activation leads to the assembly of actin:myosin filaments known as stress fibers. During cell migration, Rac and Cdc42 localize to the leading edge of the cells. Rac-induced actin polymerization is believed to be important for membrane protrusion at the leading edge, whereas Cdc42 regulates polarity and direction of cell movement. Rho, acting at the rear of the cell, generates contractile actin:myosin filaments that regulate cell retraction at the tail end of the migrating cell.

Plant cells, being surrounded by rigid cell walls, do not move or grow through the protrusion of a leading cell edge. Instead, plant cell expansion is driven by turgor pressure and controlled by cell wall properties and the cytoskeleton [44,45]. A growing body of evidence also points to ROP as a key player in the generation of cell polarity in plant cell growth and morphogenesis. ROPs have been shown to regulate polar growth and cell morphogenesis in several cell systems including pollen tubes, developing root hairs, and leaf pavement cells [46-54].

Arp2/3 is a protein complex consisting of seven subunits; the Arp2 and Arp3 subunits share similarity with conventional actins. The Arp2/3 complex associates with the sides of existing actin filaments and initiates a new filament branching out at an angle of 70 ° relative to the orientation of the parent filament. Rac and Cdc42 regulate Arp2/3 activity through the related WAVE and WASP families, respectively. WASP and WAVE proteins all contain a C-terminal domain, VCA, which initiates actin polymerization by binding actin monomers and the Arp2/3 complex, thereby bringing them together [55, 56]. The diverging N-terminal regulatory domains of WASP and WAVE domains account for their different modes of regulation. WASP homologs have not been identified in plants, and will not be discussed further. In mammals, Rac and the adapter protein Nck regulate the three WAVE family members through a pentameric protein complex called the WAVE or SCAR complex [57]. In addition to WAVE, the complex includes PIR121/Sra-1, Nap1/Hem1, Abi1/Abi2 and HSPC300.

Homologs of all subunits of the Arp2/3 and WAVE complex have been identified in *Arabidopsis* [58, 59]. A plant Arp2/3 complex has yet to be shown biochemically to exhibit the same properties as its counterparts in other eukaryotic organisms. However, *Arabidopsis* homologs of the Arp3 and ArpC2 subunits were able to complement mutants for the corresponding genes in yeast [60, 61]. Mutants of *Arabidopsis* ARP2, ARP3, APRC2a, ARPC5, NAP1 and PIR121/SRA1, as well as BRK1 RNAi plants, all show highly similar phenotypes, characterized by cell

expansion defects in different epidermal cell types [46, 60-68]. Furthermore, T-DNA knockout mutants for one of four *Arabidopsis* WAVE/SCAR homologs show similar, but milder phenotypes [69, 70]. The most striking phenotype is seen in trichomes, which are large, unicellular structures protruding from the leaf epidermis. Mutant trichomes display randomized cell expansion in these mutants, resulting in phenotypes that are mimicked by treatment with actin-depolymerizing drugs [71, 72]. Yeast two-hybrid experiments show that ROP2 interacts with PIR121/SRA1, suggesting that WAVE activity in plants may be regulated by ROP [46]. The fact that this interaction is conserved between animals and plants supports the hypothesis that ROP GTPases originated from a Rac-like ancestor. Overexpression of *CA-rop2* results in a trichome phenotype resembling the ARP2/3 and WAVE complex mutants [48], but the functional relationship between ROP and the WAVE-ARP2/3 pathway remains to be established.

The Arp2/3 and WAVE complex mutant phenotypes are surprisingly subtle compared with corresponding mutants in other organisms. Disruption of Arp2 and ARPC1 in budding yeast is lethal [73, 74]. Mutation or RNAi suppression of Arp2/3 or WAVE complex subunits in *Drosophila* and *C. elegans* all result in embryolethality [75, 76]. Actin structures produced by the ARP2/3 complex apparently plays a non-essential role in plants, only being important for proper morphogenesis of cells with complex growth patterns. Instead, formins may act as the major actin-nucleating protein class in plants and might be regulated by ROPs. It is also possible that plants have evolved unique ROP-mediated mechanisms for actin nucleation.

Formins are actin-nucleating proteins with the ability to stimulate de novo polymerization of actin filaments [77, 78]. They are conserved throughout eukaryota, and are recognized by the presence of a highly conserved formin homology 2 (FH2) domain, which is essential for actin filament nucleation. Most formins contain a proline-rich, profilin-binding FH1 domain adjacent to the FH2 domain. A subclass of formins, the Diaphanous-related formins (Drfs), contains an N-terminal Rho GTPase-binding domain. Binding of Rho GTPases releases autoinhibitory interactions, thereby activating Drf.

The formin family appears to be expanded in plants compared to animals; the *Arabidopsis* genome encodes 21 putative formin homologs [79], whereas only 9 genes encoding formins have been identified in mammalian genomes so far [80]. Most of the plant formins contain a FH1 domain in addition to the FH2 domain. However, no plant formins appear to have a GTPase-binding domain, suggesting that ROP GTPases are not directly involved in regulation of formin activity. Still, one cannot exclude the possibility that ROP participates in formin regulation through a plant-specific mechanism not yet discovered.

Apart from the conserved actin nucleation mechanisms, ROPs might regulate a novel mechanism for the assembly of the actin cytoskeleton [47, 50]. Evidence indicates that ROP1 activates the assembly of a dynamic form of tip-localized actin microfilaments required for pollen tube growth [49]. However, knocking out the Arp2/3 complex has no effects on pollen tube growth, suggesting an Arp2/3 complex independent mechanism is involved in the assembly of the tip actin (Li and Yang, unpublished data). Similarly, ROP2-dependent cortical diffuse F-actin remains in the pavement cells of *Arabidopsis* leaves defective in the Arp2/3 complex [66]. As discussed below, in both pollen tubes and pavement cells, the assembly of cortical F-actin is activated by a plant-specific ROP effector protein, RIC4.

RICs (ROP-interactive CRIB-containing proteins)

The CRIB motif (also known as GBD, or GTPase-binding domain) is used for GTP-dependent interaction with Rac and Cdc42 subfamily members by a number of Rho effector proteins in animal cells [81]. The Pak (p21-activated kinase) kinases are a family of CRIB motif-containing serine-threonine kinases that phosphorylate a wide variety of substrate proteins upon binding of activated Rac or Cdc42 [82, 83]. Pak kinases participate in regulation of gene expression through MAP kinase pathways, and organization of both actin and microtubule cytoskeleton. Most eukaryotes encode one or more Pak homologs, but none are found in plants. In fact, only two protein families have retained a CRIB motif in plants, none of which are conserved in animals. One is the RopGAP family discussed above. The other is a family of small, plant specific proteins called RICs (ROP-interactive CRIB-containing proteins) [84]. In *Arabidopsis*, the RIC family comprises 11 members, which based on sequence similarity can be sorted into five subfamilies based on sequence similarity [84]. RIC's function has been studied in two model systems: pollen tubes [50] and leaf pavement cells [47]. ROP1 regulates pollen tube growth through coordination of two counteracting pathways: activation of RIC4 promotes assembly of apical F-actin in the pollen tube tip, whereas activation of RIC3 leads to disassembly of the apical F-actin through a Ca^{2+}-dependent process [50]. In turn, these two pathways appear to provide positive (RIC4) or negative (RIC3) feedback signaling to ROP1, leading to an oscillation of ROP1 activity at the pollen tube tip that is correlated with tip growth oscillation [85]. The generation of the jigsaw puzzle-like shape of leaf pavement cells appears to be at least partly regulated by ROP-RIC signaling pathways [47]. RIC4 acts downstream of ROP2 and ROP4 to promote the assembly of cortical actin filaments in developing lobes, thus facilitating localized growth of these lobes. In contrast, RIC1 is

inactivated by ROP2/4 binding. RIC1 activity seems to be restricted to the neck regions, where it promotes formation of well-ordered cortical microtubules that promotes narrow neck morphology. Furthermore, RIC1-mediated microtubule arrays suppress the ROP-RIC4-actin pathway. Thus, the concerted action of these two counteracting pathways contributes to the generation of interdigitating lobes and indentations of pavement cells.

Regulation of NADPH oxidase

Production of ROS is an important part of host defense in animals. During phagocytosis of invading microbes, phagocytes produce superoxide anion [O_2^-], which is subsequently converted to other ROS; these compounds contribute to the killing of the phagocytosed microbe [86]. The enzymatic activity for production of O_2^- is provided by the protein complex NADPH oxidase. The catalytic component is the membrane protein gp91^{phox}, also termed Nox [87]. Other subunits include another membrane protein, p22^{phox}, and the cytosolic components p40^{phox}, p47^{phox} and p67^{phox}. Finally, active Rac2 is necessary for NADPH oxidase activity in phagocytes, interacting with both p67^{phox} [88] and gp91^{phox} [89]. The NADPH oxidase is expressed in many cell types, and superoxide production has been implicated in a number of cellular processes besides host defense.

Homologs of gp91^{phox} have been identified in several plant species [90-92]. The *Arabidopsis* genome encodes ten putative gp91^{phox} homologs, termed Respiratory burst oxidase homolog (Rboh) [93]. With the exception of ROP GTPases, none of the other components of the NADPH complex appears to be conserved in plants. Thus, regulation of NADPH oxidase activity is different in plants and animals. Plant Rbohs all contain a cytosolic N-terminal region with two Ca^{2+}-binding EF hands [91]; this region is absent in the phagocytic gp91^{phox}, but is found in other members of the mammalian Nox family [94]. *In vitro* assays indicate that plant Rbohs are able to produce superoxide in the absence of cytosolic factors and are stimulated directly

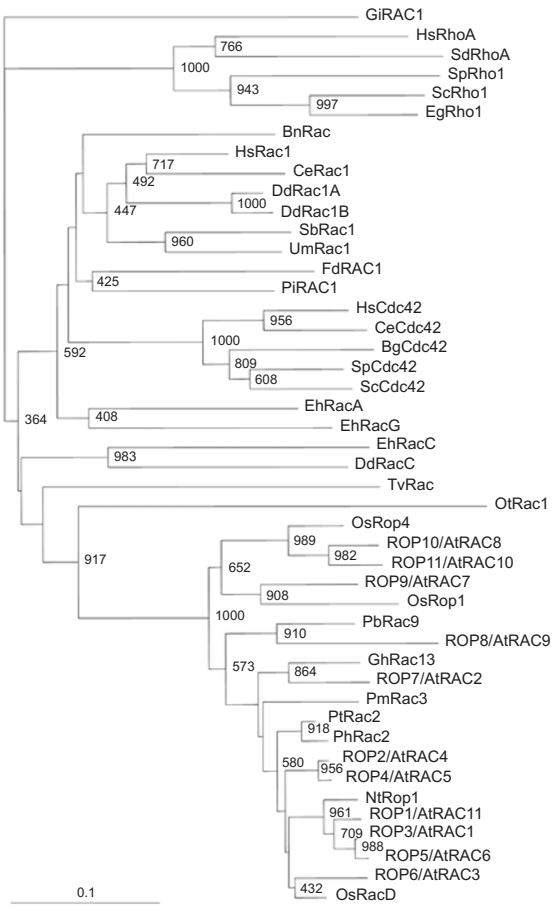

Figure 2 A phylogram of Rho GTPases in different eukaryotic organisms. The protein alignment of the Rho-family proteins was produced with the GeneDoc program version 2.4.017 and the multiple sequence file was imported into the Clustal X program. The hypervariable N- and C-terminal ends were excluded. Protein weight matrixes of the PAM series were used to calculate the distances and an unrooted neighbor-joining (N-J) tree was created using the neighbor-joining algorithm (Saitou 105). Bootstrapping of the N-J tree was done with 1000 bootstrap trials. The tree was rooted with the Rac gene from the amitochondriate protist *Giardia lamblia* and the tree was visualized using the Treeview program [106].

The figure shows the relationship between some of the characteristic protein families within the larger Rho-family. The Rac protein from the prasinophyte *Ostreococcus tauri* is an out-group for the plant RAC/ROP proteins and reinforce the close phylogenetic relationship between prasinophytes and the streptophyta. The Cdc42 and RhoA/Rho1 proteins are exclusively found within the metazoa/fungi clade and have high bootstrap support. The Rac proteins show a broader phylogenetic distribution and do also include members from the protists; stramenopiles, mycetozoa and cercozoa, suggesting that this group has the deepest root among the extended Rho-family proteins.

Abbreviations for prefixes: At: *Arabidopsis thaliana*, Bg: *Blumeria graminis*, Bn: *Bigelowiella natans* (acc: DR041190), Ce: *Caenorhabditis elegans*, Dd: *Dictyostelium discoideum*, Eg: *Eremothecium gossypii*, Eh: *Entamoeba histolytica*, Fd: *Fucus distichus*, Gi: *Giardia lamblia*, Hs: *Homo sapiens*, Nt: *Nicotiana tabacum*, Os: *Oryza sativa*, Ot: *Ostreococcus tauri* (acc: CR954206.2), Pb: *Populus balsamifera* (acc: CN550455), Ph: *Physcomitrella patens*, Pi: *Phytophthora infestans* (acc: CV947213), Pm: *Picea mariana*, Pt: *Pinus taeda*, Sb: *Suillus bovines*, Sc: *Saccharomyces cerevisiae*, Sd: *Suberites domuncula*, Sp: *Schizosaccharomyces pombe*, Tv: *Trichomonas vaginalis* (acc: AAP79439), Um: *Ustilago maydis*.

Table 1 Distribution of Rho GTPases and Rho regulatory protein domains in eukaryotic lineages

	Rho	RhoGAP	RhoGDI	RhoGEF DH	RhoGEF Dock/CZH	RhoGEF PRONE
Plants (*Arabidopsis thaliana*)	X	X	X		X	X
Chlorophyta (*Ostreococcus tauri*)	X			X?	X	
Fungi (*Aspergillus fumigatus*)	X	X	X	X	X	
Metazoa (*Homo sapiens*)	X	X	X	X	X	
Stramenopiles (*Phytophthora infestans*)	X	X	X	X	X	
Parabasalidea (*Trichomonas vaginalis*)	X	X	X			
Alveolata (*Paramecium tetraurelia*)	X	X	X			
Cercozoa (*Bigelowiella natans*)	X		X	X		
Diplomonadida (*Giardia lamblia*)	X	X	X			
Entamoebidae (*Entamoeba histolytica*)	X	X	X	X	X	
Mycetozoa (*Dictyostelium discoideum*)	X	X	X	X	X	
Euglenozoa (*Trypanosoma cruzi*)	X	X				
Haptophyceae (*Emiliania huxleyi*)		X				
Rhodophyta (*Porphyra haitanensis*)		X				

by Ca^{2+} [95]. However, ROP also appears to be involved in regulation of Rboh activity [17, 96]. In rice, a pathway leading from heterotrimeric G protein via OsRac1 to NADPH oxidase confers disease resistance towards pathogens such as rice blast fungus [96-98]. Although the precise mechanism of this pathway has not yet been resolved, Kawasaki et al. [99] mention as unpublished results that ROP regulates Rboh activity through direct interaction. It will be interesting to see whether this interaction is similar to the interaction between Rac and $gp91^{phox}$ in mammalian cells.

Novel ROP interactions in plants

Besides RIC, only a few ROP-effector interaction pairs are not conserved in other phyla. Recently, rice cinnamoyl-CoA reductase1 (OsCCR1), an enzyme involved in lignin biosynthesis, was shown to interact with OsRac1 in a GTP-dependent manner [99]. Interaction with OsRac1 induced the enzymatic activity of OsCCR1 *in vitro*. H_2O_2, a secondary product of NADPH oxidase, is required for polymerization of monolignols. OsRac1 may therefore control lignin synthesis through regulation of both OsCCR1 and NADPH oxidase.

Arabidopsis UDP-glucose transferase 1 (UGT1) is part of a protein complex including callose synthase [100]. UGT1 interacts with GTP-bound, but not GDP-bound ROP1, suggesting that UGT1 may be a ROP effector. Interestingly, yeast Rho1p regulates callose synthase activity through direct interaction with the catalytic subunit [101].

An evolutionary view of Rho GTPases and their functional partners

The Rac/Rho protein is of ancient origin and can be found in most eukaryotic lineages (Table 1). It evolved at a time when eukaryotic organisms were unicellular, and their later diversification into distinct gene families, notably the Rho and Cdc42 families, happened after the establishment of major eukaryotic divisions. Thus, the Rac/Rho proteins have developed independently within the individual eukaryotic lineages for hundreds of millions of years. Many eukaryotic species have evolved large Rac/Rho gene families; humans for instance have 22 Rac/Rho genes [8], and *Dictyostelium discoideum* and *Entamoeba histolytica* have 18 and 26, respectively [102]. The amoebozoa diverged from the animal-fungal lineage after the plant kingdom had developed, and the phylogenetic analyses of the Rac-like genes in these organisms (Figure 2) clearly show this relationship. Phylogenetic analyses of the amoebae also suggest a similar close relationship between *Dictyostelium* and *Entamoeba* [103]. The high diversity and distinct gene families within the Rac/Rho genes in *Dictyostelium discoideum* and *Entamoeba histolytica* suggest that these genes evolved at an early stage during evolution. Only one of the Rac/Rho subfamilies within these organisms, the RacC homologues, suggests a monophyletic origin; the others are unique for each organism. The appearance of Cdc42 and Rho proteins in fungi and metazoa happened at a later stage in evolution, long after the animal-fungal lineage split from the viridiplantae. Due to the early split of viridiplantae from the animal-fungal-amoebozoa lineage, Rho-family genes in plants have developed novel features

and modes of regulation. This may also explain why plants lack small GTPases and GTPase regulatory proteins found in animals and fungi. Rho-like genes do not exist in the green algae *Chlamydomonas* (Winge, unpublished data). However, in the prasinophyte *Ostreococcus tauri*, which is thought to be more closely related to plants (streptophyta), a gene encoding a GTPase related to plant RAC/ROP has recently been sequenced (Figure 2). In plants, the ROP/RAC proteins have just recently begun to split up into distinct gene families. The first terrestrial plants probably just had one single *ROP/RAC* gene. One of the first major divisions happened after the appearance of the Spermatophyta. This division was caused by a mutation in a *ROP/RAC* gene resulting in an extra C-terminal exon which destroyed the native prenylation site (ROP/RAC group II) [7,104]. Later gene duplications have resulted in several distinct sub-groups of *ROP/RAC* genes in monocotyledons and dicotyledons.

The effectors of Rho/Rac proteins, RhoGAP, RhoGDI and RhoGEF (Dbl homology type), have co-evolved with the Rac/Rho proteins over hundreds of millions of years. The DH domain RhoGEF is found in several eukaryotic lineages (Metazoa, Fungi, Mycetozoa, Entamoebidae, Cercozoa and Stramenopiles), but is missing in Streptophyta and has apparently also been lost (or did not develop) in Euglenozoa, Parabasalidea, Diplomonadida, Rhodophyta and Alveolata (Table 1). In contrast, a distinct family of RhoGEFs, RopGEFs, has co-evolved with plant ROPs/RACs [28, 29]. When comparing the evolution of the Rac/Rho regulatory proteins, there are also other distinct differences. While the RhoGDIs always exist as a single domain protein, the RhoGAPs and RhoGEFs are often integral parts of multi-domain proteins. More than 20 different domains and motifs have been found associated with RhoGAP proteins, and close to 30 domains and motifs together with RhoGEFs [14, 25]. Some of these motifs, such as PH, ArfGAP, RasGEF, DEP, BAR and myosin head, are present in both RhoGAP and RhoGEF proteins. The many and diverse RhoGAP and RhoGEF proteins in *Dictyostelium* and *Entamoeba* imply that their Rac genes evolved and diversified much earlier than in plants. Similarly, the complexity of RhoGAP and RhoGEF proteins in metazoa and fungi reflects the early diversification of Rac/Rho genes in these organisms and shows that the Rac/Rho/Cdc42 proteins began to acquire specific functions before the division between metazoa and fungi. The diversification of *ROP/RAC* genes in Streptophyta has appeared late in evolution and may explain why ROP/RAC GAPs still have a relative simple domain organization compared to the multi-domain RhoGAPs found in metazoa, fungi and amoebas. As a comparison, plants have just two described domains associated with their GAP proteins, CRIB [84] and PH (Vernoud V, Hwang J-U, Yang Z, unpublished data). However, plants most likely have evolved novel RAC/ROP regulators and pathways yet to be discovered.

Summary and perspectives

Although phylogenetic analyses suggest that ROP/RAC proteins form a distinct Rho subfamily, several of the components involved in regulation of ROP activity are conserved between plants and other eukaryotes (RhoGAP, RhoGDI, Dock-type RhoGEF). However, a putative plant specific pathway for signaling from the plasma membrane to ROP, including a receptor-like kinase and a family of RhoGEFs unique to plants, has been identified. At least one mammalian Rac effector (PIR121/Sra1, a subunit of the WAVE complex) seems to be conserved in plants and to interact with ROP. Rho GTPases in animals and plants play similar roles in certain cellular processes, such as cell polarity and morphogenesis and production of ROS, although the precise mechanisms are not always conserved. Plant ROPs also regulate plant specific processes, such as lignin synthesis.

Still, many questions remain unanswered. What kind of extracellular signals activate RopGEFs through PRK-type RLKs? Are there other RLK subfamilies with activity towards RopGEFs? And how is the Dock-type RhoGEF SPK1 regulated? Although a functional role has been assigned to a few of the ROP regulatory proteins, knowledge on most members of these families is still very limited. Among the ROP effectors, the RICs are an enigmatic group of proteins. RICs have been shown to regulate F-actin, microtubules and Ca^{2+} levels; however, very little is known about the mechanisms by which RICs perform these functions and the crosstalk between the different pathways. Also, there may still be unidentified ROP effectors with exciting roles in processes such as meristem regulation, hormone responses and plant defense and cell morphogenesis.

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