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The crystal structure of *Arabidopsis thaliana* RAC7/ROP9: The first RAS superfamily GTPase from the plant kingdom

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Abstract

Arabidopsis thaliana RAC/ROP GTPases constitute a plant specific Rho GTPase family in the RAS superfamily, which has been implicated in numerous pivotal signalling cascades in plants. Research has shown that plants in some cases have evolved different modes of regulating Rho GTPase activity as compared to the equivalent systems in animals and yeast. In order to gain structural insight into plant signaling at the molecular level, we have determined the first crystal structure of a RAC-like GTPase belonging to the RAS superfamily from the plant kingdom. The structure of AtRAC7/ROP9 bound to GDP was solved at a resolution of 1.78 Å. We have found that the structure of plant Rho GTPases is based upon a conserved G-domain architecture, but structural differences were found concerning the insert region and switch II region of the protein.

Keywords: *Arabidopsis thaliana*; Crystal structure; Ras superfamily; Rho GTPase; AtRAC7; ROP9

1. Introduction

Small (20–40 kDa) monomer GTPases belonging to the RAS superfamily of GTPases have evolved to regulate a number of cellular processes. These proteins act as molecular switches that activate numerous processes when bound to a GTP nucleotide and become inactive when GTP is hydrolyzed to GDP. RAS superfamily GTPases have similar conformations in the two nucleotide states, but have distinguishable changes in the switch I and switch II regions. It is through these nucleotide induced changes that specific interaction between effectors and regulators are achieved. These GTPases are found in all eukaryote organisms ranging from the most primitive protist to humans. The RAS superfamily is divided into five main functional families of GTPases, which are called Ras, Ran, Rab, Arf/Sar and Rho. One factor that is common to all members of the RAS superfamily is a structurally conserved G domain that facilitates nucleotide binding and hydrolysis through the interaction with GTPase regulatory proteins. (Bourne et al., 1991; Sprang, 1997; Takai et al., 2001; Leipe et al., 2002; Wennerberg et al., 2005).

Four of the main functional families in the RAS superfamily have been identified in *Arabidopsis thaliana* (Ran, Rab, Arf/Sar and Rho), with a total of 93 genes encoding monomer GTPases (Vernoud et al., 2003). However, the Ras family is absent in plants, as well as Rho sub-family members like RHO and CDC42 GTPases. This suggests that plants have evolved unique ways of regulating certain cellular processes compared to yeast and mammals (Winge et al., 1997). The Rho family in *Arabidopsis thaliana* consists of 11 RAC-like GTPases (AtRAC) and has an overall homology with the RAC (Ras related C3 botulinum toxin substrate) subfamily of yeast and animals. This plant specific Rho family has also been named Rho related proteins from plants (ROP) (Zheng and Yang, 2000; Yang, 2002).

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The *Arabidopsis thaliana* Rho family can be further divided into two main groups (I and II); group II (AtRAC7/ROP9, AtRAC8/ROP10 and AtRAC10/ROP11) has only evolved in vascular plants. Group II RAC/ROP GTPases differ from group I RAC/ROP GTPases in that they contain an additional exon at the 3′ end of the gene. This has resulted in a loss of a C-terminal prenylation motif, CaaL (a: aliphatic amino acid), which is characteristic of group I GTPases (Winge et al., 2000). However, group II GTPases have retained a C-terminal cysteine-containing motif. AtRAC7/ROP9 and homologues from dicots and monocots constitute a distinct group with a monophyletic origin, in which some members have evolved a new C-terminal prenylation motif, CTAA. This could indicate post-translational modification of the protein with farnesyl (farnesylation) instead of geranylgeranylation, which is probably the case for most of the group I AtRAC/ROP GTPases (Nambara and McCourt, 1999; Lavy et al., 2002). This lipid modification is important for membrane targeting of Rho GTPases and for the interaction with RhoGDIs.

Functional studies have shown that plant RAC/ROP GTPases coordinate numerous downstream processes in plants such as hormone responses, cell growth and differentiation, pathogen defence, stress responses (reviewed by Gu et al. (2004) and Brembu et al. (2006)). Plant RAC/ROP GTPases have possibly also evolved and adapted functions that normally are served by Ras family proteins in animalia (Winge et al., 2000). Intriguingly, this has probably resulted in plants evolving novel mechanisms for regulating RAC/ROP GTPases and transmitting signals via RAC/ROP GTPases. To support this notion, a novel family of guanine nucleotide exchange factors (GEFs) was discovered in *Arabidopsis thalina*. GEFs facilitate the otherwise slow dissociation of bound GDP that leads to the subsequent activation of GTPases by the binding of GTP. These GTPase regulating proteins in plants have been named RopGEFs and contain a novel plant specific nucleotide exchange domain named plant specific ROP nucleotide exchangers (PRONE) (Berken et al., 2005; Gu et al., 2006). The intrinsic hydrolysis of GTP within the G-domain of RAS superfamily is slow and is accelerated through the interaction with GTPase activating proteins (GAPs). The RhoGAP domain that facilitates accelerated GTPase activity is found in proteins from many organisms such as yeast, plants and mammals (Scheffzek et al., 1998). In plants, however, we find a novel combination of the GAP domain and a CDC42/RAC-interactive domain (CRIB) that in combination seem to enhance GTPase activity (Wu et al., 2000). Finally, guanine nucleotide dissociation inhibitors (GDIs) form a group of regulatory proteins that inactivate GDP bound Rho family GTPases by sequestering them from the plasma membrane into the cytosol and preventing activation by GEFs. In a recent study, *Arabidopsis thaliana* RhoGDI1 has been shown to spatially regulate the growth pattern in root hair cells, possibly through RAC/ROP mediated activation of a plant NADPH oxidase (Carol et al., 2005). A novel family of RAC/ROP downstream targets named ROP-interactive CRIB motif-containing proteins (RICs) exists in plants. These proteins function as RAC GTPase targets, which control RAC dependent pathways in plants (Wu et al., 2001). AtRAC11/ROP1 has been shown to affect tip growth in pollen tubes by regulating/balancing actin assembly and disassembly through downstream interaction with RIC4 and RIC3, respectively (Gu et al., 2005). AtRAC2/ROP7, an ancient member of RAC/ROP family in plants, has been implicated as a possible regulator of secondary cell wall development of xylem vessels (Brembu et al., 2005).

OsRAC1, an *Oryza sativa* (rice) orthologue of AtRAC7/ROP9, has emerged as a key activator of downstream defence processes upon elicitor mediated signalling. Interestingly, a constitutively activated mutant of OsRAC1 shows increased resistance to rice bacterial blight disease, due to an increased formation of reactive oxygen species (ROS) and subsequent cell death (Kawasaki et al., 1999; Ono et al., 2001). Nonetheless, no clear link between plant defence and small GTPases, similar to what is found in rice, has been reported in *Arabidopsis thaliana*.

Extensive structural studies have been performed on human members of RAS superfamily GTPases, their regulators and downstream effectors (reviewed by Vetter and Wittinghofer (2001) and Dvorsky and Ahmadian (2004)). No structural studies of the RAS superfamily of small GTPases have been conducted in plants so far. Here, we present the first structure of a small GTPase from plants. The *Arabidopsis thaliana* RAC7/ROP9 is an unique plant RAC/ROP GTPase that has evolved only in flowering plants (Anthophyta), and are not found in conifers or mosses. Homologues of AtRAC7/ROP9 can be found in monocotyledonous and dicotyledonous plants. In this study, we present the 1.78 Å crystal structure of AtRAC7/ROP9 bound to GDP and explore its structure in comparison to Rho GTPase crystal structures from humans.

2. Results and discussion

2.1. The overall AtRAC7/ROP9-GDP structure

Comparison of the four molecules in the crystallographic asymmetric unit showed that when super positioned, two pairs of monomers, molecules A and D (RMS deviation for Cα atoms of 0.29 Å) and molecules B and C (RMS deviation of 0.37 Å), were structurally more similar than the others. For other combinations, the RMS deviation was in the range of 0.44–0.67 Å. As the full extent of only molecule B is visible in electron density, the subsequent analysis is based on this monomer. The four molecules are organized into two dimers (Fig. 1), where the two molecules in each dimer form interactions in the insert region. The accessible surface area lost for one monomer upon dimerization was calculated by AreaIMol

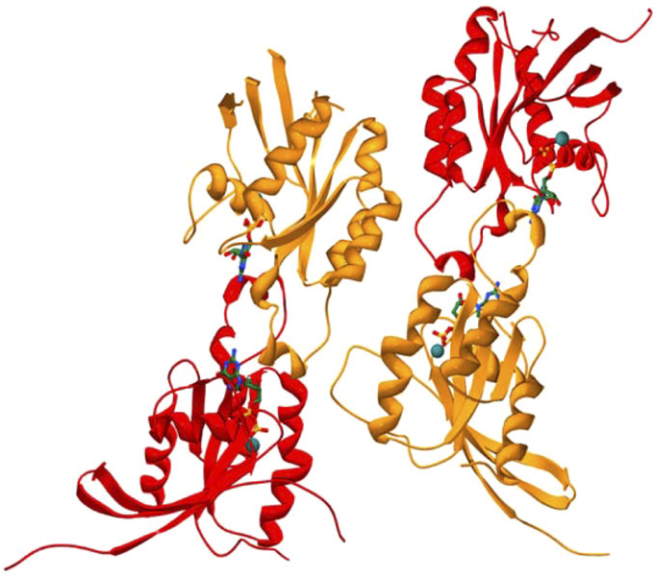

Fig. 1. The four AtRAC7/ROP9 molecules in the crystallographic asymmetric unit organized into two dimers. GDP is shown in stick representation and Mg is depicted as a green sphere. The figure was made using GlaxoSmithKline Swiss-PdbViewer v3.7 (http://ca.expasy.org/spdbv/), and visualized using the ray tracer program POV-Ray™ version 3.6.1.icl8.win.32 (http://www.povray.org). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

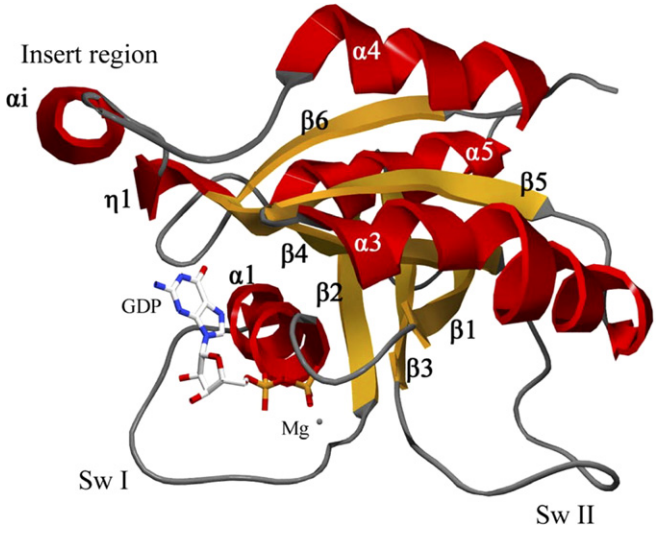

Fig. 2. The overall structure (ribbon representation) of AtRAC7/ROP9 (residues 1–181, molecule B) bound to GDP (stick representation) and Mg (grey sphere). The α-helices are in red and β-strands are shown in yellow. Secondary elements, are labelled as insert and switch (Sw) regions. αi denotes the insert helix, with the 3_{10} (η1) helix at the beginning of the insert region also indicated. The figure was made using GlaxoSmithKline Swiss-PdbViewer v3.7 (http://ca.expasy.org/spdbv/), and visualized using the ray tracer program POV-Ray™ version 3.6.1.icl8.win.32 (http://www.povray.org). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

(CCP4, 1994) to be around 650 Å^2, which is only about 7% of the total accessible surface area for one monomer. This suggests that the dimer formation may be an artefact of crystallization. Furthermore, gel filtration and attempts of cross linking AtRAC7/ROP9 with EGS showed that in solution AtRAC9/ROP9 is a monomer in solution (Results not shown).

The truncated AtRAC7/ROP9-GDP-Mg (amino acids 1–181) structure shows the basic RAS GTPase α/β fold (Fig. 2) and consists of a hydrophobic core with a six-stranded β-sheet (β1–β6), surrounded by four helices (α1–α5). Crystal structures of human RHO, CDC42 and RAC GTPases report two 3_{10}-helices (α2) in the loop between β-strands 3 and 4 (the switch II region). The AtRAC7/ROP9 crystal structure presented here shows no helical structures in switch II. The Rho family is unique in having an insertion between β-strand 5 and α-helix 4. This insertion forms a helical structure, and is referred to as the insert helix (αi). AtRAC7/ROP9 has a four amino acid deletion in the insert region compared to members of the human Rho family GTPases, but still retains a small insert helix. Similar to all Rho GTPases, AtRAC7/ROP9 has a 3_{10}-helix (η1) at the beginning of the insert region.

During construction of the expression vector, an amino acid substitution has occurred in position 3 in the primary structure. Native alanine has been substituted with valine. The position and nature of the substitution should not affect the overall structure.

Human Rho family GTPases overlaid with AtRAC7/ROP9 (Fig. 3) show a Cα RMS deviation of 0.87 Å compared with HsRHOA-GDP (pdb: 1FTN, 150 common atoms), 0.97 Å compared to HsCDC42-GDP (pdb: 1A4R, 151 common atoms) and 1.02 Å compared to

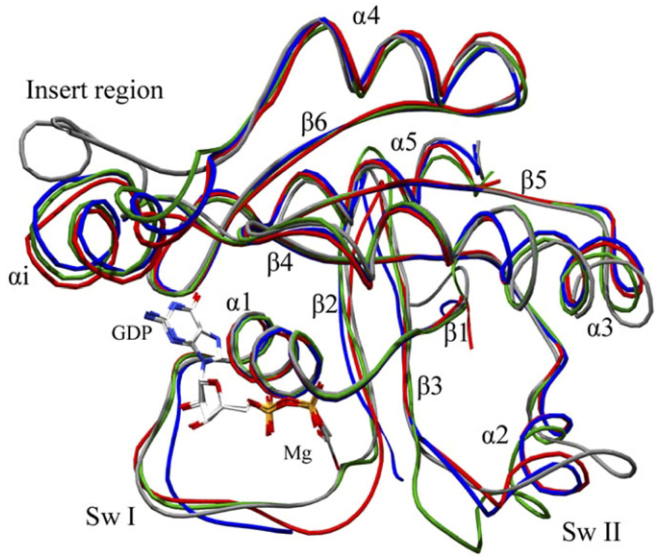

Fig. 3. The superposition of C_α-atom tracing of AtRAC7/ROP9 is shown in gray, HsCDC42 (pdb: 1A4R) in red, HsRHOA (pdb: 1FTN) in green and HsRAC3 (pdb: 2C2H) in blue. Visible secondary elements have been labeled, as well as insert and switch (Sw) regions. The figure was made using GlaxoSmithKline Swiss-PdbViewer v3.7 (http://ca.expasy.org/spdbv/), and visualized using the ray tracer program POV-Ray™ version 3.6.1.icl8.win.32 (http://www.povray.org). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

HsRAC1-GMPPNP (pdb: 1MH1, 144 common atoms). Structurally, the core of the GTPase α/β fold shows lower RMS deviation value than more exposed parts of the structure. The highest RMS deviation between human Rho GTPases and AtRAC7/ROP9 is observed in switch I/II and in the insert region.

2.2. The nucleotide binding site

Guanine nucleotides are bound to GTPases through highly conserved sequence motifs that are universal for the RAS superfamily (Wennerberg et al., 2005). These sequence motifs are termed G1–G5; the nucleotide binding motifs are for the most part conserved between human and plant Rho GTPases (Fig. 4). In AtRAC7/ROP9, the phosphate binding loop (G1) is represented with ^{13}GDGAVGKT^{20}, which is identical to human RAC and CDC42. H-bonds between the G1 residues are identical in AtRAC7/ROP9 and HsRAC2-GDI (pdb: 1DS6) with a threonine ($Thr20_{AtRAC7}$) side chain coordinating Mg^{2+}. The G2 region is located within the switch I region of GTPases and is represented by another highly conserved threonine ($Thr38_{AtRAC7}$) residue that is involved in binding Mg^{2+}. The G3 sequence is recognized by a conserved Dx_2G sequence at the N-terminal of the switch II. The sequence of G3 in AtRAC7/ROP9 (^{60}DTAG^{63}), HsRAC, CDC42 and RHOA are identical. The G4 region in AtRAC7/ROP9 is represented with ^{118}TKLD^{121} and is identical to human RAC homologues, where $Asp121_{AtRAC7}$ contacts the N1 and N2 atoms of guanine. The G5 motif shows more variation within the Rho family, and the G5 motif in AtRAC7/ROP9 (^{155}ECSSK^{159}) differs from human Rho GTPase homologues (ECSAL in HsRAC1-2 and CDC42). G5 binding of the guanine ring is achieved through main chain interactions and may explain the observed variation. The G5 region in plants contains an invariant serine ($Ser158_{AtRAC7}$) where the hydroxyl of this serine side group forms an additional H-bond to the guanine ring via a water molecule. In addition to the above contacts, $Asp125_{AtRAC7}$ from a neighbouring monomer contacts the guanine N2 atom, which may be an artefact of crystal packing. Mg^{2+} coordination within the structure shows a classical Rho GTPase-GDP octahedral conformation with three water molecules, with the hydroxyl group of Thr20 as the fourth water molecule in the equatorial plane. The apical interactions are with Thr38 within switch I and the β-phosphate of the guanine nucleotide.

2.3. Switch I

The switch I region is well defined in all four monomers. The core primary structure of switch I in AtRAC7/ROP9 (^{35}YIPTFDNF^{43}) is highly conserved between plant Rho GTPases and their human counterparts. This motif contains the invariant threonine ($Thr38_{AtRAC7}$) essential for Mg^{2+} coordination. Residues within this sequence also have the highest molecular contact frequencies with effectors and regulators of human Rho GTPases (Dvorsky and Ahmadian, 2004). The N-terminal flank of this sequence in AtRAC GTPases contains possibly functionally significant substitutions. Residues $^{33}TD^{34}_{AtRAC7}$ are conserved in higher plants, whereas human Rho GTPases show more variation. Despite this variation, an acidic and/or polar residue seems to be found in the two residues, and $Glu31_{HsRAC2/HsCDC42}$ is also involved in molecular interaction with various effectors and regulators ($p67^{phox}$, TIAM and PAK, ACK, respectively) (Dvorsky and Ahmadian, 2004). Human RHO GTPases have an aliphatic residue in this position. Further towards the N-terminal of switch I, AtRAC7/ROP9 has a surface exposed lysine (Lys30) that is specific for group II AtRAC/ROPs, whereas group I AtRAC/ROPs have a preserved threonine (Thr30).

2.4. Switch II

The switch II region, which is comprised of residues 62–80 in AtRAC7/ROP9, is generally flexible in the four molecules, and only in molecule B can the whole region be modelled into electron density due to favourable intermolecular interactions. The structure shows no helical conformation in the switch II region, a finding that has also been reported for solution structures of HsCDC42-GDP and HsCDC42-GMPPCP (Feltham et al., 1997). A helical conformation has been reported in both the crystal structure of HsRAC1-GMPPNP (Hirshberg et al., 1997) and a recently deposited crystal structure of HsRAC3-GDP (pdb#: 2C2H). These contradictory observations reflect the conformational differences between solution and crystal states of the switch II region. However, solution studies of the backbone dynamics in CDC42 indicate that the switch II region exists as a relatively ordered structure that changes between two or more structures on a millisecond timescale (Loh et al., 1999).

AtRAC7/ROP9 and other plant RAC7/ROP9 homologues have a conserved serine ($SerS68_{AtRAC7}$) in switch II, as compared to an aspartic acid ($Asp65_{HsRAC1}$) found in human Rho GTPases. This substitution might be disruptive to the formation of a helical structure in this region, as the side chain of $Asp65_{HsRAC1}$ generates ion–pair interactions with $Arg68_{HsRAC1}$ and with $Lys96_{HsRAC1}$ in the adjacent α3-helix (Fig. 5). Similar flexibility to what is observed in AtRAC7/ROP9 is also observed in the corresponding region in the crystal structure of H-RAS. In H-RAS, this is due to substitutions in the α3-helix compared to human Rho GTPases, where $Lys96_{HsRAC1}$ and $Glu100_{HsRAC1}$ are responsible for interactions that are important for the conformation of the switch II region (Pai et al., 1990; Ihara et al., 1998). The increased flexibility in the switch II region of AtRAC7/ROP9 may facilitate novel interaction mechanisms for regulating RAC7-like GTPases and signal transmission in plants.

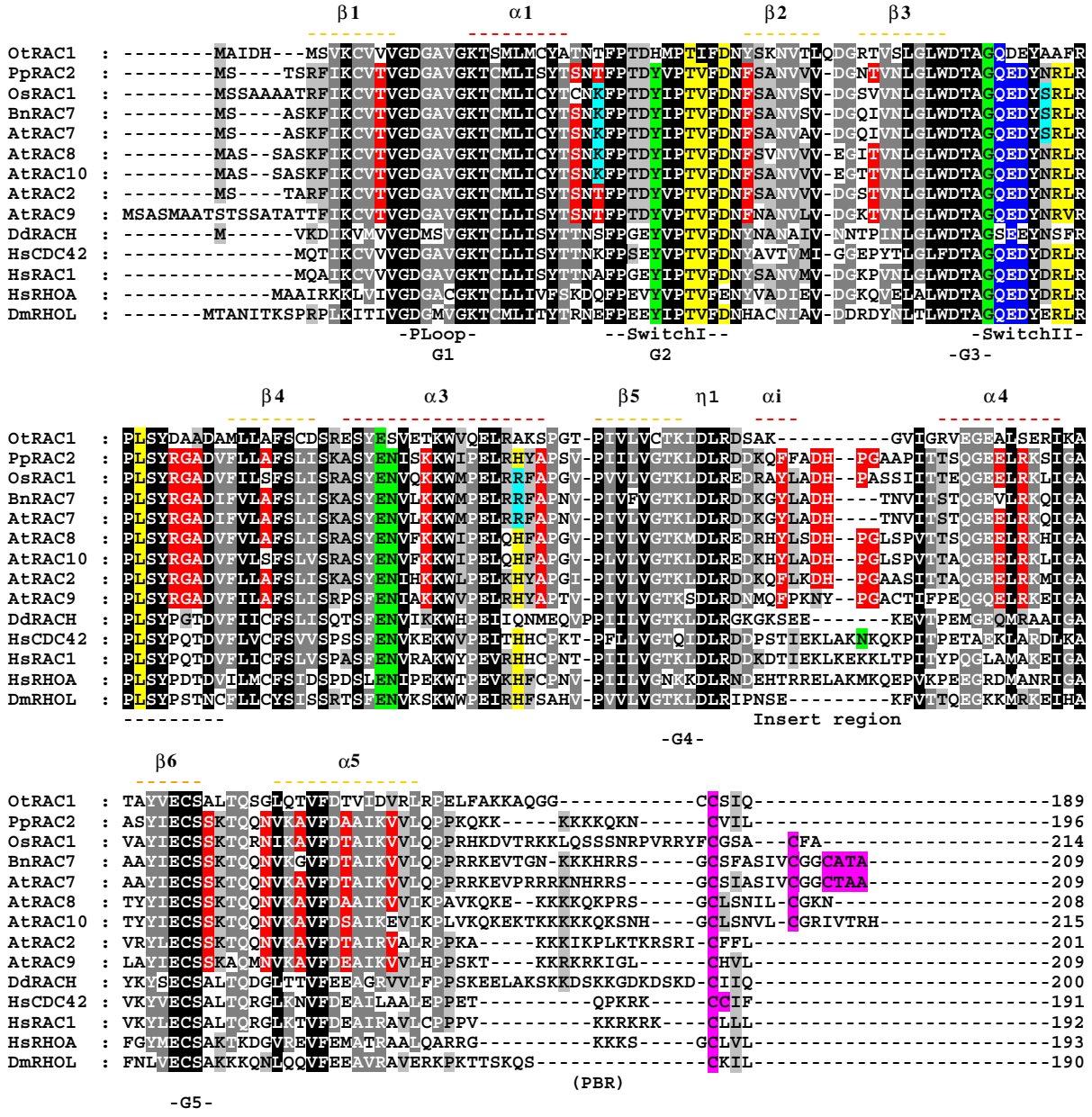

Fig. 4. An alignment of selected AtRAC/ROP proteins with homologues from different species: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum* (slime mold); Hs, *Homo sapiens*; Os, *Oryza sativa* (rice); Ot, *Ostreococcus tauri* (green algae); Pp, *Physcomitrella patens* (moss). Residues highlighted in yellow interact with GDI, green residues with GAP and blue interact with both GDI and GAP. Residues that are unique for plant RAC/ROPs are highlighted in red. A light sky blue background indicates group II or RAC7/ROP9 homologues specific residues. A magenta background indicates possible sites for lipid modification. Secondary structure elements for AtRAC7/ROP9 are noted on the top and bottom of the alignment. G domain sequence motifs are noted at the bottom of the alignment. (Accession numbers for the GTPases in the alignment can be found in materials and methods.) (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Insert region

Rho family GTPases are unique in having an 11–13 amino acid insertion between β-sheet 5 and α-helix 4 (Asp125-Ile136$_{AtRAC7}$), forming an aliphatic helical structure followed by a loop segment. This region is well defined in the crystal structure, mainly due to hydrophobic packing of the aliphatic part of the insert regions between the structures (A–B, C–D). Solution structures of HsCDC42, however, portray the region as a dynamic structure that is independent of the bound nucleotide (Feltham et al., 1997). The same structural independence of the insert region from the nucleotide binding state is also observed in the crystal structure of HsRHOA (Wei et al., 1997; Ihara et al., 1998).

In plants, RAC-like GTPases have an insert region with a two amino acid deletion, whereas AtRAC7/ROP9 and homologues in the *Brassicaceae* family are unique in hav-

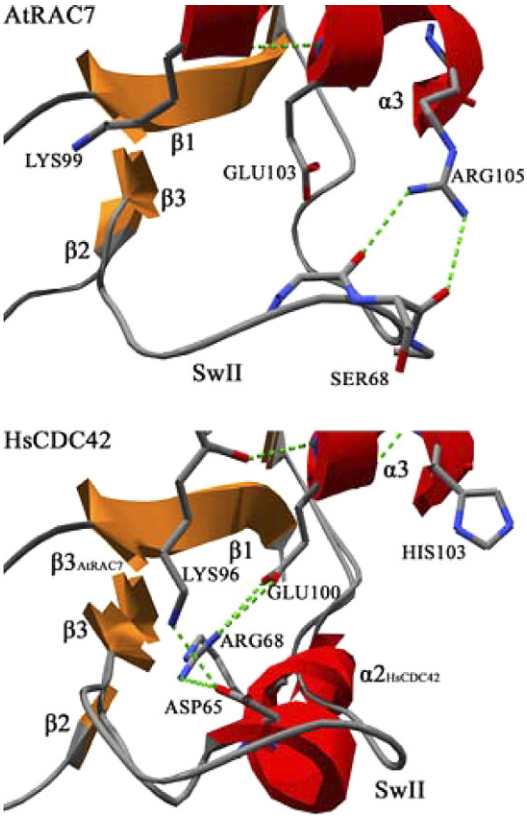

Fig. 5. The switch II region of AtRAC7/ROP9 (molecule B) and HsCDC42 (PDB: 1A4R) are shown, respectively. In addition to the ribbon presentation of secondary structures within and adjacent to the switch II region of each GTPase, side chains of key residues are also shown. Hydrogen bonds are shown with green dotted lines. In molecule B of AtRAC7/ROP9, the switch II region is partially stabilized by interactions between the backbone of switch II and Arg105 in α3. In HsCDC42 the Asp65 and Arg68 residues in switch II make stabilizing interactions with Lys96 and Glu100 in the adjacent α3 helix, which may be crucial for formation of a helical structure. The figure was made using GlaxoSmithKline Swiss-PdbViewer v3.7 (http://ca.expasy.org/spdbv/), and visualized using the ray tracer program POV-Ray™ version 3.6.1.icl8.win.32 (http://www.povray.org). (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

ing a four amino acid deletion compared to human counterparts (Winge et al., 2000). The primary structure of the insert region seems to be partially conserved in plants, starting with a 3_{10} helix that is observed in both plant and human Rho family GTPases. The N-terminal part of the insert, K/Rx_1x_2, forms a short 1 turn helix, where x_1 and x_2 are Glu and Phe for group I plant RAC/ROPs. For group II RAC/ROPs, x_1 is more variable (G/H/R/S/A) and x_2 is preferably a Tyr (see Fig. 4). The following loop region apparently has a plant specific, partially conserved sequence motif, which is ^{131}DHPG$^{134}_{\text{AtRAC2}}$. Both groups I and II RAC/ROP have a mostly complete DHPG sequence, except for plant AtRAC9/ROP8 and AtRAC7/ROP9 homologues. Overall, the insert region seems to be more variable in plants than what is observed in human Rho GTPases, thus indicating that this motif could be under relaxed evolutionary selection. The precise function of the insert region is still unclear in both plant and animals, and no proteins have been reported to interact with the region so far. However, we cannot rule out the possibility that the variation observed in primary structure is necessary for the differentiation between plant RAC/ROPs and interacting partners, or that it has essential functions during interactions with multi subunit protein complexes. It has been suggested that the insert region of RAC GTPases from human serves as a binding interface for downstream effectors, particularly those important for actin regulation (Thapar et al., 2002). The insert region is conserved in most Rho GTPases, suggesting a specific function, but there are exceptions such as *Drosophila melanogaster* RHOL and *Dictyostelium discoideum* RACH, which have deletions in the insert region. Additionally, all nine RAC genes in the alveolate *Paramecium tetraurelia* lack the insert region (http://paramecium.cgm.cnrs-gif.fr/, Accession numbers can be found in Section 4).

3. Conclusion

Our comparative analysis of the AtRAC7/ROP9 structure shows that the AtRAC7/ROP9 structure is similar to related human Rho family GTPases, and that for the most part, amino acids participating in the interaction between Rho GTPases and GAP, GDI and CRIB domains are conserved between plant Rho GTPases and human counterparts (Fig. 4). However, the recent discovery of a novel plant GEF domain shows that novel interaction mechanisms most likely have evolved in higher plants.

The AtRAC7/ROP9 structure shows interesting structural differences in two regions of the G-domain. Firstly, the αi helix in plants seems to be smaller than in human counterparts. Since no function has yet been assigned to the insert region in general, we chose not to speculate further as to whether the observed differences are significant or not. The switch II region in AtRAC7/ROP9 is, however, unequivocally more flexible in AtRAC7/ROP9 than in human counterparts. This flexibility is most likely due to an invariant serine residue (Ser68$_{\text{AtRAC7}}$) in plant RAC7/ROP9 homologues that prevent the formation of a stable secondary structure in switch II. The increased flexibility of the switch II region may be important for function and specificity in protein interactions and invite the speculation that the AtRAC7/ROP9 may have evolved to facilitate a necessary specific interaction in certain pathways, possibly interacting with novel plant proteins. Further investigation is required to elucidate these unresolved questions.

4. Materials and methods

4.1. Engineering of the recombinant expression vector

For the structure determination of *Arabidopsis thaliana* RAC7/ROP9 (GeneBank Accession # At4g28950), a con-

struct coding for residues 1–209 (wild-type) were amplified using PCR, integrating an NdeI (3′) and an EcoRI (5′) restriction site. The product was cloned into pET28a (Novagen) *Escherichia coli* expression vector. A new stop codon was introduced in the reverse primer to truncate the expression product 28 amino acids upstream from the native stop codon. The truncated region includes the poly basic region (PBR) and the part of the protein undergoing post-translational modifications. This flexible region was removed to facilitate formation of crystals. The fusion peptide with 6 times Histidine and a trombine protease cleavage site was positioned at the N-terminus of the recombinant protein.

4.2. Expression and purification

An *E. coli* BL21 (DE3) codon$^+$ strain (Stratagene) was used as an expression host. An expression was done by inoculating 1 l 2 × YT media containing 2% (v/v) Glucose, 50 μg ml^{-1} Kanamycin, 34 μg ml^{-1} Chloramphenicol with 2–4 ml of overnight culture. Cells were grown to an A_{600} of 0.8–1.0 at 37 °C and temperature was lowered to 28 °C. The cells were induced by adding isopropyl-β-1-thio-galactopyranoside to a final concentration of 0.35 mM. Cells were harvested after 4 h by centrifugation before storage at −80 °C. Cell pellets were resuspended in lysis buffer (50 mM Tris–HCl pH 7.2; 250 mM NaCl; 5 mM $MgCl_2$; 10 mM imidazole) and lysozyme was added to a final concentration of 1 mg ml^{-1}. After 30 min incubation on ice, Triton-X100 was added to the lysis solution to a final concentration of 1% (v/v), and DNAse and RNAse were added to reduce the viscosity of the sample. Finally, β-mercaptoethanol to a final concentration of 20 mM was added and the insoluble fraction was removed by centrifugation (20 min at 20,000*g*).

Before performing affinity chromatography, the sample was filtered through a 0.2 μm filter (Sarstedt). Immobilized metal (Ni^{2+}) affinity chromatography was conducted using a 5 ml Ni sepharose column (GE Healthcare) equilibrated with run buffer (50 mM Tris–HCl, pH 7.2; 250 mM NaCl; 5 mM $MgCl_2$; 10 mM imidazole; 4 mM β-mercaptoethanol). Lysate with recombinant protein was applied to the column and bound protein was eluted using a stepwise imidazole gradient to 500 mM imidazole. Recombinant AtRAC7/ROP9 was eluted at approximately 300 mM imidazole. Fractions containing recombinant AtRAC7/ROP9 was dialyzed against 2 l thrombine cleavage buffer (20 mM Tris–HCl, pH 7.5; 150 mM NaCl; 5 mM $MgCl_2$; 2 mM $CaCl_2$; 1 mM DTT) at 4 °C for 2 h. Any precipitation was then removed before human thrombine (Novagen) was added to approximately 0.5–1 U mg^{-1} of recombinant protein. Enzymatic removal of the His_6-tag was done basically as described in Smith and Rittinger (2002). After removal of the His_6-tag the sample was dialysed for 2 h against 2 l of cation exchange buffer (20 mM MES, pH 6.3; 10 mM $MgCl_2$; 2 mM DTT). The sample was then loaded onto a column packed with 6 ml Resource 15S media (GE Healthcare). Recombinant AtRAC7/ROP9 was eluted in a linear NaCl gradient. For final purification the protein was gel filtered through a Hi-prep 26–60 Sephacryl S200 (GE Healthcare) column. Fractions containing recombinant AtRAC7/ROP9 were concentrated to >10 mg ml^{-1} and stored at −20 °C. This approach yielded ample amounts of pure recombinant AtRAC7/ROP9 bound to GDP.

The Hi-Prep 26–60 gel filtration column was calibrated using a LMW Gel Filtration calibration kit (GE Healthcare) to ascertain the molecular conformation of AtRAC7/ROP9 in solution.

4.3. EGS cross-linking

Buffer exchange from a Tris-based buffer to a HEPES-based buffer was performed using a HiPrep 26/10 desalting column (GE healthcare). AtRAC7/ROP9 was concentrated to approx 5 mg ml^{-1} and EGS cross linker (Pierce) dissolved in DMSO was added to final concentrations of 0.25 mM, 0.5 mM and 1 mM. Solutions were then incubated for 40 min at RT. Samples were run on SDS-PAGE to visualise any possible dimerization of AtRAC7/ROP9 in solution.

4.4. Crystallization

AtRAC7/ROP9 was crystallized using the hanging drop vapour diffusion method. The best crystals were grown by mixing 2 μl drops of an 11 mg ml^{-1} protein solution and a solution containing 50 mM BisTris, pH 6.5; 10 mM $MgCl_2$; 5 mM DTT; 200 mM KCl; 10% (w/v) polyethylene glycol (PEG) 2000 and 10% (w/v) PEG 3350. The drops were equilibrated at room temperature, with crystals generally appearing within a month. These crystals were of sufficient quality for data collection. 10% (v/v) glycerol added to the reservoir solution sufficed as a cryo-protectant for flash-cooling crystals in liquid nitrogen. A data set (see Table 1) was collected at the macromolecular crystallography beamline BL14.1 at BESSY, with a final resolution of 1.78 Å.

4.5. Structure determination and refinement

The data were indexed, integrated and scaled using the XDS program package (Kabsch, 1993), before they were converted to structure factors using the CCP4 program TRUNCATE (CCP4, 1994). The data collection statistics are presented in Table 1. The crystals were monoclinic, with unit cell parameters of $a = 76.8$ Å, $b = 30.2$ Å, $c = 139.4$ Å and $\beta = 100.1°$. The lack of systematic absences in the data set collected identified the space group as *P*2. The solvent content was estimated to be around 37%, with a Matthews Coefficient of 1.9 Å^3 kDa^{-1}, assuming four protein molecules per asymmetric unit. The crystal structure of AtRAC7/ROP9 was determined by molecular replacement methods using PHASER (Mccoy et al., 2005),

Table 1
Data collection and refinement summary

A. Data collection	
Resolution range (Å)	38.18–1.78 (1.88–1.78)
Number of unique reflections	61,628
Redundancy	4.1 (4.1)
R_{merge} (%)[a]	8.5 (44.5)
Completeness (%)	100.0 (100.0)
Mean $I/\sigma(I)$	12.3 (3.2)
B. Refinement statistics	
R-value (%)	18.7
Free *R*-value (%)	22.7
Deviation from ideal geometry	
Bond lengths (Å)	0.017
Bond angles (°)	1.633
ESU (Å)[b]	0.087
Average *B* values (Å^2)	
Main chain atoms (A/B/C/D/All)	17.6/15.1/20.6/20.9/18.5
Side chain atoms (A/B/C/D/All)	20.5/17.0/23.0/23.5/21.1
GDP (A/B/C/D/All)	10.8/9.3/14.4/12.2/11.7
Mg^{2+} (A/B/C/D/All)	10.0/9.7/12.7/11.6/11.0
Water molecules (296)	25.9
All atoms	19.9
Ramachandran plot (%)	
Most favoured	90.3
Additionally allowed	8.8
Generously allowed	1.0

Numbers in parenthesis are for the resolution shell 1.88–1.78 Å.

[a] $R_{merge} = (\sum_h \sum_i |I_i(h) - \langle I(h)\rangle|)/(\sum_h \sum_i I(h))$, where $I_i(h)$ is the *i*th measurement of reflection *h* and $\langle I(h)\rangle$ is the weighted mean of all measurements of *h*.

[b] Estimated overall coordinate error from REFMAC5 based on maximum likelihood.

using the crystal structure of human RAC1 (1MH1; Hirshberg et al., 1997) as the search model. Automated model building with ARP/wARP (Perrakis et al., 1999) including all reflections to 1.78 Å built 649 out of a possible 736 amino acid residues into electron density, where 641 residues were correctly assigned to the sequence. After a manual intervention using O (Jones et al., 1991), the model was refined in REFMAC5 (Murshudov et al., 1999). Subsequent cycles of refinement interspersed with manual rebuilding gave final R_{work} and R_{free} values of 18.7% and 22.7%, respectively, with acceptable protein geometry. The final model of AtRAC7 consists of a total of 699 amino acid residues describing the four monomers in the asymmetric unit comprising 178, 184, 159 and 178 residues from monomers A to D, respectively. Each AtRAC7/ROP9 monomer binds GDP; a magnesium ion and 296 additional water molecules have been modelled. The coordinates have been deposited in the Protein Data Bank with accession codes PDB **2j0v** along with the structure factors. For an overview of the refinement statistics, see Table 1.

4.6. Accession numbers

Fig. 4 (from top to bottom): CR954206.2; AAD26198; BAA84492; CD827872; At4g28950; At3g48040; At5g62880; At5g45970; At2g44690 AAG45133; NP_008839; NP_426359; NP_001655; AAB05666.

Paramecium tetraurelia: CAI44493.1; CAI44536.1; CAI44570.1; CAI39257.1; CAI39324.1; CAI39295.1; CAI44517.1; CAI44551.1; CAI39268.1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.08.011.

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