Physcomitrella patens: a model to investigate the role of RAC/ROP GTPase signalling in tip growth

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Abstract

Polarized cell expansion plays an important role in plant morphogenesis. Tip growth is a dramatic form of this process, which is widely used as a model to study its regulation by RAC/ROP GTPase signalling. During the dominant haploid phase of its life cycle, the moss Physcomitrella patens contains different types of cells that expand by tip growth. Physcomitrella is a highly attractive experimental system because its genome has been sequenced, and transgene integration by homologous recombination occurs in this plant at frequencies allowing effective gene targeting. Furthermore, together with the vascular spikemoss Selaginella moellendorffii, whose genome has also been sequenced, the non-vascular moss Physcomitrella provides an evolutionary link between green algae and angiosperms. BLAST searches established that the Physcomitrella and Selaginella genomes encode not only putative RAC/ROP GTPases, but also homologues of all known regulators of polarized RAC/ROP signalling, as well as of key effectors acting in signalling cascades downstream of RAC/ROP activity. Nucleotide sequence relationships within seven different families of Physcomitrella, Selaginella, Arabidopsis thaliana and Nicotiana tabacum (tobacco) genes with distinct functions in RAC/ROP signalling were characterized based on extensive maximum likelihood and Neighbor–Joining analyses. The results of these analyses are interpreted in the light of current knowledge concerning expression patterns and molecular functions of RAC/ROP signalling proteins in angiosperms. A key aim of this study is to facilitate the use of Physcomitrella as a model to investigate the molecular control of tip growth in plants.

Key words: Physcomitrella, polarity, RAC/ROP signalling, tip growth.

Introduction

Small GTPases of the Rho family have key functions in the control of cellular polarization in all eukaryotes, and play important roles in the regulation of cell motility, division, and directional growth (Etienne-Manneville and Hall, 2002). Animal and fungal Rho GTPases have been grouped into three subfamilies called Rho, Rac, and Cdc24, whereas plants contain a single, clearly distinct Rho GTPase subfamily (Boureux et al., 2007). Members of the plant Rho GTPase family are most closely related to non-plant Rac GTPases, and are referred to as RAC (Winge et al., 1997) or ROP (Rho of plant; Li et al., 1998) GTPases. All Rho GTPases are thought to act in a similar manner as molecular switches in signalling pathways (Etienne-Manneville and Hall, 2002; Kost, 2008) (Fig. 1). Most members of this protein family are associated with the plasma membrane as a consequence of post-translational prenylation at the C-terminus. Rho GTPases interact with effector proteins to stimulate downstream signalling when they are in the GTP-bound state, whereas they are inactive in the GDP-bound conformation. Different groups of regulatory proteins directly interact with Rho GTPases to regulate their signalling function. GTase-activating proteins (RhoGAPs) stimulate the low intrinsic GTPase activity of Rho proteins, and...
thereby inactivate their signalling function. Guanine nucleotide exchange factors (RhoGEFs) activate Rho GTPase-dependent signalling by promoting exchange of GDP for GTP. A hydrophobic binding pocket, which can accommodate the prenyl tails of Rho proteins, enables guanine nucleotide dissociation inhibitors (RhoGDIs) to translocate target Rho GTPases from the plasma membrane to the cytoplasm, where the two proteins form inactive heterodimers (Etienne-Manneville and Hall, 2002; Kost, 2008).

Plant cells are enclosed within relatively rigid cell walls and are unable to change shape rapidly or to migrate within developing tissues. Directional cell expansion therefore plays a key role in morphogenesis and development particularly in plants (Kost et al., 1999b). Angiosperm root hairs and pollen tubes undergo a dramatic form of polarized cell expansion called tip growth. These highly elongated cells grow rapidly and exclusively at one end based on massive, tip-directed targeted secretion of cell wall material. Both cell types are widely used as model systems to investigate polarized cell growth (Hepler et al., 2001). Extensive cell biological and genetic evidence demonstrates that RAC/ROP GTPases accumulate at the plasma membrane at the apex of root hairs and pollen tubes, and play key roles in the control of tip growth (Kost et al., 1999a; Li et al., 1999; Cheung and Wu, 2008; Kost, 2008; Yalovsky et al., 2008; Yang, 2008). Excess RAC/ROP activity depolarizes tip growth and results in apical ballooning, whereas RAC/ROP inactivation inhibits this process. Regulators of RAC/ROP activity in tip-growing cells belonging to the RhoGAP, RhoGEF, and RhoGDI families have been functionally characterized and a substantial number of effectors acting downstream of this activity have been identified (Cheung and Wu, 2008; Kost, 2008; Yalovsky et al., 2008; Yang, 2008). Despite all these efforts, the molecular and cellular mechanisms underlying the control of tip growth by RAC/ROP signalling are still only understood to a limited extent. Further progress in this area of research is hindered by substantial limitations associated with the most commonly used experimental systems, which include: (i) lack of a protocol that reproducibly allows normal Arabidopsis thaliana pollen tube growth in vitro; (ii), absence of complete genome sequences of species for which pollen tube culture is well established [Nicotiana tabacum (tobacco), Lilium longiflorum (lily)]; (iii) extensive redundancy within angiosperm gene families coding for proteins involved in RAC/ROP signalling; and (iv) unavailability of methods enabling routine generation of genomic fluorescent protein fusions to investigate protein localization.

Physcomitrella patens combines features that make this moss a highly attractive alternative and complementary model for the investigation of tip growth in plants (Bezanilla and Perroud, 2009). After spore germination, Physcomitrella undergoes a phase of filamentous growth, during which it forms protonema (Fig. 2A). Protonemal tissue is composed of only two types of filaments: caulonema (develop from chloronema, contain relatively few chloroplasts and sometimes brown pigments, and cross-walls between neighbouring cells are oblique with respect to the growth axis; Fig. 2C) and chloronema (formed immediately upon spore germination, rich in chloroplasts, and cross-walls between neighbouring cells are perpendicular to the growth axis; Fig. 2D). Caulonema and chloronema form single cell files with an apical cell at the end, which has been

![Fig. 1. Protein families involved in Rho GTPase signalling. Rho GTPases interact with effector proteins and stimulate downstream signalling when bound to GTP, whereas they are inactive in the GDP-bound conformation. A C-terminal prenyl tail anchors most Rho GTPases in the plasma membrane. RhoGAPs increase the Rho GTPase activity, and inactivate the signalling function, of Rho proteins. RhoGEFs promote nucleotide exchange and stimulate Rho signalling. RhoGDIs transfer GDP-bound Rho GTPases from the plasma membrane to the cytoplasm, where the two proteins form inactive heterodimers.](image)

![Fig. 2. Tip-growing cells of Physcomitrella protonema and gametophores. (A) A 20-day-old wild-type colony displaying protruding branched protonemal filaments (arrowheads) and gametophores at various developmental stages. The diameter of the colony is ~20 mm. (B) An isolated gametophore with a stem, leaf-like structures, and basal rhizoids (arrowhead). (C) The tip of a caulonemal filament. (D) The tip of a chloronemal filament. (E) The tip of a rhizoid. Scale bars: 100 μm.](image)
demonstrated to elongate by tip growth (Menand et al., 2007). Subapical filament cells can form side-branch initials, which also elongate by tip growth to form new branches (Fig. 2A). Different environmental conditions can induce protonema to develop buds, which subsequently grow into gametophores (leafy shoots). From these structures, rhizoids emerge at the basal end (basal rhizoids) and below leaf–stem junctions (midstem rhizoids) (Fig. 2B). Rhizoids resemble caulonemal filaments and are also single cell files ending in an apical cell that elongates by tip growth (Fig. 2E). In contrast to protonema, rhizoids branch rarely. Both protonemal filaments and gametophore rhizoids can easily be grown in culture in large quantities and are highly amenable to microscopic imaging. Efficient methods have been developed to generate transiently and stably transformed Physcomitrella protonema, which can be indefinitely propagated in vitro, or maintained as frozen stocks (Schaef er and Zryd, 2001; Cove, 2005; Quatrano et al., 2007). Both protonema and gametophores are haploid gametophytic structures, which facilitates genetic manipulation. Sexual reproduction can be induced to cross different Physcomitrella genotypes (Schaef er and Zryd, 2001; Cove, 2005; Quatrano et al., 2007).

A complete draft sequence of the Physcomitrella genome supported by expressed sequence tag (EST) collections is available on the website of the ‘Joint Genome Institute’ (JGI, Walnut Creek, CA, USA; Rensing et al., 2008). Coordinated international efforts are underway to improve the quality of the draft genome assembly, as well as of gene model predictions and functional annotations (Moss 2009: The Annual International Conference for Experimental Moss Research, St Louis, USA). Physcomitrella is the only well developed plant model in which transgene integration by homologous recombination occurs at a frequency sufficient for effective gene targeting (Quatrano et al., 2007). Based on the available genome sequence and on high frequency homologous recombination, individual Physcomitrella genes can be effectively eliminated (knock-out) to investigate their functions. Furthermore, it is possible to insert DNA fragments (knock-in) coding for non-invasive visible marker proteins [e.g. green fluorescent protein, GFP] into target genes to generate genomic fusions, such that these target genes will express tagged proteins under the control of endogenous expression signals. These tagged proteins can be ideal reporters of intracellular localization and gene expression pattern, particularly if it is possible to demonstrate that they are fully functional as the only expressed member of the protein family to which they belong.

Using Physcomitrella as a model to investigate tip growth in plants is not only attractive because of the availability of the unique toolkit described above, but also because of its interesting phylogenetic position within the plant kingdom (Pryer et al., 2002). The non-vascular moss Physcomitrella belongs to the land plants together with all vascular plants, and provides an evolutionary link between the green algae and ancient vascular plants such as the spikemoss Selaginella moellendorffii (Fig. 3), whose genome has also been sequenced (http://genome.jgi-psf.org/Selmo1/Selmo1.home.html; Banks, 2009). A comparison between the molecular mechanisms underlying the control of tip growth in Physcomitrella and in vascular plants can improve our understanding of how these mechanisms have evolved.

In this study, Physcomitrella and Selaginella genes coding for homologues of proteins involved in RAC/ROP signalling have been identified. The sequences of these genes were compared with those of families of related angiosperm genes. Genes of the vascular spikemoss Selaginella were included in the analysis because of their potential to provide a link between gene families of the non-vascular moss Physcomitrella and of higher vascular plants. The original, previously unpublished data generated by these efforts are discussed in the context of an extensive review of current knowledge concerning expression patterns and molecular functions of angiosperm Rac/Rop signalling proteins. This discussion reveals that Physcomitrella and Selaginella contain genes encoding members of all major classes of proteins with important roles in RAC/ROP signalling in angiosperms. Furthermore, it provides insights that will facilitate the functional characterization of Physcomitrella RAC/ROP signalling proteins with the aim of enhancing our understanding of the molecular control of tip growth.

**Sequence analysis procedures and methods**

Full-length Arabidopsis and tobacco coding domain sequences (CDS) of genes involved in RAC/ROP signalling were obtained from ‘The Arabidopsis Information Resource’ (TAIR; http://www.Arabidopsis.org/index.jsp) or ‘GenBank’ (http://www.ncbi.nlm.nih.gov/Genbank/) in September or October 2009 (Supplementary Table S1 available at JXB online). In the case of AtRic8 (At1g03982), the CDS sequence as reported by Wu et al. (2001) was used, which deviates from the one deposited in TAIR. To discover Physcomitrella and Selaginella homologues, amino acid sequences of Arabidopsis and tobacco RAC/ROP signalling proteins translated from the collected CDS (full length or conserved domains) were used as queries in extensive tBLASTn searches (Altschul et al., 1990) against the ‘allmaskedPhyscomitrella_patens.1_1’ (/http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html) and ‘Selmo1_assembly_scaffolds’ (/http://genome.jgi-psf.org/Selmo1/Selmo1.home.html) databases at the DOE Joint Genome Institute (JGI, Walnut Creek, CA, USA). In some cases, obviously incomplete or low quality Physcomitrella or Selaginella gene models identified in these BLAST searches were manually corrected based on available EST data, or on sequence similarity with well characterized homologous genes (Supplementary Table S1). The majority of the Physcomitrella and Selaginella RopGEF gene models extracted from the JGI databases were incomplete at one or both ends. Because information required for the manual correction of these gene models was not available, four Physcomitrella and all Selaginella RopGEF sequences used in this study are truncated upstream of the conserved PRONE domain (Fig. 7B). A complete predicted 3’ end is available for all Physcomitrella RopGEF genes, but only for one of the homologous...
Selaginella sequences (Fig. 7B). As the Selaginella genome database contains a mixture of sequence data from two haplotypes (http://genome.jgi-psf.org/Selmo1/Selmo1.info .html), only one of two nearly identical copies of each Selaginella gene identified in BLAST searches was included in this analysis.

Coding sequences of Physcomitrella and Selaginella RAC/ROP signalling genes identified and edited as described above, together with homologous Arabidopsis and tobacco sequences (Supplementary Table S1), were converted to amino acid sequences and aligned using default settings for the MUSCLE algorithm (Edgar, 2004) in Seaview v.4.2 (Galtier et al., 1996; Supplementary alignment 1 at JXB online). Resulting alignments were trimmed to remove long stretches of non-conserved sequence and subsequently converted to corresponding nucleotide sequence alignments (Supplementary alignment 2 at JXB online).

Based on these nucleotide sequence alignments, phylogenetic trees were generated using the maximum likelihood (ML) method implemented in ‘GARLI’ (Zwickl, 2006; http://garli.nescent.org). A heuristic search was repeated a total of 100 times to reduce the probability of inferring a suboptimal likelihood solution. The optimal evolutionary model was estimated as the ‘GTR+I’ model of evolution using ‘FindModel’ (Posada and Crandall, 1998; http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). ML bootstrap support (100 iterations) was generated using the ‘RAxML Black Box’ web server (Stamatakis et al., 2008; http://phylobench.vital-it.ch/raxml-bb/index.php) that implements a novel rapid bootstrapping algorithm.

The highest scoring heuristic tree resulting from the analysis of each gene family is presented (Figs. 5A–11A) and discussed below. In some gene families (e.g. RopGAP and RIC), conserved regions are relatively short. In the case of the RIC family, they constitute <30% of the full-length sequence of each member. Interestingly, trees generated based on the full-length coding sequences of these gene families correlated very well to those obtained using just conserved regions (DME, EMS, and BK, unpublished).

All best ML trees (Figs. 5A–11A) were compared with Neighbor–Joining (NJ) trees (Saitou and Nei, 1987), which were generated in ‘Phylogenetic Analysis Using Parsimony’ (PAUP*) version 4.0 (Swofford, 2002) based on the same nucleotide sequence alignments and bootstrapped 2000 times. The distance matrices for the NJ analysis were calculated using the ‘GTR’ model. In all cases, corresponding NJ and ML trees displayed an essentially identical topology (DME, EMS, and BK, unpublished).

‘FigTree v1.2.3’ (Uhttp://tree.bio.ed.ac.uk/software/figtree/) was used to display tree data graphically. Graphical displays showing the domain structures of all members of each analysed RAC/ROP signalling protein family are also presented below (Figs. 5B–11B). These displays were drawn to scale in PowerPoint based on full-length amino acid sequence alignments created as described above, and on the consensus sequences of characterized functional domains as listed in Supplementary Table S2 at JXB online.

RAC/ROP GTPases

The Physcomitrella genome contains four genes encoding putative RAC/ROP GTPases (PpROP1–4; Table 1), which share with characterized angiosperm RAC/ROP GTPases (Sormo et al., 2006; Berken and Wittinghofer, 2008) extremely highly conserved regions that mediate guanine nucleotide binding and GTPase activity (G-box motifs G1–G5), interaction with regulators and effectors (switch I and II, and the Rho insert), or lipid modification and membrane association [the C-terminal polybasic region (PBR) or the CAAX/GC-CG domain] (Fig. 4). PpROP1–4 are therefore typical RAC/ROP GTPases expected to function in a similar manner as their homologues in angiosperms. Among themselves, PpROP1–4 display a very high degree of overall sequence identity at the amino acid level (99–100%). Amino
acid sequence variability between PpROP1–4 is restricted to two adjacent positions 148 and 149 within a region that is folded into the α4-helix of these proteins (Fig. 4). Although the α4-helix is exposed on the surface of 3-D structures of RAC/ROP GTPases (Sormo et al., 2006; Berken and Wittinghofer, 2008), it has not been specifically implicated in the binding of these proteins to cofactors (guanine nucleotides and Mg2+), regulators, effectors, or the plasma membrane. Based on current knowledge, all four PpROP GTPases are therefore likely to be functionally essentially identical. Developmentally and/or physiologically controlled differential expression could explain the existence of four genes coding for nearly identical RAC/ROP GTPases in Physcomitrella. Consistent with this hypothesis, the promoter sequences upstream of the four PpROP open reading frames, which were obtained from the JGI database, are divergent as determined by MUSCLE alignment (Supplementary Fig. S1 at JXB online). Efforts are currently underway in our laboratory to characterize the expression pattern of each of the four Physcomitrella RAC/ROP GTPases based on genomic YFP (yellow fluorescent protein) fusions and quantitative reverse transcription-PCR (RT-PCR).

In Arabidopsis, 11 RAC/ROP genes have been indentified (Table 1), which have been divided into four groups based on sequence comparison (Zheng and Yang, 2000; Christensen et al., 2003). For each of these genes, the Genevestigator database (Zimmermann et al., 2004) provides a detailed anatomical expression pattern based on compiled microarray analyses. Furthermore, many Arabidopsis RAC/ROP proteins have been functionally characterized at least to some extent (Kost et al., 1999a; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002; Cheung et al., 2003; Bloch et al., 2005). Valuable information concerning the expression pattern and function is also available for a number of tobacco RAC/ROP genes (Cvrckova and Zarsky, 1999; Kieffer et al., 2000; Tao et al., 2002; Chen et al., 2003; Morel et al., 2004; Klahre et al., 2006).

Nucleotide sequences of the complete RAC/ROP gene families in Physcomitrella (PpROP) and Arabidopsis (AtROP), of the two homologous genes identified in Selaginella (SmROP), and of selected tobacco homologues were compared using ML algorithms. This analysis established that the four Physcomitrella genes tightly cluster together in a separate group in the RAC/ROP family tree (Fig. 5). If branches supported by bootstrap values <50 are disregarded, the PpROP branch shares a node with four other branches, each of which is formed by one of the following single genes: SmROP1, SmROP2, AtROP7, and AtROP8. All other members of the RAC/ROP gene family appear to be more distantly related to the Physcomitrella homologues. Little is known about the functions of AtROP7 and AtROP8 (Brembu et al., 2005; Mane et al., 2007). AtROP7 is specifically expressed in terminally differentiating xylem cells and was proposed to be involved in secondary cell wall biogenesis (Brembu et al., 2005). The expression of AtROP8 is down-regulated under drought stress in wild-type plants, but up-regulated under the same conditions in plants with reduced phospholipase D2δ1 activity (Mane et al., 2007).

The overall structure of the ML RAC/ROP tree shown in Fig. 5 is in good agreement with results of previously reported phylogenetic analyses (Zheng and Yang, 2000; Christensen et al., 2003). The four distinct groups of RAC/ROP GTPases identified in these studies also form separate clusters in the tree presented here (Fig. 5). Interestingly, the clustering of Arabidopsis and tobacco RAC/ROP GTPases based on sequence comparison is also strongly supported by available expression and functional characterization data. RAC/ROP GTPases closely related based on sequence analysis generally display highly similar expression patterns and have been shown to be involved in the control of the same biological processes. In contrast, unique expression patterns are typical of family members without close homologues.

In the context of this study, RAC/ROP GTPases regulating tip growth are of particular interest. Genevestigator data indicate that AtROP1, 3, and 5 are highly and specifically expressed in pollen, whereas AtROP2 and 4 share a common, less restricted expression pattern with a clear peak in the hair-forming zone of roots. RT-PCR, in situ hybridization, and promoter-GUS (β-glucuronidase) fusion results are consistent with these data (Li et al., 1998; Jones et al., 2002; Cheung et al., 2003). NtRAC5 is also specifically expressed at high levels in tobacco pollen and pollen tubes as demonstrated by northern analysis (Klahre et al., 2006), whereas NtRAC1 (Chen et al., 2003) and NtROP1 (Cvrckova and Zarsky, 1999) were reported to be expressed in pollen. Consistent with these expression patterns, AtROP1 and 5 (Kost et al., 1999a; Li et al., 1999), NtRAC1 (Chen et al., 2003), and NtRAC5 (Klahre et al., 2006) are well characterized regulators of tip growth.

Table 1. Relative sizes of Physcomitrella, Selaginella, and Arabidopsis RAC/ROP signalling gene families

<table>
<thead>
<tr>
<th></th>
<th>RAC/ROP</th>
<th>SPK1</th>
<th>RopGEFs</th>
<th>REN</th>
<th>RopGAP</th>
<th>RopGDI</th>
<th>RIC</th>
<th>ICR</th>
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<tbody>
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<td><strong>Total</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>1.3</td>
<td>3</td>
<td>0.3</td>
<td>5</td>
</tr>
<tr>
<td>Selaginella</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Physcomitrella</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>1.5</td>
<td>6.5</td>
<td>2</td>
<td>0.5</td>
<td>6</td>
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<td><strong>Ratio</strong></td>
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<tr>
<td>Arabidopsis</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td>14</td>
<td>1.3</td>
<td>3</td>
<td>0.3</td>
<td>5</td>
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<tr>
<td>Selaginella</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>4</td>
<td>2</td>
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<tr>
<td>Physcomitrella</td>
<td>4</td>
<td>1</td>
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<td>2</td>
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Total, total number of all genes in the indicated gene families. Ratio is the total number of all genes in the indicated gene families divided by the number of all RAC/ROP genes in the same plant species.
in pollen tubes, whereas AtROP2 and 4 have the same function in root hairs (Molendijk et al., 2001; Jones et al., 2002). The control of tip growth appears to be a major task of angiosperm RAC/ROP GTPase families. Five of the 11 Arabidopsis family members, all of them belonging to group IV, seem to have specialized on this task, and as many as three of them are exclusively expressed in pollen tubes.

Taken together, these observations suggest that all RAC/ROP GTpase functions in the relatively simple non-vascular plant Physcomitrella are covered by four nearly identical proteins, which are most closely related to the group III angiosperm RAC/ROP GTpases. Physcomitrella may express multiple functionally non-distinct RAC/ROP proteins under the control of divergent regulatory sequences, which could enable flexible control of RAC/ROP activity in response to developmental and environmental cues. In contrast, RAC/ROP GTpase families in Arabidopsis and other complex vascular plants are composed of a larger number of more diverse members, which appear to have evolved specialized functions and seem to be specifically expressed where these functions are needed. The ancient vascular plant Selaginella contains a small family of RAC/ROP genes that are more diverse than those of Physcomitrella, which may represent an intermediate situation.

Fig. 4. RAC/ROP GTpase family: functional domains and variable amino acid residues. MUSCLE alignment of the full-length amino acid sequences of all Physcomitrella and Arabidopsis RAC/ROP GTpases. Thick lines above aligned sequences indicate highly conserved regions mediating essential Rho GTpase functions: G-box motifs G1–5, switch (sw) I and II, Rho insert (ins), polybasic region (PBR), and C-terminal lipid modification domains: CAAX in PpROP1–4 and AtROP1–8 or GC–CG in AtROP9–11 (underlined in grey within the alignment). Thin lines below the aligned sequences indicate secondary structural elements: α-helices (α1, 3, 4, and 5) and β-strands (β1–6). Sites of amino acid variability between PpROP1–4 (residues 148 and 149) are indicated by asterisks.
Rho guanine nucleotide exchange factors (RhoGEFs)

RhoGEFs are considered key regulators of the activity of all Rho GTPases including members of the RAC/ROP subfamily (Berken et al., 2005; Rossman et al., 2005). In response to stimulation by plasma membrane-associated receptor proteins, animal and fungal RhoGEFs activate the signalling function of their target Rho GTPases by promoting GDP for GTP exchange (Fig. 1). Two distinct, structurally unrelated families of RhoGEFs called Dbl (diffuse B-cell lymphoma) and CZH [CDM (Ced-5, Dock180, Myoblast city)-Zizimin homology] are found both in animals and fungi (Meller et al., 2005; Rossman et al., 2005).

The only homologue of these two families of animal and fungal RhoGEFs identified in Arabidopsis is AtSPK1 (Table 1), which is distantly related to CZH RhoGEFs and is encoded by a single copy gene (Qiu et al., 2002; Fig. 6A). The Dbl family of RhoGEFs does not seem to be represented in plants. Full-length AtSPK1, as well as the isolated conserved C-terminal DHR2 domain of this protein (Fig. 6b), display RhoGEF activity towards AtROP proteins in vitro (Basu et al., 2008). Arabidopsis mutants lacking AtSPK1 activity fail to develop beyond the seedling stage and show severe defects in epidermal cell shape and adhesion, as well as in organ morphology (Qiu et al., 2002; Basu et al., 2008). Consistent with these observations, Genevestigator data suggest that AtSPK1 is expressed in a wide range of tissues and cell types. Interestingly, AtSPK1 transcripts appear to accumulate to particularly high levels in the hair-forming region of roots, but are absent from pollen and pollen tubes. In agreement with this expression pattern, pollen tube tip growth is not affected by the absence of AtSPK1 activity (Qiu et al., 2002). In contrast, this activity is likely to play a role in normal root hair growth, although a root hair phenotype of Atspk1 mutants has not been reported yet.

Recently, a plant-specific novel family of RhoGEFs called RopGEFs, has been identified (Berken et al., 2005; Gu et al., 2006). All RopGEFs contain a conserved PRONE (plant-specific ROP nucleotide exchanger) domain, which is exclusively found within this protein family. Full-length Arabidopsis RopGEFs and isolated PRONE domains promote GDP for GTP nucleotide exchange on Arabidopsis RAC/ROP GTPases in vitro (Berken et al., 2005; Gu et al., 2006), but do not show activity towards non-plant Rho GTPases (Berken et al., 2005). The Arabidopsis genome encodes 14 RopGEFs (Berken et al., 2005; Gu et al., 2006; Table 1), whose activity has been proposed to be regulated by members of a large family of receptor-like kinases [RLKs; >600 homologues in Arabidopsis (Shiu and Bleecker, 2001)]. Available evidence for physical and functional interactions between a RopGEF and a RLK in Arabidopsis pollen tubes is discussed below.

Genes coding for homologues of Arabidopsis AtSPK1 were identified both in Physcomitrella and in Selaginella. Remarkably, Physcomitrella contains six genes encoding AtSPK1 homologues, of which at least three are expressed based on available EST data, whereas in Selaginella this gene family is represented by a single member, as it is in Arabidopsis (Table 1). All Physcomitrella, Selaginella, and Arabidopsis SPK1 proteins share a similar domain structure with each other (Fig. 6B), as well as with the related animal and fungal CHZ RhoGEFs (Meller et al., 2005). However, comparison by ML analysis shows that the nucleotide sequences coding for these plant proteins are quite diverse.
It is interesting to note that the single copy SPK1 genes of Selaginella and Arabidopsis appear to be more closely related to each other than to the corresponding family of Physcomitrella genes (Fig. 6A). Together, these observations suggest that the SPK1 protein family in the non-vascular moss Physcomitrella, like the related CHZ RhoGEF families in animals and fungi, plays a more complex role in the control of RAC/ROP GTPase signalling as compared with the single SPK1 homologues in the vascular plants Selaginella and Arabidopsis.

To determine whether the protein families responsible for RhoGEF activity in Physcomitrella are generally more similar to the corresponding families in vascular plants, or in animals and fungi, a search was conducted for Physcomitrella and Selaginella homologues of PRONE domain RopGEFs and Dbl RhoGEFs. It was discovered that like Arabidopsis, Physcomitrella and Selaginella contain in addition to SPK1 genes a family of genes coding for PRONE domain RopGEFs. Six of these RopGEFs were identified in Physcomitrella, and two in Selaginella (Table 1). In contrast, genes encoding Dbl homologues, which are absent from angiosperm genomes (see above), were not found in the Physcomitrella or Selaginella genomes either. RhoGEF function in the non-vascular moss Physcomitrella therefore appears to be mediated by a set of proteins (extended CHZ RhoGEF and RopGEF families) that is intermediate in composition between the corresponding protein sets of vascular plants (single CHZ RhoGEF and extended RopGEF families) and of non-plant organisms (extended CHZ RhoGEF and Dbl RhoGEF families).

ML analysis of nucleotide sequence relationships between Physcomitrella, Selaginella, and Arabidopsis RopGEF genes, which all code for structurally similar proteins with PRONE domains (Fig. 7B), established that all Arabidopsis
genes cluster separately from their homologues in _Physcomitrella_ and _Selaginella_ (Fig. 7A). The RopGEF tree shows a number of remarkable similarities with the RAC/ROP tree discussed above (Fig. 5). Like the PpROP1–4 genes, four _Physcomitrella_ genes coding for PpRopGEF1, 2, 5, and 6 tightly group together in a separate cluster (Fig. 7A). Because some of the gene models coding for these PpRopGEFs appear to be incomplete at the 5' end (Fig. 7B), no attempt was made to compare promoter sequences to assess whether these genes may be differentially expressed. Like _Selaginella_, _Arabidopsis_, animals, and fungi (Meller et al., 2005; Rossman et al., 2005), _Physcomitrella_ contains more genes coding for RopGEFs than for RAC/ROPs (Table 1). Two more RopGEF genes (PpRopGEF3 and 4) in addition to those belonging to the cluster described above are present in the _Physcomitrella_ genome. These two RopGEF genes also share a very high degree of sequence similarity with each other and form a separate cluster (Fig. 7A). As the RAC/ROP gene family, the RopGEF family comprises more diverse genes in _Selaginella_ than in _Physcomitrella_, and contains a considerably increased number of members in _Arabidopsis_, which fall into different subgroups that are supported by Genevestigator expression data (Fig. 7A). Interestingly, like the RAC/ROP tree, the RopGEF tree contains a large cluster of closely related genes that are highly and specifically expressed in pollen tubes. This cluster comprises AtRopGEF8, 9, 11, 12, and 13. In addition, both trees contain a smaller subfamily of genes that display a less restricted expression pattern with a clear peak in the hair-forming root zone. This subfamily contains AtRopGEF3 and 4 in the RopGEF tree. However, the _Arabidopsis_ pollen tube and root hair RopGEF gene subfamilies each are closely linked to an additional gene with a divergent expression pattern. AtRopGEF10 groups together with the pollen tube subfamily (Fig. 7A), but appears to be expressed exclusively and at low levels in root hairs. In contrast, AtRopGEF2 is tightly linked to the root hair gene cluster (Fig. 7A), but displays a unique pattern of low level expression in pollen, root hair-forming tissues, and other cell types. These variations in the expression patterns of closely related genes may reflect the high complexity of the AtRopGEF gene family, which contains a larger number of more diverse members as compared with the AtROP family.

Some members of the _Arabidopsis_ pollen tube AtRopGEF subfamily (AtRopGEF8, 9, and 12) were shown to accumulate at the plasma membrane specifically at the apex of tobacco pollen tubes when transiently overexpressed in these cells fused to GFP (Gu et al., 2006). This observation is consistent with a key function of RopGEFs in the

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**Fig. 7.** RopGEF family: nucleotide sequence relationships and domain structures. (A) Unrooted tree obtained by maximum likelihood analysis of the nucleotide sequences encoding the PRONE domains of all _Physcomitrella_ (Pp), _Selaginella_ (Sm), and _Arabidopsis_ (At) RopGEFs. The indicated bootstrap values are based on 100 iterations and were generated using ‘RAxML Black Box’. Scale bar: nucleotide substitutions per site. (B) Domain structures of all proteins encoded by the genes shown in (A) as determined based on MUSCLE alignment and comparison with consensus sequences of characterized functional domains. The PRONE domain is shown as grey blocks. Structures are drawn to scale such that the first amino acids of the PRONE domains are vertically aligned. Vertical lines indicate presumably incomplete ends of gene models. Asterisks denote a conserved serine residue found in all _Arabidopsis_ pollen tube proteins, and in all _Physcomitrella_ and _Selaginella_ proteins with intact C-termini, which corresponds to S510 of AtRopGEF12. Black bars indicate additional conserved potential phosphorylation sites. Scale bar: 100 amino acids.
polarization of RAC/ROP activity at the apex of tip-growing cells. The C-termini downstream of the PRONE domain are well conserved within RopGEF subfamilies encoded by genes that cluster together in the ML tree, but are substantially divergent between these subfamilies (Fig. 7b; Supplementary Fig. S2 at JXB online). This is interesting because these C-termini appear to have important regulatory functions. The C-terminus of the tomato RopGEF LeKPP specifically interacts with the cytoplasmic kinase domain of the tomato RLK LePRK2 (Kaathien et al., 2005). A similar mode of interaction has also been reported for AtRopGEF12 and the RLK AtPRK2a, which are both expressed in Arabidopsis pollen tubes (Zhang and McCormick, 2007). Within the C-termini of all Arabidopsis pollen tube RopGEFs including AtRopGEF12 (AtRopGEF8, 9, 11, 12, and 13), as well as of the related homologues AtRopGEF10 and 14, three conserved putative phosphorylation target sites have been identified (Zhang and McCormick, 2007; Fig. 7B, Supplementary Fig. 2A). Interestingly, overexpression of full-length AtRopGEF12 does not strongly affect tobacco pollen tube tip growth, whereas massive tip swelling is induced by the overexpression of forms of this protein missing the C-terminus altogether, or carrying a point mutation in this region that mimics phosphorylation of one of the conserved target sites (S510D). A similar tip swelling of tobacco pollen tubes is induced by co-overexpression of full-length AtRopGEF12 together with the RLK AtPRK2, or by RAC/ROP overexpression. Together, these observations suggest that RLKs may stimulate RAC/ROP signalling in Arabidopsis via the phosphorylation of RopGEF C-termini, which relieves autoinhibition of RhoGEF activity by these domains (Zhang and McCormick, 2007). Phosphorylation of autoinhibitory domains is also thought to underlie receptor-mediated activation of Dbl RhoGEFs in animals and fungi (Rossman et al., 2005).

Although the three phosphorylation sites described above are missing in the C-termini of the proteins encoded by the AtRopGEF5–7 gene cluster, other conserved amino acids, including possible phosphorylation sites, are found exclusively in these domains (Fig. 7B, Supplementary Fig. S2B). Some of these conserved amino acids also appear to be present in the C-terminus of AtRopGEF1, which has an otherwise rather unique sequence (Fig. 7B, Supplementary Fig. S2B). The proteins encoded by the AtRopGEF2–4 gene cluster, which includes the two genes coding for putative root hair isoforms, are completely missing C-termini downstream of their PRONE domains, suggesting that they are either constitutively active or regulated by alternative mechanisms (Fig. 7B, Supplementary Fig. S2B).

Interestingly, the C-termini of all Physcomitrella RopGEFs, and of the only Selaginella homologue with a complete putative 3′ gene model, contain the conserved serine residue corresponding to S510 in AtRopGEF12, which is phosphorylated by upstream RLK activity (Fig. 7B, Supplementary Fig. S2A at JXB online). In addition, these proteins contain at least one of the two other conserved phosphorylation sites found in the C-termini of Arabidopsis pollen tube RopGEFs and of their closest homologues (Fig. 7B, Supplementary Fig. S2A). Furthermore, several of the conserved amino acids specifically identified in the C-termini of proteins encoded by the AtRopGEF5–7 cluster and by AtRopGEF1 are also present in all Physcomitrella RopGEFs and in the single Selaginella RopGEF with a complete available C-terminus (Fig. 7B, Supplementary Fig. S2B). Together, these observations suggest that the activity of Physcomitrella RopGEFs may be regulated by molecular mechanisms conserved in all land plants, which targeted the C-termini of these proteins and appear to involve phosphorylation of this domain by RLK homologues. They also indicate that each of the closely related members of the small Physcomitrella RopGEF family may be regulated in the same way by multiple upstream signalling pathways, whereas each of these pathways in Arabidopsis appears to control only the activity of specific RopGEF subfamilies with specialized functions and expression patterns.

In summary, like Selaginella and Arabidopsis, Physcomitrella contains two types of proteins with RhoGEF activity: SKP1-related CHZ family RhoGEFs, which are also found in animals and fungi, and members of the plant-specific PRONE domain RopGEF family. The Physcomitrella CHZ gene family displays a similar complexity to corresponding families in animals and fungi, whereas single CHZ genes are found in Selaginella and Arabidopsis. This places the non-vascular moss Physcomitrella between vascular plants and non-plant organisms with respect to the composition of the protein families responsible for RhoGEF activity. Like the RAC/ROP family, the PRONE domain RopGEF family is highly expanded in Arabidopsis as compared with Physcomitrella, and appears to contain separate groups of proteins specialized in the control of tip growth in pollen tubes and in root hairs. Interestingly, structural comparisons of RopGEF C-termini suggest that the different subfamilies of Arabidopsis RopGEFs are regulated by distinct upstream signalling pathways, which may act together in Physcomitrella to modulate the activity of each of the closely related PpRopGEFs.

Rho GTPase-activating proteins (RhoGAPs)

In animals and fungi, large families of RhoGAPs are present, which all share a conserved RhoGAP domain (Lamarche and Hall, 1994) that contains an invariable arginine (R) residue required for catalytic activity (Rittinger et al., 1997; Graham et al., 1999). The RhoGAP domain of these proteins can inactivate the signalling functions of target Rho GTPases by enhancing their low intrinsic GTPase activity (Fig. 1). In addition to the RhoGAP domain, many animal and fungal RhoGAPs contain a variety of other domains, which display either enzymatic activity (e.g. RhoGEF or protein kinase activity) or affinity for specific lipids or proteins. Individual members of this protein family can contain up to 11 different functional domains (Bernards and Settleman, 2004; Tcherkezian and Lamarche-Vane, 2007). Animal proteins with inactive RhoGAP domains have also been identified, which are thought to act as
scaffolds for the formation of signalling complexes containing Rho GTPases along with some of their regulators and effectors (Chiang et al., 2003). It is increasingly appreciated that members of the RhoGAP protein family, in part based on their ability to integrate multiple signalling pathways, play roles in the regulation of Rho signalling equally important to those of RhoGEFs (Kost, 2010).

In plants, RhoGAP domains with the conserved catalytic R residue are found in a smaller and less diverse group of proteins, which all contain only one additional distinctive functional domain. Plant RhoGAPs fall into two different families, RENs and RopGAPs, based on the nature of the additional domain, which in all cases is located in close proximity upstream of the RhoGAP domain (Figs 8B, 9B). The REN family of plant RhoGAPs contain a PH domain, which is also present in a number of RhoGAPs from other organisms (Tcherkezian and Lamarche-Vane, 2007). PH domains bind to phospholipids (Maffucci and Falasca, 2001) and have been shown to be required for the membrane association and correct subcellular targeting of animal RhoGAPs (Ren et al., 2001). However, the intracellular localization of AtREN1, an Arabidopsis REN family RhoGAP, does not seem to depend on its PH domain (Hwang et al., 2008). Interestingly, members of the second plant RhoGAP family called RopGAPs contain a CRIB domain, which is not present in any animal or yeast RhoGAP. CRIB domains have been shown to mediate the specific binding of many downstream effectors to activated forms of plant and non-plant Rho GTPases (Pirone et al., 2001). Although the functions of these domains in RopGAPs are not entirely understood, they have been proposed to modulate the activity of RopGAPs by contributing to the binding of these proteins to target RAC/ROP GTPases (Klahre and Kost, 2006). Consistent with this hypothesis, RhoGAP and CRIB domains appear to interact with non-overlapping regions of Rho GTPases (Berken and Wittenhofer, 2008).

Full-length proteins of the plant REN and RopGAP families, as well as their isolated RhoGAP domains, have been shown to enhance the GTPase activity of RAC/ROP GTPases in vitro (Wu et al., 2000; Klahre and Kost, 2006; Hwang et al., 2008). Members of both protein families play essential roles in the maintenance of the polarity of RAC/ROP signalling and cell expansion at the tip of elongating pollen tubes, although based on completely different mechanisms. AtREN1 appears to be associated with cytoplasmic vesicles at the apex of Arabidopsis pollen tubes and globally down-regulates RAC/ROP signalling activity. Pollen tubes of Atren1 mutants display depolarized growth (Hwang et al., 2008). In contrast, in tobacco pollen tubes the RopGAP protein NrRhoGAP1 is associated with the plasma membrane specifically at the flanks of the tip, where it promotes the GTPase activity of RAC/ROP proteins and thereby spatially restricts RAC/ROP signalling to the apex. NrRhoGAP1 contains a phosphorylation-sensitive consensus binding site for 14-3-3 proteins, and interacts with a member of this protein family called Nt14-3-3b1. Binding to Nt14-3-3b1 modulates membrane association of NrRhoGAP1. NrRhoGAP1 overexpression strongly inhibits pollen tube growth, whereas overexpression of an inactive mutant version of this protein missing the conserved catalytic R residue has dominant-negative effects and depolarizes this process (Klahre and Kost, 2006). Arabidopsis mutants defective in the expression of the NrRhoGAP1 homologue AtRopGAP1 have been reported to show normal pollen tube growth (Hwang et al., 2008; Yang, 2008), presumably because four additional closely related AtRopGAPs are expressed at similar levels in pollen (see below).

The Arabidopsis genome contains three AtREN and six AtRopGAP genes (Table 1), of which the one called AtRopGAP6 appears to be a pseudo gene and was excluded from all analyses described below. All genes coding for proteins with RhoGAP domains which have been identified in the genomes of Physcomitrella and Selaginella are closely related to one of these two gene families. With two and five members, respectively, the Physcomitrella REN and RopGAP gene families are similar in size to the corresponding Arabidopsis families (Table 1). In contrast, Selaginella only contains a single REN gene and two RopGAP homologues (Table 1). Amino acid sequence alignments (DME, EMS, and BK, unpublished) established that the arginine residue required for catalytic activity is conserved within the RhoGAP domains of all REN and RopGAP family members in Arabidopsis, Physcomitrella, and Selaginella. Interestingly, a complete putative binding site for 14-3-3 proteins matching the consensus sequence R/K-X-X-pS/T-X-P (Ferl, 2004), which is involved in the regulation of membrane association of NrRhoGAP1 (Klahre and Kost, 2006), is present in AtRopGAP2 and 3, and in both Selaginella RopGAP homologues, but could not be detected in any of the Physcomitrella homologues. This indicates that 14-3-3 proteins may regulate intracellular targeting of different RopGAPs in vascular plants, but not in the non-vascular moss Physcomitrella.

Nucleotide sequence relationships within the REN (Fig. 8A) and the RopGAP (Fig. 9A) gene families of Arabidopsis, Physcomitrella, and Selaginella were investigated by ML analysis. The tobacco gene coding for NrRhoGAP1 was included in the analysis of the RopGAP family because of its well characterized function in pollen tube tip growth (Klahre and Kost, 2006). In the ML trees of both gene families all genes of each of the three species Arabidopsis, Physcomitrella, and Selaginella appear to form separate clusters (Figs. 8A, 9A). AtREN1, which plays an important role in control of tip growth in pollen tubes (Hwang et al., 2008), is highly and specifically expressed in pollen as suggested by Genevestigator data. In contrast AtREN3 shows a broad expression pattern with an elevated level of expression in the hair-forming root region, whereas no data concerning AtREN2 expression are currently available. AtREN3 is quite divergent from AtREN1 and is much more closely related to AtREN2, with which it clusters together in a separate group (Fig. 8A). Because most members of the Arabidopsis RAC/ROP and RopGEF gene families with similar functions and expression patterns cluster together in ML trees (see previous sections), AtREN2 may be predicted
to be expressed and to function in a wide range of vegetative cells like AtREN3. However, within the RopGAP gene family, sequence similarity does not strongly correlate with expression pattern and function. In the ML tree of this family, the Arabidopsis and the Physcomitrella genes are divided into two subfamilies, whereas the Selaginella genome only contains two homologues (Fig. 9A). Each of the Arabidopsis genes displays a rather unique expression pattern. AtRopGAP1 and 5, which belong to the Arabidopsis subfamily most closely related to the tobacco pollen tube homologue NtRhoGAP1, and AtRopGAP3, which belongs to the other subfamily, display elevated expression in the hair-forming root zone. All AtRopGAP genes display low level expression in pollen, as does NtRhoGAP1. NtRhoGAP1 has been shown to display high enzymatic activity and to maintain polarized RAC/ROP signalling in pollen tubes by catalysing RAC/ROP inactivation at a subapical domain of the plasma membrane (Klahre and Kost, 2006).

In the course of the analysis of the plant RhoGAP gene families, an additional related Arabidopsis gene (At5g61530) was discovered which has received little attention in the literature to date (Berken et al., 2005). The protein encoded by this gene contains a RhoGAP domain, which is missing the conserved arginine residue required for catalytic activity. Although additional functional domains, such as a PH or a CRIB domain, cannot be clearly identified, the protein appears to be structurally somewhat related to the RopGAP family (Fig. 9B) and was therefore tentatively named AtRopGAPlike1. It is possible that AtRopGAPlike1 has a function in the modulation of RAC/ROP activity, perhaps by promoting the formation of signalling complexes, like animal proteins with inactive RhoGAP domains (Chiang et al., 2003), or by acting as a dominant-negative inhibitor of RhoGAP activity. Interestingly, AtRopGAPlike1 appears to be a single-copy gene in Arabidopsis, which is highly and specifically expressed in pollen based on Genevestigator data. It was possible to identify homologues of this gene in other angiosperms such as rice and poplar, but not in Physcomitrella or in Selaginella. Interestingly, ML analysis of the nucleotide sequences coding for the RhoGAP domains of AtRopGAPlike1 and all Arabidopsis, Selaginella, and tobacco members of the REN or RopGAP

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**Fig. 8.** REN family: nucleotide sequence relationships and domain structures. (A) Unrooted tree obtained by maximum likelihood analysis of the full-length coding domain sequences of all Physcomitrella (Pp), Selaginella (Sm), and Arabidopsis (At) REN genes. The indicated bootstrap values are based on 100 iterations and were generated using RAxML Black Box. Scale bar: nucleotide substitutions per site. (B) Domain structures of all proteins encoded by the genes shown in (A) as determined based on MUSCLE alignment and comparison with consensus sequences of characterized functional domains. In addition to the distinctive PH and RhoGAP domains, two putative coiled-coil protein interaction motifs (cc) were identified in the C-terminal half of all RENs. All domains and motifs are shown as grey blocks. Structures are drawn to scale such that the first amino acids of the PH domains are vertically aligned. Scale bar: 100 amino acids.
protein families suggests that AtRopGAPlike1 is roughly equally distantly related to both these families (Supplementary Fig. S3 at *JXB* online).

Altogether, the analysis of plant genes coding for proteins with RhoGAP domains shows that *Physcomitrella* contains rather large gene families encoding REN and RopGAP proteins (Table 1), the two types of RhoGAP proteins also found in other plants. All *Physcomitrella* REN and RopGAP proteins contain RhoGAP domains that appear to be functional based on the presence of a conserved arginine residue required for catalytic activity. However, in contrast to RopGAPs in vascular plants including *Selaginella*, all RopGAPs in the non-vascular plant *Physcomitrella* are lacking a consensus binding site for 14-3-3 proteins. These proteins are therefore unlikely to modulate RopGAP membrane association in *Physcomitrella*. *Physcomitrella*, like *Selaginella*, is also missing homologues of proteins with inactive RhoGAP domains, which may play a role in the regulation of RAC/ROP signalling in angiosperms. These observations suggest that vascular plants may have evolved

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**Fig. 9.** RopGAP family: nucleotide sequence relationships and domain structures. (A) Unrooted tree obtained by maximum likelihood analysis of the nucleotide sequences encoding the CRIB domain, the RhoGAP domain, and the highly conserved region connecting these two domains of all *Physcomitrella* (Pp), *Selaginella* (Sm), and *Arabidopsis* (At) RopGAPs, as well as the only identified tobacco RopGAP. The indicated bootstrap values are based on 100 iterations and were generated using ‘RAxML Black Box’. Scale bar: nucleotide substitutions per site. (B) Domain structures of all proteins encoded by the genes shown in (A) as determined based on MUSCLE alignment and comparison with consensus sequences of characterized functional domains. The CRIB and RhoGAP domains are shown as grey blocks. Structures are drawn to scale such that the first amino acids of the CRIB domains are vertically aligned. The domain structure of AtRopGAPlike1 is included for comparison. Scale bar: 100 amino acids.
additional levels of complexity in the control of RAC/ROP activity based on proteins with RhoGAP domains, which are not required in *Physcomitrella*.

**Rho guanine nucleotide inhibitors (RhoGDIs)**

Only a few RhoGDIs have been identified in each of the eukaryotic organisms for which a complete genome sequence is available. The human genome encodes large families of Rho GTPases, RhoGEFs, and RhoGAPs, but contains just three RhoGDIs, two of which are specifically expressed in only a few cell types (DerMardirossian and Bokoch, 2005). A single RhoGDI is present in the yeast *Saccharomyces cerevisiae* (Masuda *et al.*, 1994), whereas three *Arabidopsis* homologues called AtRopGDI1, 2a, and 2b have been identified (Carol *et al.*, 2005; Kost, 2010; Table 1). RhoGDIs from all organisms are small proteins, share a high degree of sequence homology, and have a very similar structure (Kost, 2010; Fig. 10B). They contain a large C-terminal immunoglobulin-like (IG-like) domain with a hydrophobic binding pocket that can accommodate the prenyl tail of target Rho GTPases. This domain is responsible for the ability of RhoGDIs to extract Rho GTPases from the plasma membrane, and to form cytoplasmic heterodimers with them (Fig. 1). Within these heterodimers, the regulatory arm (RA) located just upstream of the IG-like domain of all RhoGDIs directly interacts with Rho GTPases and blocks nucleotide release, RhoGAP, and RhoGEF activity, as well as effector binding (Hoffman *et al.*, 2000; Scheffzek *et al.*, 2000; DerMardirossian and Bokoch, 2005). Upstream of the RA, Rho GDIs contain a highly variable N-terminal domain with unknown functions, which is slightly longer in plant proteins than in their animals and fungal homologues (Klahre *et al.*, 2006; Kost, 2010).

Based on their ability to form inactive cytoplasmic heterodimers with target Rho GTPases, RhoGDIs are generally thought to act as negative regulators of Rho signalling (DerMardirossian and Bokoch, 2005). Consistent with this view, RhoGDI overexpression has been shown to inhibit Rho signalling and Rho-dependent cellular processes in a variety of cell types (e.g. Masuda *et al.*, 1994; Lin *et al.*, 2003; Klahre *et al.*, 2006). However, a few reports have suggested that RhoGDIs can promote Rho signalling in animal cells by translocating target Rho GTPases in the active GTP-bound form between different plasma membrane domains (Del Pozo *et al.*, 2002). An essential function of RhoGDIs in the promotion of polarized RAC/ROP signalling has been clearly demonstrated in tip-growing plant cells. *Arabidopsis* root hairs are formed as a single outgrowth at the basal end of root epidermal cells. RAC/ROP GTPases accumulate at the plasma membrane specifically at the site of root hair outgrowth already before root hair formation, and remain associated with the apex of elongating root hairs (Molendijk *et al.*, 2001; Jones *et al.*, 2002). In *Arabidopsis* mutants lacking AtRopGDI1, RAC/ROP GTPases display enhanced association with extended
regions of the plasma membrane of root hair-forming cells. As a consequence, these cells develop multiple root hair buds, which fail to elongate (Carol et al., 2005). Based on these observations it can be concluded that AtRopGDI1 is required for the establishment and maintenance of a single polarized site of RAC/ROP activity at the plasma membrane of root epidermal cells, which controls root hair formation and growth. The maintenance of polarized RAC/ROP activity at the pollen tube tip also depends on RopGDI function (Klahre et al., 2006). In tobacco pollen tubes, a mutant version of the RAC/ROP GTPase NtRAC5 specifically disrupted in its ability to interact with the RopGDI NtRhoGDI2 strongly associates with the plasma membrane laterally, but not at the apex. Furthermore, in contrast to wild-type NtRAC5, mutant NtRAC5 does not depolarize tip growth when overexpressed (Klahre et al., 2006). These findings strongly suggest that NtRAC5 accumulation and activation at the pollen tube apex depends on NtRhoGDI2, which appears to recycle NtRAC5 back to the apex after its inactivation by NtRhoGAP2 at the flanks of the tip (Kost, 2008). Consistent with an essential function of NtRhoGDI2 in the maintenance of apical NtRAC5 activity, RNAi (RNA interference) constructs targeting NtRhoGDI2 expression inhibit tobacco pollen tube tip growth without depolarizing this process (Kost, 2010).

The Physcomitrella genome encodes three RopGDIs, whereas two genes coding for homologous proteins were identified in Selaginella (Table 1). These five proteins share a very similar domain structure with RhoGDIs of angiosperms (Fig. 10B), and of other organisms (Kost, 2010). However, it is interesting to note that the N-terminal variable domain is longer in Physcomitrella PpRopGDIs than in all RopGDIs of vascular plants including Selaginella (Fig. 10B), which in turn have longer N-terminal variable domains than animal and yeast homologues (Klahre et al., 2006; Kost, 2010). ML analysis showed that the Arabidopsis and tobacco RopGDI genes cluster together separately from the Physcomitrella and Selaginella gene families, each of which also forms a distinct group (Fig. 10A). Like PpROP genes, PpRopGDI genes are highly similar within the coding region, but contain divergent promoter sequences (Supplementary Fig. S4 at JXB online). As discussed above, this may enhance the ability of Physcomitrella to control the activity of the proteins encoded by these genes based on differential gene expression. Interestingly, the three Arabidopsis RopGDI genes are all expressed at very high levels in pollen and at much lower levels in most other cells and tissues based on Genevestigator data. Presumably, this reflects not only the importance of RopGDI function for tip growth, but also the very strong expression of some AtROP genes in pollen. The total molar amounts of all proteins of the Rho GTPase and RhoGDI families were found to be roughly equal in animal cells (Michaelson et al., 2001; DerMardirossian and Bokoch, 2005), which is consistent with an import role in the control of Rho signalling of heterodimer formation between these two types of proteins.

In summary, Physcomitrella contains three very similar RopGDI proteins, whose activity may be controlled in part by differential gene expression, like that of the PpROP and PpRopGEF protein families. PpRopGDIs share a highly conserved structure with all RhoGDIs from other organisms, and are therefore likely to function in an essentially similar manner. However, the Physcomitrella RopGDI family may be considered relatively large compared with the RhoGDI families in animals, fungi, and angiosperms, which contain much higher numbers of more diverse Rho GTPases (see above and Table 1). Together with the extended N-terminal domain of PpRopGDIs, the relatively large size of the PpRopGDI protein family may indicate a particularly important and complex role for this family in the control of RAC/ROP signalling in Physcomitrella.

**RAC/ROP effectors**

Activated Rho GTPases regulate and coordinate different cellular processes (actin reorganization, membrane trafficking, gene expression, etc.) to induce major changes in cell behaviour (movement, polar growth, division, etc.) (Etienne-Manneville and Hall, 2002). To achieve this, GTP-bound active Rho GTPases typically bind to multiple effector proteins that stimulate distinct downstream signalling pathways. A substantial number of proteins have been shown to act as effectors downstream of activated plant RAC/ROP GTPases (Berken, 2006; Yalovsky et al., 2008; Berken and Wittinghofer, 2008; Molendijk et al., 2008; Dorjgotov et al., 2009). Although most of these proteins have only been functionally characterized to a limited extent, it has become clear that plants have evolved a unique RAC/ROP-dependent signalling network. Members of some protein families (e.g. enzymes involved in cell wall biosynthesis, WAVE complex proteins, protein kinases, phosphatidylinositol monophosphate kinases, and NADPH oxidases) appear to act as Rho effectors not only in plants but also in other organisms (Qadota et al., 1996; Bustelo et al., 2007). However, the molecular mechanisms underlying their functions in Rho signalling are generally different in plants (Berken and Wittinghofer, 2008; Yalovsky et al., 2008). Furthermore, homologues of important effectors of animal and fungal Rho GTPases including PAKs (p21-activated kinases) and ROCKs (Rho-associated kinases) (Zhao and Manser, 2005) are absent in plants, which instead contain specific families of RAC/ROP effectors not found in other organisms, such as ICRs (interactors of constitutively active ROP; Lavy et al., 2007; Li et al., 2008) and RICs (ROP-interacting CRIB-containing; Wu et al., 2001). Here, the plant-specific ICR and RIC protein families are considered. Members of these families appear to have functions in the control of tip growth downstream of activated RAC/ROP GTPases, with which they have been shown to interact directly.

AtICR1 seems to bind both in vitro and in vivo not only preferentially to activated RAC/ROP GTPases, but also to AtSEC3 (Lavy et al., 2007). AtSEC3 is a component of the
exocyst, a plasma membrane-associated protein complex promoting polarized secretion, which in yeast is assembled in response to the activation of Rho signalling (Guo et al., 2001). Tip growth of both root hairs and pollen tubes is abolished in plant mutants lacking exocyst components (Cole et al., 2005; Wen et al., 2005; Hala et al., 2008). These observations suggest that downstream of RAC/ROP activation AtICR1 may recruit AtSEC3 to stimulate exocyst assembly and polarized secretion required for tip growth. Consistent with this view, AtICR1 overexpression depolarizes root hair expansion (Lavy et al., 2007). The growth of pollen tubes and root hairs does not seem to be affected in mutants lacking AtICR1, presumably because Arabidopsis contains four homologous proteins (Li et al., 2008; Table 1), which all are expressed in tip-growing cells based on Genevestigator data. However, these data also indicate that AtICR1 and all its Arabidopsis homologues are expressed in pollen tubes and in the hair-forming region of roots at much lower levels than in other tissues. Together with a recent report suggesting a role for AtICR1 upstream of RAC/ROP activation (Li et al., 2008), this indicates that AtICR1 functions may only be partially understood to date.

The 11 RIC proteins found in Arabidopsis all contain a CRIB domain (Fig. 11B; Table 1), but outside of this domain show little sequence similarity with each other or with other proteins. CRIB domains are responsible for the specific interaction of many animal and fungal effectors (e.g. PAKs) with activated Rho GTPases (Pirone et al., 2001), and modulate the interaction of RopGAPs with their target RAC/ROP GTPases (Klahre and Kost, 2006). These domains are located near the N-terminus of most AtRIC proteins, although in AtRIC2 and 4 they are found closer to the C-terminus (Fig. 11B). Nothing is known about the functions of the highly variable regions of AtRIC proteins outside of the CRIB domain. Based on the absence of recognizable structural elements in these regions, and on analogy with characterized animal CRIB domain proteins, the variable domains of AtRICs have been proposed to be intrinsically unstructured and to assume a structured conformation in which they may serve as scaffolds for the formation of signalling complexes only upon CRIB domain-mediated binding to an activated RAC/ROP GTPase (Berken and Wittinghofer, 2008).

AtRIC3 and 4 were proposed to act as key RAC/ROP effectors in pollen tubes based on the observation that these two proteins are the only members of the AtRIC family which depolarize tobacco pollen tube growth when overexpressed (Gu et al., 2005). Reduced levels of AtRIC3 or AtRIC4 expression in Arabidopsis mutants or RNAi lines result in lower pollen tube growth rates in vitro. FRET (fluorescence energy resonance transfer) experiments have indicated that AtROP1 interacts with AtRIC3 and AtRIC4 at the plasma membrane at the apex of tobacco pollen tubes. The effects of altering AtRIC3 and AtRIC4 expression levels in pollen tubes individually or together were analysed using in vivo markers for F-actin organization and cytoplasmic calcium concentration. These experiments led to the conclusion that AtRIC3 and AtRIC4 are essential for tip growth.
components of two different AtROP1-dependent signalling pathways with opposite effects on F-actin organization (Gu et al., 2005). AtRIC3 appears to promote F-actin disassembly via the stimulation of calcium signalling, whereas AtRIC4 seems to promote F-actin assembly by an unknown mechanism. AtROP1 was proposed to control pollen tip growth by maintaining a delicate balance between the activities of these two antagonistic pathways (Gu et al., 2005).

Extensive BLAST searches only resulted in the identification of a single RIC gene each in the genomes of Physcomitrella (PpRIC) and Selaginella (SmRIC) (Table 1). Sequences coding for ICR homologues were not found at all in these genomes (Table 1). ML analysis of the Physcomitrella, Selaginella, and Arabidopsis RIC genes showed that the two non-angiosperm genes cluster together separately from all Arabidopsis genes (Fig. 11A). Three different subfamilies can be discerned within the Arabidopsis RIC gene cluster, which are comprised of AtRIC2 and 4, AtRIC1, 3, and 5–8, and AtRIC9–11, respectively (Fig. 11A). Expression data are available in the Genevestigator database for AtRIC1, 2, 4, 6, and 10, and have been generated by semi-quantitative RT-PCR for AtRIC1–7, 9, and 10 (Wu et al., 2001). These data suggest that closely related Arabidopsis genes forming subfamilies in the RIC tree, like in the ROP and RopGEF trees, display similar expression patterns, which include specific high level expression in pollen as well as expression in a range of cell types, with a clear peak in the hair-forming root zone. Genes specifically expressed in pollen tubes are found in the AtRIC1, 3, and 5–8 subfamily, whereas genes strongly expressed in root hair-producing cells cluster within the AtRIC9–11 subfamily. The NtRIC gene, which is specifically expressed at a high level in tobacco pollen tubes and codes for a RIC homologue that preferentially binds to active forms of the pollen tube RAC/ROP GTPase NtRAC5 in vivo and in vitro (P Kapoor, M Wheeler, and B Kost, unpublished), clusters together within the Arabidopsis pollen tube subfamily (Fig. 11A).

It is interesting to note that AtRIC4, which has been proposed to have a key function together with AtRIC3 in the regulation of pollen tube tip growth (Gu et al., 2005), displays a similar expression pattern to AtRIC2 according to the Genevestigator database. AtRIC2 and 4 appear to be expressed at the highest levels in the stem, and form a separate cluster in the RIC tree (Fig. 11A). Remarkably, the AtRIC2 and 4 proteins share a unique overall structure with each other, as well as with the Physcomitrella and Selaginella RIC proteins (Fig. 11B). In these four proteins, the CRIB domain is located closer to the C-terminus, whereas in all other Arabidopsis RICs this domain is found near the N-terminus.

In brief, the two extended Arabidopsis ICR and RIC families of RAC/ROP effectors, which are thought to play pivotal roles in the control of directional cell expansion downstream of RAC/ROP activation in angiosperms, are represented only by a single RIC homologue in Physcomitrella and in Selaginella. Like the RAC/ROP and RopGEF families, the Arabidopsis RIC family contains large subfamilies of proteins with expression patterns suggesting specialized functions in the regulation of tip growth either in root hairs or in pollen tubes. Interestingly, based on overall protein structure, the Physcomitrella and Selaginella RIC homologues appear to be most closely related to AtRIC2 and 4, which do not belong to the root hair or pollen tube subfamilies. Together, these observations strongly suggest that the single Physcomitrella RIC may play a different and less complex role in the control of tip growth as compared with its Arabidopsis homologues, and perhaps functions in concert with other RAC/ROP effectors during this process. In any case, the presence of a single RIC gene in Physcomitrella provides a unique opportunity to learn more about the enigmatic molecular and cellular functions of the non-conserved regions outside of the CRIB domains of plant RIC family proteins.

General conclusions

Extensive nucleotide sequence relationship analyses have established that Arabidopsis and Physcomitrella members of RAC/ROP signalling gene families generally form separate, non-overlapping clusters in ML trees. The only exception to this role is the uncertain distinction between the Arabidopsis RAC/ROP genes AtROP7 and 8 and their Physcomitrella homologues, which is only supported by low bootstrap values (Fig. 5). It is therefore not possible to use sequence comparison with RAC/ROP signalling genes known to be involved in the control of tip growth in angiosperms to pinpoint Physcomitrella genes with possible functions in this process. Knocking-out members of Physcomitrella RAC/ROP signalling gene families and investigating their expression patterns is required to identify such genes.

Arabidopsis and Physcomitrella are predicted to contain similar total numbers of protein-coding genes [27 379 (release tair9) and 32 239 (release 1.5), respectively]. However, the relative sizes of different RAC/ROP signalling gene families in the two organisms are highly variable (Table 1). Physcomitrella, for example, contains six SPK1 genes and a single RIC gene, whereas a single SPK1 gene and 11 RIC genes are found in Arabidopsis (Table 1). Interestingly, the ratios between the sizes of the gene families coding for upstream RAC/ROP regulators and RAC/ROP GTPases appear to be highest in Physcomitrella and lowest in Arabidopsis, whereas the corresponding ratios for gene families encoding downstream RAC/ROP effectors display the opposite trend (Table 1). This suggests that the activity of the four nearly identical Physcomitrella RAC/ROPs may be regulated not only by differential gene expression, as discussed above, but also by particularly complex upstream signalling. In contrast, the downstream signalling network stimulated by activated RAC/ROP GTPases appears to be simpler in Physcomitrella than in Arabidopsis. This hypothesis is also supported by the comparably small sizes of the Physcomitrella gene families coding for
phosphatidylinositol monophosphate kinases (two and 11 homologues in Physcomitrella and Arabidopsis, respectively; Mueller-Roeber and Pical, 2002; Sousa et al., 2008; Stenzel et al., 2008; Saavedra et al., 2009) and NADPH oxidases (four and 10 homologues in Physcomitrella and Arabidopsis, respectively; DME, EMS, and BK, unpublished). Alternatively, it is possible that different families of effector proteins are acting downstream of RAC/ROP activation in Physcomitrella and in Arabidopsis. In any case, it was not possible to identify in BLAST searches Physcomitrella homologues of PAKs or ROCKs (DME, EMS, and BK, unpublished), which are also absent from angiosperms but act as key effectors of non-plant Rho GTPases (Zhao and Manser, 2005).

Interestingly, the Arabidopsis RAC/ROP, RopGEF, and RIC gene families contain separate subfamilies of genes that appear to have specialized on the control of tip growth either in pollen tubes or in root hairs (Figs 5, 7, 11). One representative of each subfamily is also found in the small AtREN gene family with only three members (Fig. 8). Typically, pollen tube subfamily members are specifically expressed at high levels in pollen, whereas their root hair homologues display less restricted expression patterns with a clear peak in the hair-forming zone of the root. In the AtROP and AtRopGEF families, and probably also in the AtRIC family, the pollen tube subfamilies are larger. Together, these observations indicate that the RAC/ROP signalling network required to control tip growth may be different and more complex in pollen tubes as compared with root hairs, perhaps because pollen tube elongation is faster and more responsive to extracellular cues (Hepler et al., 2001; Kost, 2008). Tip-growing cells at the ends of Physcomitrella filaments and rhizoids also elongate more slowly, and are less responsive to the environment, than pollen tubes. RAC/ROP signalling mechanisms in these cells are therefore more likely to resemble those in root hairs.

In summary, this study establishes that homologues of all Arabidopsis genes with key functions in the RAC/ROP-mediated regulation of tip growth, with the exception of ICR genes, are present in the Physcomitrella genome. Furthermore, interesting similarities and differences between the RAC/ROP signalling gene families in Physcomitrella and Arabidopsis are uncovered, and their implications are discussed. The work presented here provides a framework of information supporting the use of Physcomitrella as a model to investigate the role of RAC/ROP signalling in tip growth. A number of exciting opportunities offered by this approach are highlighted. Physcomitrella clearly has enormous potential as an experimental system to enhance our understanding of the molecular control of polar cell growth.

**Supplementary data**

Supplementary data are available at JXB online.

**Table S1.** Genes and gene models.

**Table S2.** Conserved domains in RAC/ROP regulators and effectors.

**Supplementary alignment 1.** Full-length amino acid alignments.

**Supplementary alignment 2.** Nucleotide alignments for ML and NJ phylogenies.

**Fig. S1.** Alignment of 5′ UTR and promoter sequences of Physcomitrella RAC/ROP genes.

**Fig. S2.** Conserved putative phosphorylation sites in RopGEF C-termini.

**Fig. S3.** Nucleotide sequence relationships between AtRopGAPlike1 and genes coding for RopGAP and REN proteins.

**Fig. S4.** Alignment of 5′ UTR and promoter sequences of Physcomitrella RopGDI genes.

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