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Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in *Arabidopsis thaliana*

Thesis for the degree of Philosophiae Doctor

Trondheim, January 2011

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Never memorize what you can look up in books.

- Albert Einstein

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Last but not least my wonder woman Carina deserves a lot of thanks for support, patience and soon we will begin a new chapter of our lives together.

Christopher G. Sørmo

Trondheim, January 2011

Biology has at least 50 more interesting years.

- James Dewey Watson, Dec 1984

List of papers

Paper I
C. G. Sørmo, I. Leiros, T. Brembu, P. Winge, V. Os and A. M. Bones (2006)
The crystal structure of Arabidopsis thaliana RAC7/ROP9: the first RAS superfamily GTPase from the plant kingdom
Phytochemistry **67**: 2332-2340

Summary:

In this study we aimed to produce and purify recombinant AtROP9 to achieve adequate amounts for structural studies. Purified AtROP9 bound to GDP was then crystallized and a three-dimensional structure was obtained in collaboration with the Norwegian structural biology center (NorStruct). AtROP9 was chosen for this work since it is a unique plant specific RAC-like GTPase that has only evolved in flowering plants. Structural analysis showed that like human Rho GTPases, AtROP9 is based on an evolutionarily conserved G domain architecture. However, the AtROP9 structure shows some structural distinctness in the Rho GTPase defining insert region and the protein interacting switch II domain. The switch II region of AtROP9 is more flexible since it does not adopt a helical structure like human Rho GTPases do. The observed flexibility is most likely due to a serine residue that is conserved among plant AtROP9 orthologs and could be of importance for function and specificity in protein interactions. This opens the possibility for AtROP9 having evolved to facilitate specificity in certain signaling pathways in plants.

Paper II
C. G. Sørmo, T. Brembu, P. Winge and A. M. Bones (2011)
***Arabidopsis thaliana* MIRO 1 and MIRO 2 GTPases are unequally redundant in pollen tube growth and fusion of polar nuclei during female gametogenesis**
In press (PLoS One)

Summary:

We set out to find functional role(s) for Arabidopsis MIRO GTPase 2 during plant development. Our studies revealed that whereas *miro2* mutants do not display any observable phenotypes, additional loss of function in AtMIRO2 significantly enhances the *miro1* mutant phenotypes. In comparison to the *miro*1 mutant, the *miro1/miro2* double mutant showed increased defects in gametophyte development and function. Phylogenetic analysis showed that eudicot plant MIRO1 and MIRO2 GTPases cluster in two distinct subgroups and that they are gene orthologs that most likely originated due to a genome duplication event. These findings show that AtMIRO2 has retained a fraction of the ancestral gene function and that loss of function in AtMIRO2 is only phenotypically visible in a *miro1* background. Consequently we conclude that MIRO1 and MIRO2 GTPases are genetically unequally redundant in plant development and that this fact should be taken into consideration in future investigations of plant MIRO function.

Abbreviations

ABA	abscisic acid
ABP	actin binding protein
AFH	Arabidopsis formin homology
ARP	actin-related protein
BTB	Broad-Complex, Tramtrack and Bric-a-Brac
CBG	calcium-binding GTPase
CBK	calcineurin B-like protein
CCR1	cinnamoyl-CoA reductase 1
CDC42	cell division cycle 42
CPK	calcium-dependent protein kinase
CMT2A	Charcot-Marie-Tooth disease
CRIB	Cdc42/Rac-interactive binding
DRM	detergent resistant membrane
Drp1	dynamin-related protein
EGF	epidermal growth factor receptor
GAP	GTPase-activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine di-phosphate
GEF	GDP/GTP exchange factor
GPI	glycosylphosphatidylinositol
GRIF-1	GABA receptor interacting factor
GTP	Guanosine tri-phosphate
HUMMR	hypoxia up-regulated mitochondrial movement regulator
IAA	indole acetic acid
ICR1	interactor of constitutive active ROP
IP3	inositol-3-phosphate
IRSp53	insulin-receptor substrate p53
KHC	kinesin-1 heavy chain
mDia	mammalian diaphanous
Mfn2	mitofusin2
MIRO	mitochondrial Rho GTPase
NAP	Nck-associated protein
NADPH	nicotinamide adenine dinucleotide phosphate

OIP106	O-linked N-acetylglucosamine transferase interacting protein
PAK	p21 activated kinase
PBR	polybasic region
PDGF-β	platelet-derived growth factor receptor
PH	pleckstrin homology
PINK1	PTEN-induced putative kinase 1
PIP2	phosphatidylinositol-3,4-diphosphate
PIR	121F-specific p53 inducible RNA
PRONE	plant specific ROP nucleotide exchanger
PtdIns P-K	phosphatidylinositol monophosphate kinase
RAC	Ras-related C3 botulinum toxin substrate
RACK1	receptor for Activated C-kinase 1
Ras/RAS	rat sarcoma viral oncogene homologue
RBK	ROP binding kinase
Rboh	respiratory burst oxidase homologue
REN	ROP Enhancer
Rho	Ras homologous
RIC	RAC/ROP-interactive CRIB motif-containing protein
RIP1	ROP Interactive Partner 1
RLCK	receptor-like cytoplasmic kinases
RLK	receptor like kinases
ROCK	Rho associated coiled coil making protein kinase
ROP	Rho of plants
ROS	reactive oxygen species
TSM	triton-x soluble membrane
VAN	vascular network
WASP	Wiskott-Aldrich syndrome proteins (WASP)
WAVE	WASP family verprolin-homologous protein

Introduction

The Ras superfamily of GTPases

A large proportion of proteins that bind and utilize guanine nucleotides (GTPases) are characterized as binary molecular switches, being in an activated conformation when bound to guanosine triphosphate (GTP) and inactivated by hydrolysis of GTP to guanosine diphosphate (GDP). Among the more evolutionary conserved groups of GTPases is the Ras superfamily of GTPases which is found in all eukaryotes studied to date. Ras superfamily GTPases are instrumental in relaying intracellular signals that ultimately change cell behavior in eukaryotes.

The Ras superfamily of GTPases has evolved immensely in eukaryotes and were until recently classified into five functionally distinct families; Ras, Rab, Rho, Ran and the Arf family [1]. However the recent discovery of mitochondria associated GTPases adds a "new" GTPase family to the Ras superfamily. Initially discovered through a database search for novel proteins with Rho consensus motifs, these proteins where classified as Rho GTPases and was therefore named as mitochondrial Rho GTPases [2]. Phylogenetic analysis shows that Miro GTPases branched out before Rho GTPases and therefore constitute a Ras family of their own. This is further supported by the overall sequence divergence of Miro GTPases from Rho family GTPases and their lack of the Rho family specific insert region [3-4].

The six families of Ras superfamily GTPases (including Miro) have each evolved to regulate specific signaling tasks in cell life: Ras GTPases regulates cell proliferation, Rho GTPases regulate actin organization/gene regulation/cell cycle progression, Rab and Arf GTPases regulate vesicle trafficking, Ran GTPases regulate nuclear trafficking/cytokinesis and Miro GTPases regulate mitochondrial transport and morphology (Figure 1) [1, 5].

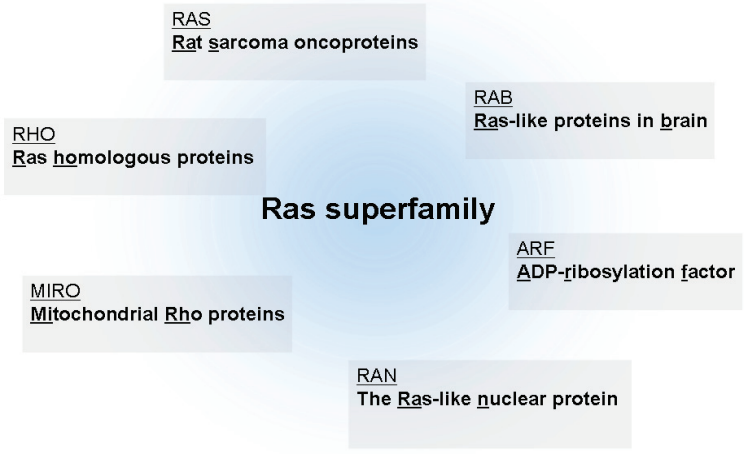

Figure 1: Overview of the Ras superfamily in eukaryotes and their nomenclature

The Rho family of GTPases

Rho family GTPases are only found in eukaryotes and have primarily expanded in the metazoan lineages. A recent phylogenetic analysis of 26 eukaryotic genomes showed that members of this family fall into 4 distinct clusters (I-IV) with 8 subfamilies; (I) Rho, Rnd and RhoD/RhoF, (II) Rac/RhoG, CDC42/RhoJ/RhoQ and RhoU/RhoV, (III) RhoH and (IV) RhoBTB1-2 (Table 1). The presence of all 8 eight subfamilies have to date only been found in mammals. However, Rac-like GTPases are found in all organisms ranging from yeast to animals. Indeed, the diversification of Rho family GTPases is thought to have originated and evolved from an ancestral Rac-like GTPase [4]. It should be mentioned that Miro GTPases most likely also originates from this ancestral RAC-like GTPase and that phylogenetic analyses may have placed Miro GTPases in the root together with other divergent RHOs due to long branch attraction (P. Winge personal communication). Nonetheless, most Rho family GTPases distinguish themselves structurally from other Ras-like GTPases by having an insertion of 13 amino acid residues between the fifth β strand and the fourth α helix in the G domain which is suitably called the Rho insert region. Typical Rho family GTPases are small monomeric proteins (20-40 kD) containing a G domain that facilitates the switching mechanism. Rho family GTPases undergo post-translational modification at the C-terminal with isoprenoid lipids, which is important for membrane targeting and thus the function of the proteins. RhoBTBs are an exception regarding domain composition by containing two BTB domains in addition to the G domain. RhoBTBs, together with RhoU/V are, not subject to post-translational modification with isoprenoid lipids [3].

Table 1: The Rho subfamilies in eukaryotes

Rho family			
I	**II**	**III**	**IV**
Rho **Rnd** (Rnd for "round" [6]) **RhoD/RhoF**	**Rac/RhoG** (Ras related C3 botulinum toxin substrate) **CDC42/RhoJ/RhoQ** (Cell division control protein 42 homolog) **RhoU/RhoV**	**RhoH**	**RhoBTB** (BTB: Broad-Complex, Tramtrack and Bric-a-Brac [6])

Rho family GTPase cycle: activation/inactivation

The intrinsic GTPase activity of Rho family GTPases is slow, and inactivation of the G domain by hydrolysis of GTP to GDP is accelerated by GTPase activating proteins (GAPs) (Figure 2). Activation of the G domain by dissociation of GDP and the subsequent binding of GTP are accelerated by guanine nucleotide exchange factors (GEFs). In the GTP bound state the G domain is in an activated conformation and thus able to interact with downstream effectors[7]. Rho and Rab-family GTPases are regulated by a third class of regulatory proteins in addition to GAPs and GEFs, the guanine nucleotide dissociation inhibitors (GDIs). Rho-GDIs act on Rho GTPases in three way: (1) they prevent the dissociation of GDP by GEFs and maintain the inactive conformation of the G domain, (2) GDIs can also interact with the GTP-bound form of the G domain, preventing both intrinsic and GAP-mediated GTP hydrolysis and simultaneously preventing interaction with downstream effectors, and (3) GDIs regulate the cycling of Rho family GTPases between the membrane and cytosol, thereby inactivating the GTPase by sequestering it in the cytosol [8].

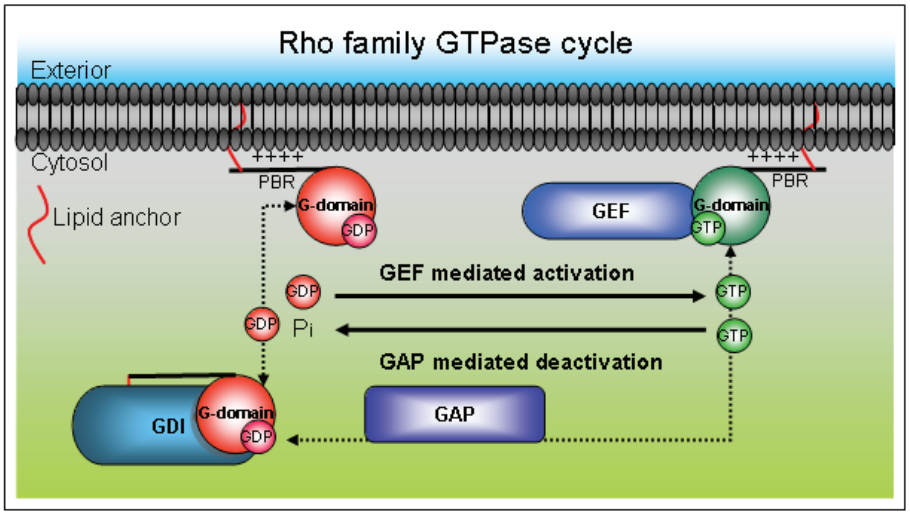

Figure 2: A schematic overview of Rho family G domain activation and inactivation by Rho regulatory proteins. The Rho G domain is anchored to cellular membranes via a lipid modification on the C-terminal. The C-terminal polybasic region (PBR) contains positively charged basic residues that interact with negatively charged membrane lipids to further enhance membrane association.

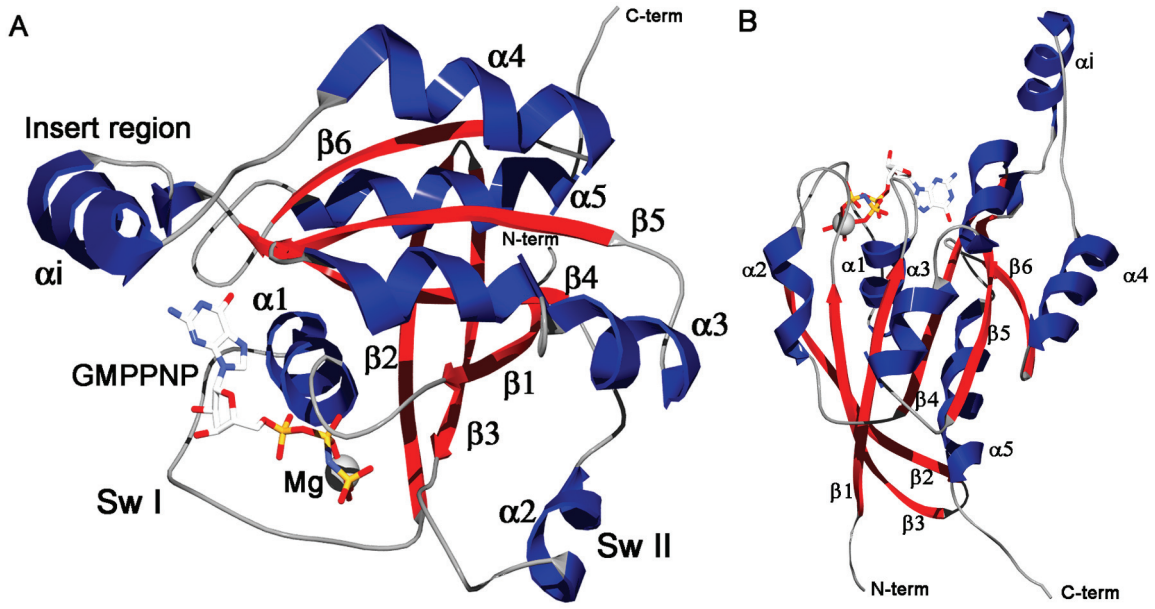

Figure 3: The overall structure of human Rac1 [9] (pdb: 1MH1) bound to a non-hydrolysable form of GTP, guanosine-5'-(beta gamma-imino)triphosphate (GMPPNP). A-B) The 1-5 α-helices (blue) surrounds the six β-sheets (red) that together constitute the G domain. The αi helix between β-sheet 5 and α4-helix defines the Rho specific insert region. The switch regions are labelled Sw I and Sw II. B) Different perspective of the human Rac1 crystal structure. Note the α2 helix in the switch II region and the αi helix protruding from the G domain.

Rho family G domain structure

The G domain fold (Figure 3A-B) is a conserved protein domain shared by Ras superfamily members, heterotrimeric G protein α-units and translation elongation factors. The G domain itself consist of a hydrophobic core with a six stranded β-sheet surrounded by hydrophobic loops and α-helices (alpha/beta structure). Five loops of the G protein structure are instrumental in GDP- to GTP-exchange, GTP-induced conformational change and GTP-hydrolysis. These polypeptide loops are designated G-1 to G-5 and contain highly conserved sequences that define the G domain protein superfamily (Figure 4). The G1 region or P-loop connects the β1 strand with α1 helix and is recognized by a GxxxxGK(S/T) motif (aka Walker A motif) that contacts the α- and β-phosphate on the guanine nucleotide through main-chain interactions and the amino group of the conserved lysine residue. The G2 region is found at the N-terminal of the β-2 strand and the preceding loop; the sequence motif of this region is highly conserved within each GTPase family. The G2 sequence contains a conserved threonine residue involved in Mg^{2+} coordination which is important for GTP hydrolysis.

The G3 motif (aka Walker B motif) at the N-terminal of the α2 helix contains a DxxG sequence where the conserved aspartate binds Mg^{2+} through a water molecule and the conserved glycine binds to the γ-phosphate of GTP. The G4 region is recognized by a conserved NKxD motif found between the β-5 strand and the α-4 helix. The conserved aspartate contacts the guanine ring of the nucleotide. The G5 region located between the β-6 strand and the α-5 helix and has a E(A/C/S/T)(C/S)A(K/L) motif. This motif supports guanine base recognition mainly trough main chain interactions, hence the observed variation in primary structure.

It should be noted that the Walker A and B motifs are also found in other nucleotide binding proteins that are not G-protein homologues [10-11].

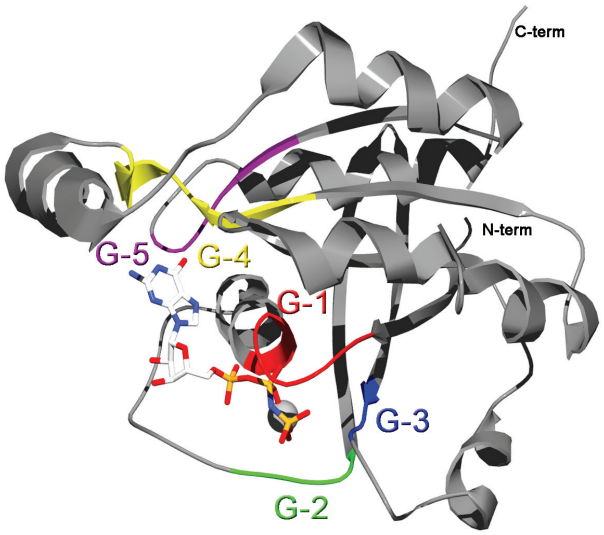

Figure 4: The position of the polypeptide loops G1 to G5 in human Rac1 (pdb: 1MH1) that contains the highly conserved sequence motifs that are universal for the Ras superfamily of GTPases.

The switch mechanism and G domain protein-protein interactions

In most cases, the structural differences between the GTP and GDP bound states within members of the Ras superfamily are small and for the most part confined to two sections called the switch regions (switch I and II)(Figure 3A). The switch I region is identical to the G2 loop of the G domain. This section is also referred to as the effector loop. The switch II section is comprised of the G3 and the following α2-helix. The switch regions show large variation in the GDP conformation, whereas the GTP conformation is more ordered and similar between Ras superfamily members [7, 11]. The γ-phosphate oxygen of GTP is

positioned through hydrogen bonds to invariant Thr and Gly residues in switch region I and II respectively. The conformational change upon GTP binding has been described as a loaded spring mechanism where hydrolysis of GTP releases the switch regions into a more "relaxed" state [7].

An apparent feature of small GTPases is their ability to interact with different proteins in both the GDP and GTP state. Structural analyses of Rho proteins in complex with their respective regulators and effectors have revealed that all proteins interacting with the G domain do so mainly through the switch regions. Both regulators and effectors bind to conserved elements of the flexible switch regions and are able to adopt different conformations upon protein interactions. Regulators that interact with the "relaxed" GDP form of the G domain induce substantial induced fit on the switch regions, whereas interaction with GAP and effectors do not induce large changes upon interaction. The malleability of the switch regions thus enables a single G domain to adopt conformations that complements binding sites of various regulators and effectors [12].

The Rho insert region

The key structural feature that distinguishes Rho family GTPases from other members of the Ras superfamily is a 13 amino acid helical insertion between β-5 and α-4 (Figure 3 A-B), which has solvent exposed and highly charged structure. The amino acids that constitute the region are different in the Rho, Rac and CDC42 subfamilies, which imply that the insert region is important for specificity towards Rho, Rac and CDC42 targets, respectively. In contrast, structural studies show that the insert region is not influenced by the nucleotide state of the G domain [13].

Studies performed on human Rac1 without the insert region showed that the remaining G domain retained all its intrinsic GTPase functions, performed nucleotide exchange by GEFs and interacted with GDIs. Also, the overall structure was not affected in the absence of the insert region. However, the insert-less Rac1 was not able to induce lamellipodia formation in NIH3T3 cells, thus implying a role for the insert region during Rac1-mediated regulation of the actin cytoskeleton [14]. Several studies have also showed that the insert region is important for Rac1-mediated activation of Nox-family NADPH oxidases [15-17]. In contrast to these findings, a recent study showed that activation of Nox-family NADPH oxidases are not dependent on the insert region. Observations reported in previous studies may be due to

the removal of the insert helix, which leads to incorrect protein folding, subsequently resulting in reduced effector binding and activation capacity. The same study also showed that the insert region of Rac1 is not necessary for proper membrane targeting [18].
RhoA has been shown to bind with Rho kinase in an insert independent manner, although kinase activation however seems to be dependent on the insert region [19]. RhoA is also shown to directly contact its target mDia, a formin related protein, partially through the insert region [20]. Similar to RhoA activation of Rho kinase, CDC42 activation of phospholipase D1 (PLD1) is dependent on the insert helix and a serine residue in the insert is critical for activation of PLD1 [21].

Post-translational modification and membrane localization of Rho GTPases

An important biochemical feature of most of the Rho family GTPases (as well as other Ras superfamily members) is post-transitional modification by lipids, which is important for correct sub-cellular membrane localization (Figure 2). Rho GTPases have a C-terminal C*aaX* motif (C; Cys. *a*; aliphatic side group, *X*; any amino acid) which is recognized by either farnesyltransferase and/or geranylgeranyltransferase type I. The transferases covalently attach a farnesyl or a geranylgeranyl isoprenoid to the cysteine of the C*aaX* motif. Next the *aa*X is proteolytically cleaved off and finally the prenylated cysteine is methylated. In addition, some Rho GTPases (RhoB, RhoQ) undergo post-translational modification at one or two cysteine residues upstream of the C*aaX* motif by covalent addition of the fatty acid palmitate [22].
In addition to lipidation, another sequence element plays a secondary role for membrane association and is therefore functionally important. Preceding the C*aaX* motif, clusters of adjacent lysine or arginine residues make up the so called polybasic region (PBR). Positively charged basic residues in PBR interact with negatively charged phosphatidylinositide lipids and provide additional specificity to the overall membrane association of Rho GTPases (Figure 2) [23-24].

Biological functions of metazoan Rho GTPases

Metazoan Rho GTPase have been studied extensively because of their pivotal roles in fundamental processes of cell biology and some Rho GTPases are implicated in cancer development which naturally has triggered extensive research on their role in this context. Most of the biochemical and structural knowledge acquired to date is based on the highly conserved Rho GTPases; RhoA, Rac1 and CDC42. The main strategy for elucidating biological function of Rho GTPases has come from studying the effect of overexpression of dominant negative and constitutive active forms of Rho GTPases in various model systems. CDC42 has a conserved role in regulating the actin cytoskeleton and cell polarity through activation of several downstream effectors. CDC42 induces formation of filopodia by activating actin related protein 2/3 (ARP2/3) through the Wiskott-Aldrich syndrome protein (WASP), Insulin-receptor substrate p53 (IRSp53) kinase and the mammalian formin diaphanous-2 (mDia2).

Rac regulates the actin cytoskeleton and the formation of lammellipodia by activating ARP2/3 through WASP-family verprolin-homologous protein (WAVE) complex and possibly through mDia2.

Both Rac and CDC42 regulate actin polymerization by activating p21-activating kinase (PAK) that phosphorylates LIM-kinase (LIMK), which inhibits cofilin and subsequently regulates actin turnover. In neuronal development both CDC42 and Rac are instrumental in regulating the actin cytoskeleton to promote axon growth, filopodia formation and axon guidance respectively. Rac is also instrumental in membrane ruffling and lammellipodia formation that facilitates phagocytosis. During phagocytosis of bacteria, reactive oxygen species are produced to facilitate bacterial killing, and Rac is important both for assembly and activation of the NADPH oxidase complex on the phagosome membrane.

Rho subfamily GTPases are involved in stress fiber formation through the actin cytoskeleton and also regulate endocytotic vesicle trafficking. Constitutive activated or dominant negative RhoB changes both endocytotic trafficking of epidermal growth factor receptor (EGF) in epidermal cells and endosomal trafficking of platelet-derived growth factor receptor (PDGF-β) in smooth muscle cells. [Rho GTPase functions in metazoa are reviewed in 25, 26-27].

Mutated forms of Ras GTPases (constitutively activated) are found in 15% of human tumors. The Rho GTPases, on the other hand, are rarely mutated in human cancers, but in some forms of cancer several Rho GTPases show upregulated expression levels and increased activity. Since Rho GTPases are involved in regulation of the cytoskeleton, they most likely assist in

cancer cell migration and invasion. Furthermore, Rho GTPases may affect tumors through regulation of gene transcription, cell division, cell survival, intracellular transport or cell-to-cell interaction [28].

The Rho family of GTPases in plants

In plants only five of the six functionally distinct families of the Ras superfamiliy GTPases have been identified: Rab, Rho, Ran, Arf and the Miro family, with the Ras family being absent in plants. In addition, the Rho family in plants lacks the Cdc42 and Rho sub-families Instead plants have evolved a novel group of Rac-like GTPases that most likely act as *bona fide* signaling molecules to the Ras family and the Cdc42 and Rho subfamilies in metozoan counterparts [29].

The Rac-like GTPases found in plants are most likely from a monophyletic origin, where an ancestral Rac-like gene evolved into plant specific Rac-like GTPases. The Rho and Cdc42 subfamilies on the other hand have only evolved in fungal and metozoan lineages after the split from an ancestral organism that eventually evolved into terrestrial plants [30]. The Rac-like GTPases in Viridiplantae have only recently diversified into new subfamilies/groups. The major diversifications, which occurred in spermatophyta (seed plants) before the split into monocotyledons and dicotyledons, divided the Rac-like GTPases into two major subgroups based on the primary structure of the C-terminal motif. The Group 1 Rac-like GTPases have a geranylgeranylation/farnesylation motif: C*aaX*. In vascular plants, an ancestral Rac-like GTPase gene acquired an additional intron at the far 3' end of the gene, thereby generating a new subfamily of Rac-like GTPases. As a result the the C*aaX* motif was lost, but these genes have retained a cysteine-containing motif, suggesting a different type of C-terminal modification for the Group 2 of Rac-like GTPases. Futhermore, several gene/genome duplications have resulted in the formation of several other distinct sub-groups of Rac-like GTPases in both monocots and dicotyledons [30-31].

Interestingly, the fact that the Ras-family is absent from Viridiplantae may indicate that the Rac-like GTPases in plants have evolved to function in processes that is otherwise regulated by Ras family GTPases in metozoa, fungi, mycetozoa and entamoeba. This may have been a part of the selection pressure in the evolution of a Rac-like multigene family in plants [30].

In the dicotyledonous plant *Arabidopsis thaliana* the genome contains 93 small GTPases belonging to the Ras superfamily (96 members if Miro GTPases are included) [32], of which

11 Rac-like GTPases constitute the Rho family (8 Group I GTPases and 3 Group II GTPases) [29-30]. Mainly two nomenclatures have been used for Rho GTPases in plants since they were first characterized in Pisum sativum (Garden pea) [33]. The terminology ROP (Rho like GTPases from plants) was first used by Lin and colleagues [34] and Winge and colleagues used AtRAC nomenclature based on the protein sequence similarities with human Rac GTPases [29]. For clarity, the more widely used ROP nomenclature will be used henceforth and the corresponding RAC numbering is shown in table 2.

Table 2: AtRAC vs. ROP nomenclature (Group II RACs/ROPs are underlined)

AtRAC1	AtRAC2	AtRAC3	AtRAC4	AtRAC5	AtRAC6	AtRAC7	AtRAC8	AtRAC9	AtRAC10	AtRAC11	Winge et al. [29-30]
ROP3	ROP7	ROP6	ROP2	ROP4	ROP5	ROP9	ROP10	ROP8	ROP11	ROP1	Li et al. [35], Yang et al. [36]

Arabidopsis ROPs: activation/inactivation

Extracellular signals are thought to activate receptor-like kinases (RLK) in the plasma membrane of plant cells. RLKs activate ROPGEFs that in turn activate ROPs through nucleotide exchange (Figure 5).

Plant RLKs generally contain an N- terminal extracellular domain that recognize specific ligands and a C- terminal serine/threonin kinase-like intracellular domain. Upon activation the RLKs mediate signaling to downstream target proteins. The Arabidopsis genome encodes more than 600 RLK family members, including receptor-like cytoplasmic kinases (RLCK). RLKs vary greatly in their domain architecture and are phylogenetically divided into 45 subfamilies based on these differences [37-38]. The abundance and expansion of RLKs in plants is in sharp contrast to human receptor tyrosine kinases (RTK), of which there are 58 known members categorized into 20 subgroups [39].

ROP GTPases play a significant role in establishing cell polarity in various cell types in plants, especially during pollen tube growth. Studies have shown that AtROPGEF12, through its C-terminus, interacts with a pollen-specific receptor kinase; AtPRK2a. A model has been proposed to explain how PRK2a regulate pollen specific ROPGEFs leading to the subsequent activation of ROP (Figure 5). The C-terminal of ROPGEF is thought to act in an auto-inhibitory manner on the nucleotide exchange properties of GEF, preventing an interaction

with ROP. The intracellular kinase domain of PRK2a interacts and phosphorylates an invariant serine residue at the C- terminal domain of ROPGEF. This exposes the catalytic domain of the ROPGEF and facilitates activation of ROP, ultimately leading to polarized pollen tube growth [40].

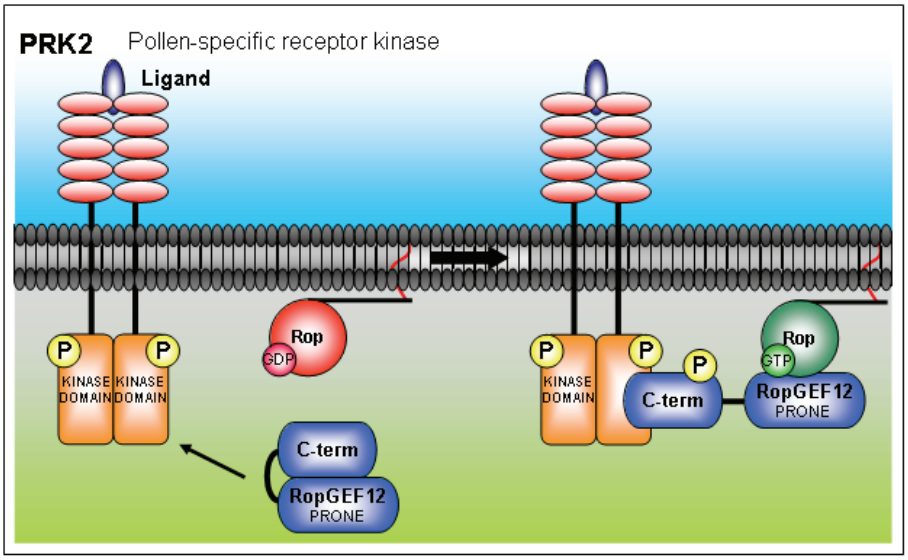

Figure 5: A model suggesting how AtPRK facilitates activation of AtROPGEF12 that subsequently leads to ROP nucleotide exchange. Putatively, the activated PRK kinase domains phosphorylates the C-terminal domain of ROPGEF, which exposes the catalytic GEF (PRONE)–domain [40].

Guanine nucleotide exchange and activation of ROP is facilitated by a plant-specific ROP nucleotide exchanger domain (PRONE) that share no homology with RhoGEFs found in animals [41-42]. In Arabidopsis there are 14 ROPGEFs that share a central PRONE domain. In addition one ortholog to the more widely conserved Dock180 GEF domains exists in Arabidopsis, namely SPIKE1 (Figure 6) [43]. Recently, it was demonstrated that SPIKE1 has GEF activity towards ROPs [44].

The molecular mechanisms behind how the PRONE domain catalyses the nucleotide exchange has been studied extensively. The crystal structure of ROP4•GDP in complex with the PRONE domain of ROPGEF8 revealed that two PRONE domains form a constitutive dimer with two ROPs. The two ROP•GDP GTPases makes contacts with both PRONE domains, which explain why dimerization is necessary for catalytic activity. The structure also gave insight into how GEFs in general weakens nucleotide affinity through removal of the Mg^{2+} ion and through remodeling of the two switch regions in the G domain [45]. Another structural study of PRONE in complex with a nucleotide-free ROP showed that the complex facilitates the interaction between a conserved lysine in the ROP P-loop and a conserved glutamate in switch II. This interaction replaces the P-loop lysine interaction with the β-

phosphate on the guanine nucleotide, resulting in low nucleotide affinity and subsequent loss of GDP. The nucleotide-free state is stabilized by a conserved WW-motif in the PRONE domain, which most likely also promotes the association with GTP. Taken together, these two studies show the molecular steps of the GEF reaction by the PRONE domain [46].

Like yeast and metozoan Rho GTPases, the intrinsic GTPase activity of ROPs is low and is enhanced several fold by plant ROP GTPase activating proteins (ROPGAPs). Compared to yeast and metozoa, where the number of RhoGAPs is 2-3 times larger than the number of RhoGTPases [47], there are relatively few ROPGAPs compared to the number of ROPs in plants. In addition, the diversity of functional domains associated with the GAP domain is greatly limited in plants compared to animals (Figure 6).
In Arabidopsis 9 ROPGAPs exists, which are divided into 2 subfamilies [31]. One subfamily with 6 members of ROPGAPs has an unusual domain composition that is not found in yeast or animals. ROPGAP1-6 has an N-terminal Cdc42/Rac interactive binding (CRIB) domain that in animals usually is associated with Cdc42/Rac effectors like PAK and WASP.
The AtROPGAPs show about 27% sequence homology to RhoGAPs in yeasts and animals, and the GAP domain itself is more similar to p50 RhoGAP, which preferably activates Cdc42 GTPases [48]. The ROPGAP domain is functionally similar to RhoGAPs from yeast and animals by containing a conserved catalytical arginine residue (a.k.a. the arginine finger) that is inserted into the active site that facilitates hydrolysis of GTP [7]. How the CRIB-domain functions in association with the ROPGAP domain is not yet fully known. In Cdc42/Rac effectors like PAK and WASP, the CRIB domain is a part of a larger auto-inhibitory switch domain (IS). Binding of Cdc42/Rac•GTP to these CRIB-domains invokes structural changes to the IS-domain that results in effector activation [49]. *In vitro* studies show that the ROPGAP CRIB domain enhance the GAP activity, demonstrating that the N-terminal part of the ROPGAP containing the CRIB-domain does not inhibit the GAP activity like the PAK and WASP IS domain, but rather that the CRIB domain is necessary for maximum GAP activity. Further studies suggested that the explanation for this observation might be that the ROPGAP CRIB domain assists in the formation of or stabilization of the transitional state of ROP•GTP, which is important for the overall GAP activity [48, 50]. How the GAP and CRIB domains interact together towards ROPs is not known, but several interesting scenarios may exist. Structural studies have shown that the CRIB domain mainly interacts with the β2 and α5 region of Rho GTPase, as well as the switch regions [49, 51]. This fact may allow for the binding of two ROPs to ROPGAP where possibly one ROP binds to the CRIB domain,

recruiting ROPGAPs to the plasma membrane, where the GAP domain deactivates another ROP. The CRIB domain of ROPGAP can also bind to GDP-bound ROP [48] which may function as a negative regulator of the GAP domain. Biochemical and structural studies with ROP in complex with ROPGAP are needed to clarify the function for this plant specific domain combination.

The second group of 3 GAPs in Arabidopsis contains a conserved RhoGAP domain in combination with an N-terminal Pleckstrin homology (PH) domain, which is also observed in several RhoGAPs ranging from yeast to humans [31, 47]. These PH-GAPs have also been named ROP Enhancer (REN) [52]. PH-domains have the ability to bind to various forms of phosphoinositides on the plasma membrane or other structural membranes. Upon activation of cell surface receptors, phosphoinositides act as second messengers, transiently recruiting signaling proteins to the plasma membrane where they perform their function [53]. Several lipid binding domains are found in Arabidopsis, and 53 proteins are predicted to contain a PH domain together with other functional domains including ArfGAP, phospholipase D or phosphatidylinositol 3/4 kinases [54]. Interestingly, ArfGAPs in both mammals and Arabidopsis have a phosphoinositide-dependent activation of the GAP domain through allosteric regulation by a PH domain [55]. The Arabidopsis VAN3 ArfGAP is in addition to being allosterically regulated through the PH domain, deactivated by binding of inositol triphosphate (IP_3) [56]. It remains to be investigated if a similar allosteric regulatory mechanism exists for ROP(PH)GAPs in Arabidopsis.

Rho guanine nucleotide dissociation inhibitors (RhoGDI) are in general single domain proteins and not as abundant as other Rho GTPase regulatory proteins in eukaryotes. In humans only three RhoGDI family members are found, whereas several other eukaryotic organisms have only one copy of RhoGDI, suggesting a more general regulatory role for these proteins [57]. The Arabidopsis genome encodes 3 RhoGDIs. One of the three GDIs, RhoGDI1 is expressed ubiquitously in all plants tissues and stages, but shows especially high expression in pollen during development and germination. The two other RhoGDIs in Arabidopsis (At1g62450 and At1g12070) shows very low expression during sporophytic growth, but relatively high (albeit lower than RhoGDI1) expression in pollen development and germination (data obtained from Arabidopsis eFP Browser [58]). The Arabidopsis RhoGDIs are predicted to have the same domain structure as RhoGDIs in other species, which facilitates binding of the G domain and the C-terminal end containing the PM-interacting prenyl lipid moiety of the GTPase (usually geranylgeranyl). This prevents interaction with the

PM as the lipid moiety becomes shielded in a hydrophobic pocket in the GDI domain. RhoGDI interaction leads to the release of the Rho GTPase from the PM into the cytosol, restricting the Rho GTPase from accessing GAPs, GEFs and downstream effectors.
The N-terminal part of the RhoGDI contains a region that is referred to as the regulatory arm. Upon G domain binding, this region forms a stable helix-loop-helix motif containing residues that form contacts with key amino acids in the switch regions of the GTPase. These interactions can maintain the G domain in either nucleotide conformation and prevent G domain interaction with other proteins. The C-terminal part of the RhoGDI contains an immunoglobulin-like fold (IGL-fold) that upon binding to the Rho GTPase forms a cavity between two β-sheets that is lined with conserved hydrophobic residues that can accommodate the prenyl moiety [57, 59].
The mechanisms behind the subsequent dissociation of RhoGDI from ROP GTPases and the re-association of the ROP GTPase with the PM have not been investigated in plants. However based on biochemical and *in vitro* studies on mammalian RhoGTPase-RhoGDI complexes, the dissociation may involve active displacement by putative proteins (GDFs: GDI displacement factors), membrane lipids and/or phosphorylation of GDI [reviewed in 8].

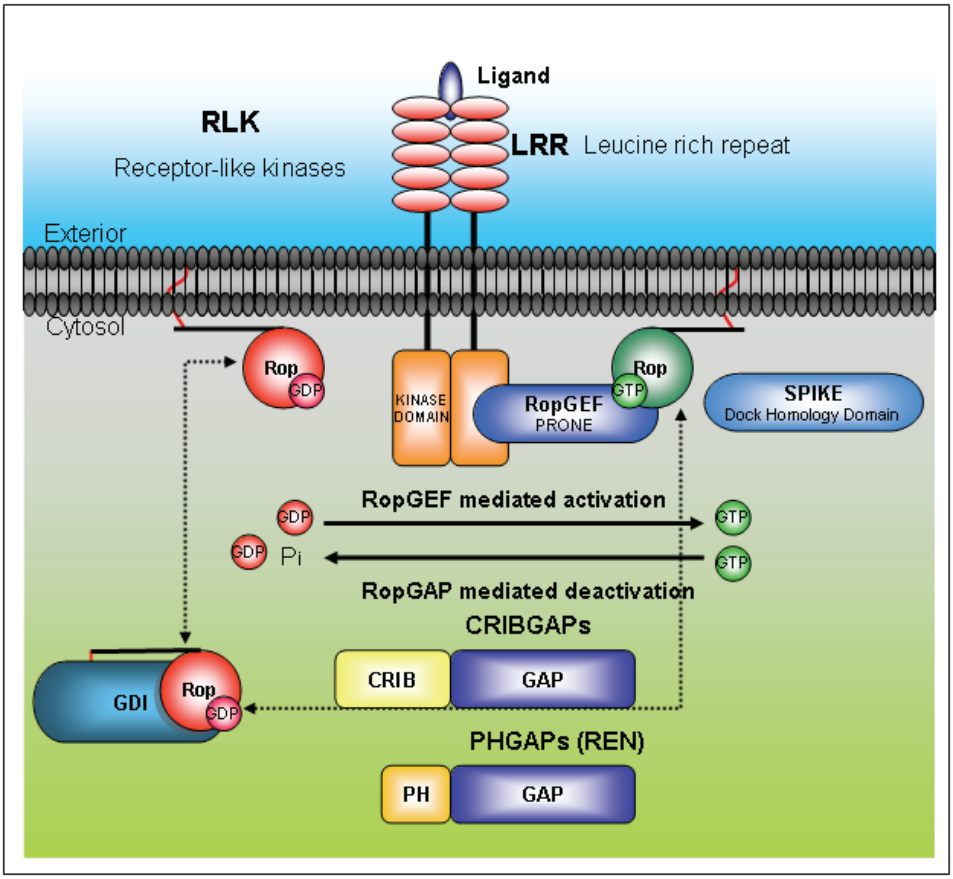

Figure 6: A schematic overview of Rop family G domain activation and inactivation by Rop regulatory proteins in plants.

ROP structure

ROP GTPases, like their orthologs in yeast and animals, are approximately 20-24 kD and consist of around 200 amino acids. Within the ROP primary structure, the highly conserved G1-G5 loops are present in addition to the hypervariable polybasic region and the C-terminal cysteine containing motif. The three dimensional crystal structure resolved for Arabidopsis ROP9 (Figure 7 A-B) shows that the structure of ROPs is based upon a conserved G domain architecture that is basically the same as in human Rho family orthologs. There are, however, some distinct structural features for AtROP9 in switch II region and the insert region (Paper I).

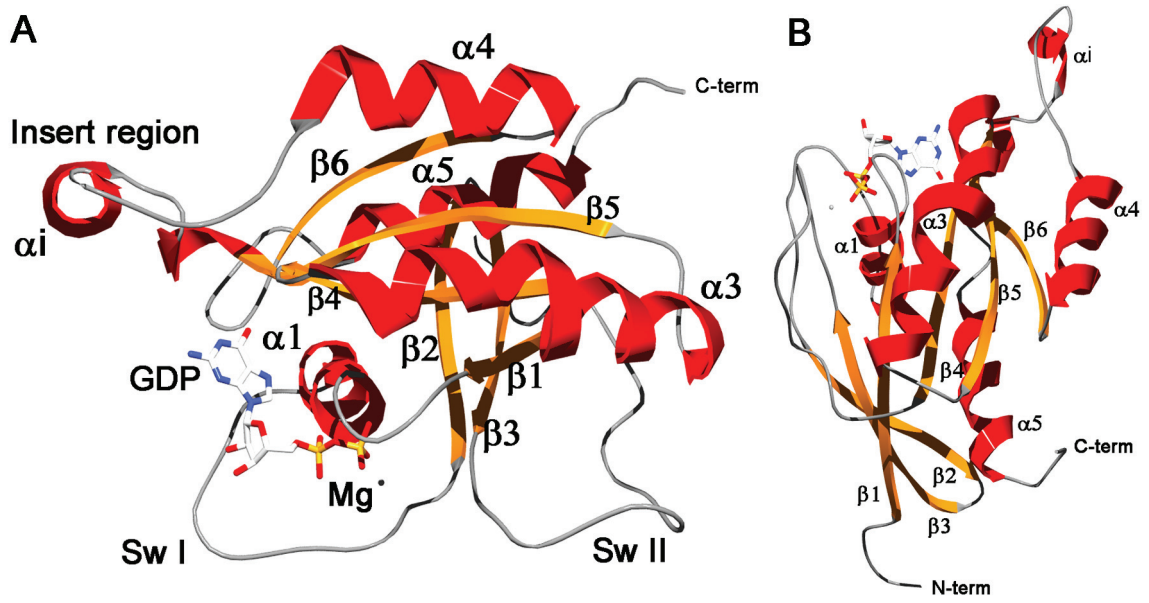

Figure 7: The overall structure of Arabidopsis ROP9 (pdb: 2J0V) bound to GDP. A) The plant G-domain is basically the same as in human Rho family orthologs. In the ROP9 structure, 4 α-helices (red) surrounds the 5 β-sheets (orange) which makes up the G-domain. Note the missing α-2 helix in the switch II (Sw II) region of the ROP9 structure. B) Different perspective of the ROP9 structure. Note the smaller αi helix in the ROP9 structure compared to the human Rac1 structure in Figure 3.

Unlike crystal structures of its human orthologs, the switch II region of the ROP9 crystal structure does not adopt a helical conformation, but a loop-shaped structure (Figure 7). The switch II region could only be modeled into density in one of the four ROP9 molecules in the crystallographic asymmetric unit. Similar flexibility of the switch region has only been observed in solution structures of Rho subfamily GTPases, and this suggests that the switch II region in AtROP9 is more flexible compared to human orthologs (Paper I). However, it has been argued that the observed lack of structure in switch II of ROP9 may be an artifact of molecular interactions within the crystallographic unit [51]. In paper 1, we discuss that a serine residue ($Ser68_{ROP9}$) in switch II, which is conserved in plant ROP9 orthologs, is

disruptive in formation of a helical structure in this region (Figure 8 B). Serine68 in ROP9 substitutes an aspartic acid residue ($Asp65_{HsRAC1}$) in human Rho GTPases and aspargine residue ($Asn68_{ROP5}$) in non ROP9 orthologs in plants. In HsRAC1 (Figure 8 A), Asp65 makes ion pair interactions with $Arg68_{HsRAC1}$ and with $Lys96_{HsRAC1}$ and $Glu100_{HsRAC1}$ in the adjacent α4-helix, which may be crucial for helix formation and stabilization. In a crystal structure of AtROP5, there is a helical structure in switch II, where Asn68 make stabilizing interactions similar to those found in HsRAC1 (Figure 8 C). This supports the idea that the conserved serine residue is disruptive for a helix conformation in switch II. Serine is also considered to have low helix formation propensities compared to aspargine and aspartic acid [60]. The lack of an α-helix in ROP9 switch II results in the region being more flexible than other ROPs and human Rho orthologs. The higher flexibility of switch II in ROP9 is supported by its higher intrinsic GTPase capabilities, where ROP9 showed a 2.5-fold higher intrinsic GTPase activity compared to ROP4 [61].

This flexibility may be important for specificity in downstream effector interactions and suggests that ROP9 orthologs have evolved to perform similar roles in other plants (Paper I).

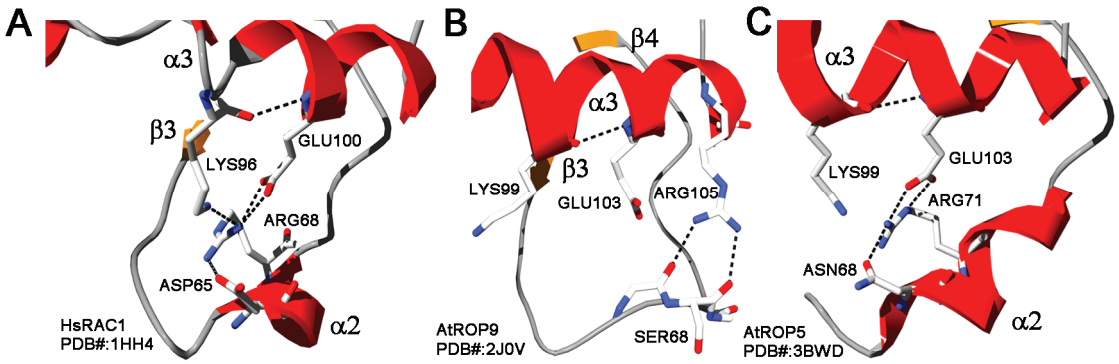

Figure 8: The switch II region and adjacent α3 helix of A) HsRAC1, B) AtROP9 and C) AtROP5. Hydrogen bonds are shown in black dotted lines. Note how ASP65 and ASN68 in HsRAC1 (A) and AtROP5 (C) respectively, forms helix-stabilizing interactions with an arginine residue in the α2 helix and a glutamate residue in the α4 helix. In AtROP9 (B) these helix-forming interactions are most likely disrupted by a conserved serine residue (SER68).

ROP insert region

The insert region in ROPs has a two amino acid deletion compared to their human counterparts, whereas AtROP9 orthologs within the *Brassicaceae* family have a four amino acid deletion [30]. Structurally, the ROP insert consists of less charged amino acids and the AtROP9 insert helix is slightly displaced (Figure 7) compared to human counterparts [51]. Overall the primary structure is more variable in plant ROPs, suggesting that it is necessary for specificity in protein-protein interactions. To date, only a few studies of ROPs and their interacting proteins support a function for the ROP insert helix in protein-protein interaction and effector activation.

The specificity of the ROPGEF PRONE domain towards ROPs is in part attributed to interactions with the insert helix. This was experimentally shown by making a HsRAC1/AtROP4-insert (Asp65Asn/Pro73Arg) chimera that functioned as a true substrate for PRONE8 with acceleration of nucleotide exchange. Notably, the conserved aspargine (Asn68) together with a conserved arginine (Arg76) in the switch II region are also important for substrate specificity and nucleotide exchange. The HsRAC1/AtROP4-insert chimera alone did not accelerate nucleotide exchange; instead, a deceleration of nucleotide exchange below intrinsic rates was observed. This indicates that the PRONE domain binds to the chimera, but that the interaction does not induce structural change in the G domain and thereby actually slows the intrinsic nucleotide exchange. The importance of the insert helix in PRONE specificity was further demonstrated by mutating serine 68 with aspargine in AtROP9, which did not increase the PRONE8 mediated nucleotide exchange above intrinsic rates. The result suggested that binding does not occur, possibly since the shorter AtROP9 insert helix does not structurally fit into PRONE8 [61].

Two receptor-like cytoplasmic kinases named RBK1 and RBK2 (ROP binding kinase, RLCK class VI), together with a cysteine rich receptor kinase (NCRK) have been found to be potential downstream effectors of ROPs in Arabidopsis. Unlike animal and yeast Rho GTPases that interact with kinases through a CDC42/RAC interactive binding (CRIB) domain, plant ROPs seem to interact directly with the kinase domain [62]. A *Medicago truncatula* ROP6^{CA}/HsRAS-insert chimera interacted with MtRRK1 (Rop-interacting receptor-like kinase) in a yeast two-hybrid system, but the MtROP6 chimera could not activate the kinase *in vitro*. The ROP insert region has been implicated in activation of RLCK [63]. Based on currently available data, it seems that the ROP insert region may have evolved to facilitate

protein interaction specificity through activation of the target protein. In theory it could function as a third switch region.

Post-translational modification of ROPGTPases and membrane association

Intracellular localization studies show that ROP GTPases are localized to the plasma membrane due to post-translational modification with hydrophobic side groups.
As mentioned earlier, ROP GTPases are divided into two subgroups based on the primary structure of the C-terminal motif. Group I ROPs have a geranylgeranylation/farnesylation motif; C*aaX* . Group II ROPs have lost their C*aaX* motif but have a cysteine-containing box, GC-CG. The two cysteins are flanked by glycine residues and separated by five to six resides (mostly alipahtic). Both of the cysteins most likely undergo stable S-acylation and results in attachment of acyl lipids that facilitate PM association [64-65].
The cysteine residue in the C*aaX* motif of group I ROPs are primarily prenylated in the cytosol by geranylgeranyltransferase I, which results in a covalently bound geranylgeranyl (C20) isoprenoid lipid. After prenylation, group I ROPs are most likely targeted to the endoplasmatic reticulum where they undergo further C*aaX* processing. The first step involves proteolytic removal of the three last amino acids by C*aaX* proteases and finally the prenylated cysteine is methylated [Reviewed in 66].
Sorek and colleagues have investigated the relationship between the nucleotide state of ROPs and plasma membrane localization. Their studies have revealed that in addition to being prenylated, ROPs are also subjected to transient S-acylation upon GTP binding and activation. Plants overexpressing constitutively activated AtROP6 showed that AtROP6$^{\mathbf{CA}}$ undergoes S-acylation by addition of palmitate (C16) or stearate (C18) to a conserved cysteine residue in the G domain (AtROP6$^{\mathbf{C156}}$). Importantly, the S-acylation resulted in accumulation of AtROP6 in detergent-resistant membranes (DRM), whereas AtROP6$^{\mathbf{CA/Cys156Ser}}$ was accumulated in Triton-X soluble membranes (TSM). These results show that group 1 ROPs are most likely S-acetylated and possibly relocated to PM microdomains (a.k.a. “lipid rafts”) upon GTP binding and activation [67]. Two other highly conserved cysteine residues are found in ROPs and their orthologs (AtROP6$^{\mathbf{C9/C21}}$), and recently it was discovered that a subpopulation of AtROP6$^{\mathbf{CA}}$ are S-acylated at Cys21. Whether S-acylation occurs simultaneously on both cysteins within the G domain or whether there exists two subpopulations of ROPs S-acylated at either cysteine is not known. Functional studies

revealed that replacing the conserved cysteins with serine in ROP6 (AtROP6$^{CA/C21S+C156S}$) reduced the deleterious effect of over expressing constitutively active AtROP6 on cell polarity in leaf pavement cells and root hair growth. Thus, S-acylation appears to be necessary for ROP signaling. Analyses showed that loss of S-acylation increases the membrane-to-cytosol exchange rate compared to WT and ROP6^{CA}, indicating that an S-acylation switch stabilize PM interactions, presumably by directing ROPs into lipid rafts (Figure 9) [68].
Group II ROPs are associated with the PM solely through S-acylation of the two cysteins in the C-terminal GC-CG motif. Compared to group I ROPs, the S-acylation is thought to be stable [65]. The GC and CG pairs are separated by five to six non-polar and hydrophobic residues that may be instrumental in forcing the acyl group into the lipid bilayer, thereby making the S-acylation stable by preventing removal by S-acyl protein thioesterases [69]. Studies of group 2 AtROP10 showed that modification of the GC-CG motif or removal of the adjacent PBR region resulted in accumulation of AtROP10 in the nucleus, showing that both the PBR and GC-CG motifs are necessary for PM localization of group II ROPs [65].
S-acylation of ROPs may prevent interaction and regulation of RhoGDIs since the C-terminal IGL fold of RhoGDI only accommodates prenyl groups. This indicates that activated group I ROPs are not regulated by RhoGDIs and/or that S-acylation of group I ROPs functions as a RhoGDI displacement factor. However group II ROPs, which are exclusively palmiotylated are not regulated by RhoGDIs and are possibly permanently situated in DRMs suggesting that membrane dynamics may be regulated by other plant specific mechanisms [66].

Biological functions of ROPs in plants

Since the first cloning and characterization of a RAC-like GTPase from garden pea back in 1993 [33], the Rho GTPases of plants have received much scientific attention. Most of our knowledge about ROPs originates from research on the model organism *Arabidopsis thaliana*, but functional characterization of ROP GTPases in other plant species have also contributed significantly in our understanding of ROP GTPase function. Cumulatively, the scientific evidence shows that ROPs are pivotal regulators of many important processes in plant cells which will be described below (Figure 9 summarizes some of the known effector interactions of ROPs). Experimentally, ROP function has been studied through reverse genetics using both T-DNA knockout lines and by making transgenic plants overexpressing various forms of the ROP of interest. In particular, overexpressing constitutively active (CA) form of the G domain

where usually a conserved glycine (G15 ROP9) in the P-loop (G1) is usually exchanged with a valine residue that abolishes the GTPase activity of the G domain. The G domain can also be oveexpressed as a dominant negative (DN) form where a conserved threonine (T20 ROP9) in the P-loop is usually exchanged with aspargine, which renders the G domain unable to perform nucleotide exchange [51, 66].

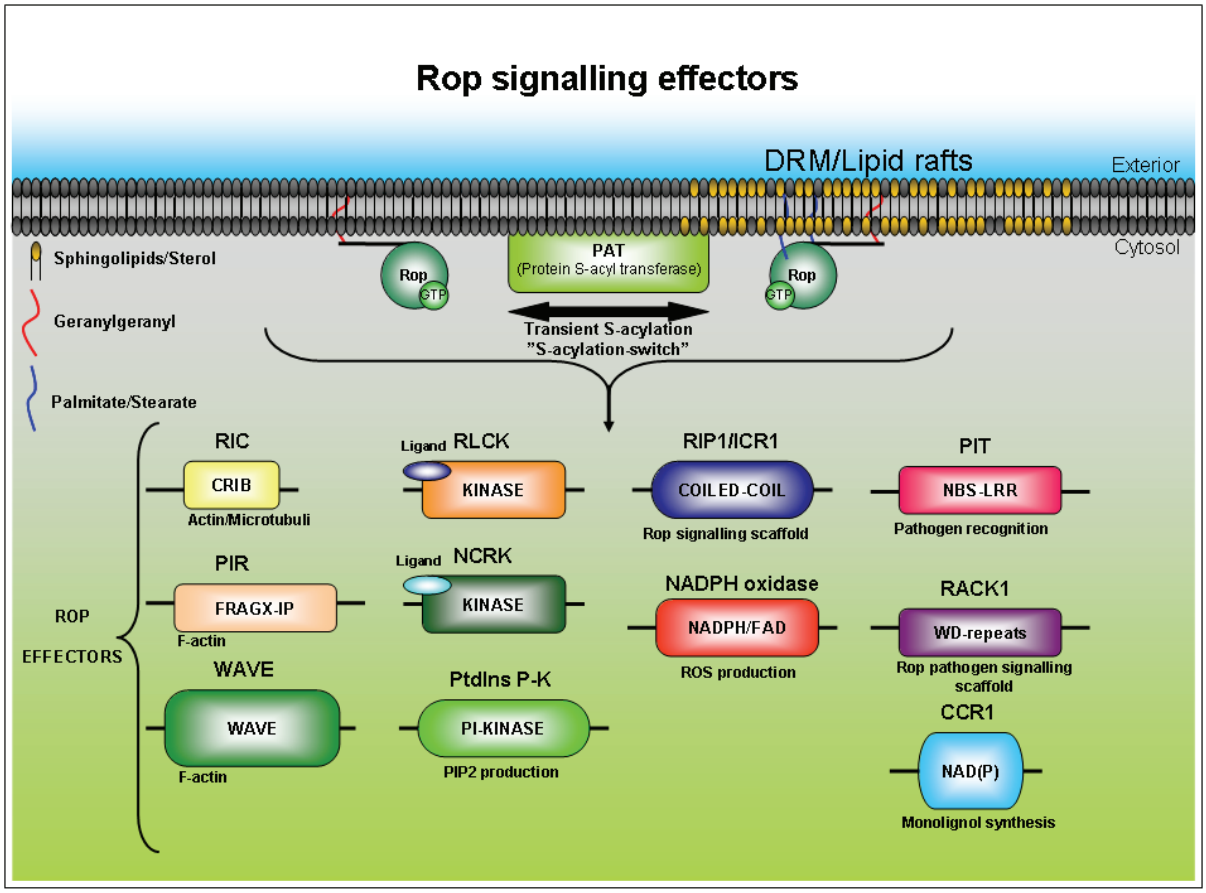

Figure 9: Overview of known plant ROP interacting effectors. Upon activation, ROPs are subjected to transient S-acylation of conserved cysteine residues in both the G domain itself and at the C-terminal of the protein. The S-acylation presumably enhances the PM association of ROPs by directing the protein into detergent resistant membranes (DRMs). The transient S-acylation is necessary during ROP signaling, possibly by allowing interactions between ROPs and DRM residing proteins to occur. Therefore the "S-acylation switch" is most likely an important regulatory step during ROP signaling.

Membrane association of ROPs

Through prenylation and S-acylation, ROPs function is tightly associated with the plasma membrane in plant cells. It is therefore pertinent to mention current theory on PM composition and organization and the possible functional implications this has for proteins associated with the PM in eukaryotic cells. It was originally perceived that protein lipid modification was as a way of recruiting proteins to cell membranes and that the PM itself was a homogenous mix of proteins and lipids.

However, an increasing body of evidence indicates that the PM composition is heterogeneous by forming so called transient lipid rafts or membrane microdomains. PM lipid rafts are hypothesized to be transiently occurring sphingolipid-cholesterol rich assemblies that can recruit specific membrane proteins while excluding other membrane proteins, thereby promoting specific protein-protein interactions. Protein clusters formed in these microdomains are thought to have functional roles connected to the proteins that are embedded in the lipid rafts. Isolation and analysis of detergent resistant membranes (DRMs) reflect the existence and the constituents harboring lipid rafts in PM [reviewed in 70]. Analyses of PM lipid composition in Arabidopsis have shown that like their counterparts in animals and yeast, Arabidopsis DRMs contain significantly higher ratios of sterols and sphingolipids to proteins compared to TSMs. Proteins associated with DRMs were investigated, and a plant ortholog to Flotillin, a mammalian DRM protein, was identified as well as glycosylphosphatidylinositol (GPI) anchored proteins which are also constituents of yeast and animal DRMs. These findings suggests that lipid rafts contain conserved components in eukaryotic cells [71]. Furthermore, DRMs analyzed from tobacco BY-2 cells contained Ras superfamily GTPases like Rab, Arf and more importantly ROPs [72] [for reviews on lipid rafts in plants see 73, 74-75].

As mentioned earlier, group I ROPs associate with DRMs or lipid rafts in an activation dependent manner through transient S-acylation, whereas group II ROPs associate with the PM through stable S-acylation. Transient protein S-acylation (Palmitoylation) of target proteins increase the membrane affinity, but more importantly it allows for spatiotemporal separation of proteins into distinct membrane compartments, presumably lipid rafts. The change in membrane affinity is of functional importance and has an important regulatory role in several cellular processes by modulating target protein activity. In plants, several PM-associated proteins besides ROPs such as heterotrimeric G proteins, calcium-dependent protein kinases (CPKs), calcineurin B-like proteins (CBKs) and tubulin are modulated by

transient S-acylation [review on palmitoylation in plants 76], [review on lipid modification in plants 77].
Current data indicates that group I ROPs function is dependent on transient S-acylation, which may lead to the formation of transiently occurring lipid rafts containing "ROP signaling protein complexes". The so-called S-acetylation switch [68] and the following partitioning into DRMs should therefore be included in the ROP signaling model [77]. Notably, it has recently been demonstrated that polyphosphoinositides are enriched in plant DRMs and that 60% of the phosphatidylinositol 4,5 bisphosphate (PIP2) present in the PM was visualized in approximately 25 nm sized clusters on the surface of the PM [78].

ROPs and polar cell growth

By studying ROP expression in cotton fibers back in 1995, Delmer and colleagues suggested that like their metazoan counterparts ROPs are also involved in cytoskeleton organization [79]. Lin et al. (1996) [34] discovered that Pea ROP1Ps is expressed in the apex of the growing pollen tube. When anti-ROP1Ps antibodies were injected into the pollen tube, growth was arrested, suggesting that ROPs play an important role during pollen tube elongation [35]. Since then, polar tip growth in pollen has become a popular model system for studying ROP-mediated regulation of the actin cytoskeleton as well as other cellular processes regulated by ROPs. Pollen tubes and root hairs exhibit polar growth (tip growth) by vesicle targeting and exocytosis to the growth site [80].
In Arabidopsis, ROP1 is exclusively expressed in mature pollen in addition to a non-exclusive and low expression of ROP3 and ROP5 (Data obtained from Arabidopsis eFP Browser [58]). GFP-ROP fusion proteins have been shown to accumulate in the PM of the growing pollen tube tip. Overexpression of dominant negative (T20N) forms of ROP1 and ROP5 arrested pollen tube growth, whereas overexpression of wild type ROP1 and ROP5 lead to depolarized pollen tube growth. Overexpression of constitutively active (G15V) ROP1 and ROP5 in pollen resulted in an enhanced depolarized growth phenotype [81-82]. Injection of anti-ROP1 antibodies also disrupted the intracellular tip-focused Ca^{2+} gradient in growing pollen tubes and arrested further growth, suggesting that ROP also regulate Ca^{2+} influx and Ca^{2+} gradient maintenance [81].
Pollen ROP also affects the activity of phosphatidylinositol monophosphate kinase (PtdIns P-K). Phosphatidylinositol 4,5 bisphosphate (PIP2), the product of PtdIns P-K, accumulates at

the tip together with ROP in the PM, which is necessary for pollen tube growth [82]. The PBR of ROPs interact with phosphatidyl phosphoinositides to stabilize PM interactions, indicating that ROP and PtdIns P-K (with the production of PIP2) could form a positive feedback loop that enhances recruitment of ROP to the pollen PM tip and strengthens ROP PM (lipid raft) interactions [66]. These results imply that ROP regulates several downstream processes during polar growth of pollen tubes.

Sexual reproduction in flowering plants requires precise pollen tube guidance towards the ovule. Increasing evidence points towards chemotropism as a way of guiding the growing pollen tube. Sporophytic cells inside the pistil are believed to produce molecules (morphogens) that attract or repel the growing pollen tube, and in the final stages, guidance is controlled by the female gametophyte [83]. Characterization of the interaction between the cytoplasmic domain of pollen specific receptor protein kinase (PRK) and the PRONE domain of ROPGEFs gives an insight into how ROPs may initialize and maintain cell polarity [40, 84]. PRKs are thought to bind extracellular ligands (morphogens) originating from surrounding sporophytic tissues. Upon ligand binding, the cytosolic domain recruits and possibly phosphorylates the C-terminal domain of ROPGEFs, which results in exposure of the catalytic domain of the ROPGEFs. Subsequently, ROPs are activated and accumulate at a distinct region of the cell membrane, possibly towards an increasing gradient of ligands that will direct tube growth [40, 83]. Another ROP-associated protein involved in polar cell growth is the scaffold-like protein ROP Interactive Partner 1 (RIP1/ICR1). In mature pollen, RIP1/ICR1 localizes to the nucleus. Upon pollen hydration, RIP1 re-localize from the PM to the cell cortex at the future pollen tube initiation site. RIP/ICRs contain a C-terminal ROP binding motif, and bind and target cytosolic ROP to the PM of the tube initiation site. Overexpression of RIP1/ICR1 leads to depolarized growth similar to ROP1 overexpression, possibly by (over)recruiting ROP to the PM. Therefore, RIP1/ICR1 may in part function as a ROP regulatory protein during pollen tube growth through positive feedback regulation of ROP localization to the PM. The mechanisms that mediates the re-localization of RIP1/ICR1 from the nucleus to the the PM of the tube initiation site are not known [85].

Maintaining polarized growth involve ROPGEF proteins and other ROP regulatory proteins. The Arabidopsis ROP PH-GAP named REN1 is localized to exocytic vesicles and the PM tip in growing pollen tubes. T-DNA knockout of REN1 resulted in enhanced depolarized growth of pollen tubes similar to overexpressing constitutively active AtROP1. REN1 inhibits lateral spreading of ROP1 in the PM, thereby restricting the distribution of activated ROP1 to the pollen tube tip. Localization of REN1 at the tip is dependent on ROP1 signaling targets such

as the actin cytoskeleton and exocytic vesicles, thereby forming a negative feedback loop of ROP1 signaling [52]. Interestingly, the PH-domain of REN1 may bind PIP2 to localize REN1 to ROP signaling complexes on lipid rafts at the pollen tube tip, which could enhance spatial specificity.

Transient overexpression of NtRhoGDI2 in tobacco pollen resulted in tube growth being inhibited by transferring inactivated NtROP5 from the flanks of the growing tip into the cytosol. Cytosolic inactivated ROP is further thought to be recycled towards the apex of the tip [86]. Thus both ROPGAP and ROPGDI play an important role in restricting ROP (spatial) activity and accumulating ROP to the pollen tube tip.

It has been demonstrated that ROPs regulates actin structures at the tip during growth. The actin cytoskeleton in wild type pollen tubes consists of thick longitudinally oriented actin bundles and fine filamentous (F-actin) structures towards the growing tip. Transient overexpression of constitutively active ROP in Arabidopsis pollen tubes resulted in thick transverse helical actin bundles, whereas dominant negative AtROP resulted in finer, less organized actin filaments. These observations imply that the dynamics of apical F-actin is dependent on ROP regulation during polar growth [82, 87]. It was later discovered that ROP1 controls the F-actin dynamics through balancing two counteracting pathways by interacting with a novel family ROP effectors termed RICs (ROP-interactive CRIB containing proteins)[88]. These proteins share a conserved CRIB, domain but there is little sequence homology outside this domain. In Arabidopsis, the RIC family consists of 11 members that are considered to be ROP effectors that link active ROPs to various downstream processes in plants [89]. In pollen tube tips it was demonstrated that ROP1 interacts with either RIC3 or RIC4 to promote Ca^{2+}-mediated actin disassembly and actin assembly, respectively. The ROP-mediated balanced regulation of the two opposing pathways downstream of RIC3 and RIC4 is proposed to be crucial for temporal regulation during tip growth [88]. Pollen tubes grow in an oscillatory manner; ROP1 activity precedes the growth phase during tip growth by interacting with RIC4 which facilitates F-actin assembly. F-actin assembly is thought to be important for accumulation of vesicles containing PM and cell wall materials for the expanding tip. Paradoxically, the F-actin may function as a barrier that prevents fusion of vesicles to the PM. RIC3-mediated Ca^{2+} influx lags behind RIC4-mediated actin assembly, which is possibly due to a longer or slower signaling cascade compared to the ROP1/RIC4 pathway. The RIC3-mediated increase in cytosolic Ca^{2+} generates a tip-focused Ca^{2+} gradient which ultimately results in F-actin disassembly and docking/fusion of vesicles to the PM. The two opposing pathways most likely affect (feedback) ROP1 activity and may cooperatively

set the pace for tip growth oscillation [90]. Proteins that act downstream of RIC3 and RIC4 to regulate Ca^{2+} influx and F-actin assembly in pollen tube growth remains to be indentified.
As mentioned earlier (page 30), Rac and CDC42 GTPases in mammalian cells regulate actin nucleation by activating ARP2/3 through WAVE and WASP proteins respectively. In Arabidopsis, both WAVE and ARP2/3 orthologs are present, and several ROPs have been shown to interact directly with WAVE proteins [91]. However, none of the 5 WAVE paralogs in Arabidopsis are noticeably expressed in pollen (data from the Arabidopsis eFP Browser [58]), suggesting that WAVE/ARP2/3 does not mediate actin nucleation during pollen tip growth. Formins, which are another group of actin nucleating proteins regulated by Rho GTPases in animals, also exists in plants. In Arabidopsis, at least 21 formin like proteins exist and 6 formins are expressed in mature pollen [92]. Interestingly, overexpression of Arabidopsis formin homology 1 (AFH1) in tobacco pollen tubes lead to depolarized growth by formation of thick actin cables protruding from the tip PM into the cytosol [93]. AFH5 has been shown to mediate actin assembly from the sub-apical PM that provides actin filaments for vesicular transport [94]. However, it remains to be demonstrated if ROP (possibly through RIC4) modulate F-actin formation through formins.
A study on actin binding protein 29 (ABP29) in lily pollen tubes showed that ABP29 binds and fragments F-actin in the presence of Ca^{2+}, whereas PIP2 inhibits this process. Overexpression of ABP29 in lily pollen tubes lead to tube growth inhibition by disrupting the actin cytoskeleton, possibly in a Ca^{2+}-dependent manner. Therefore it is not unlikely that ABP29 could act downstream of ROP1/RIC3 signaling during growth [95].
It has been shown that ROP GTPases in rice (*Oryza sativa*) bind and activates NADPH oxidases [96]. The formation and accumulation of reactive oxygen species (ROS) at the pollen tube tip is important for pollen tube growth [97]. In root hair cells, ROS are thought to activate Ca^{2+} channels that are important for the formation of a tip-focused Ca^{2+} gradient [98], which may be the case in pollen tubes as well. Research has also shown that Ca^{2+} activates NADPH oxidases in pollen and root hairs, possibly forming a positive feedback loop to fortify the tip-focused Ca^{2+} gradient [97, 99]. Interestingly, a recent study on NADPH oxidases in *Picea meyeri* (Meyer spruce) pollen showed that NADPH oxidases partly localize to DRMs in the pollen tube tip and that NADPH activity is dependent on the micro-domain sterol composition. Chemically induced sterol sequestration in growing pollen tubes led to dissipation of the Ca^{2+} gradient and arrested further growth. The recruitment of ROP and NADPH oxidase to PM lipid rafts in the pollen tube tip, may therefore be spatially important in forming a ROS gradient that in turn facilitates a tip-focused Ca^{2+} gradient [100].

Root hair cells have also been used as a model to study polar growth in plants, and not surprisingly, ROP signaling is also involved during root hair initiation and elongation. Immunolocalization of ROP4 and ROP6 in roots showed that ROP localize to growth sites before budding of root hairs in the PM of trichoblasts (root hair forming cells), indicating that ROP have a role in root hair initiation. Unlike pollen tip growth, a tip-focused Ca^{2+} gradient is not present during budding of root hairs, suggesting that ROP localization is independent of a Ca^{2+} gradient. Overexpression of constitutively active ROP 4/6 lead to tip swelling and delocalized Ca^{2+} gradients, indicating that ROP alone is not enough to establish a Ca^{2+} gradient in root hairs [101]. Accumulation of ROS is also important during root hair development, as Arabidopsis with disruption of an NADPH oxidase (AtRbohC/RHD2) shows defects in root hair growth. ROS accumulation is thought to mediate Ca^{2+} influx through PM Ca^{2+} hyper-polarization channels [98]. Ca^{2+} has been demonstrated to stimulate NADPH oxidase activity, thereby forming a positive feedback loop fortifying the Ca^{2+} gradient and maintaining the polarity [99]. Arabidopsis ROP2 affects ROS production in root hairs through indirect activation of RBOHC/RHD2. Overexpression of WT or CA ROP2 increased ROS production, which is decreased in DN ROP2 plants [102]. Cumulatively, these results imply that the formation of ROS by NADPH oxidase activity is dependent on ROP regulation during root hair growth. In support of this hypothesis, it was discovered that *rhoGDI1* mutant plants were unable to spatially restrict ROP activity leading to depolarized growth and enhanced ROS production in trichoblasts [103].

ROPs and diffuse cell growth

In contrast to pollen tube and root hair growth, epidermal cells like trichomes and leaf pavement cells exhibit diffuse growth to increase cell surface by remodeling specific regions of the cell wall [80]. Mature Arabidopsis pavement cells form a jigsaw puzzle-like pattern in leaves, where lobes protruding from one cell fit into groves between lobes of neighboring cells. During initiation of lobe formation, AtROP2 localize to the specific regions in the PM from where the lobe will expand, and persists in the PM during lobe formation. Overexpression of CA AtROP2 leads to isotropic growth (analogous to depolarized growth in pollen), whereas overexpression of DN AtROP2 inhibited lobe expansion. Early stages of lobe expansion are associated with localized formation of F-actin in the cortical region of the expanding site. DN ROP overexpression inhibites cortical F-actin formation, indicating ROP

also has an important role during diffuse growth [104]. Unlike polar growth in pollen and root hairs, the downstream actors of ROP mediated F-actin nucleation has been identified in pavement cells and trichomes.

Trichomes are non-functional unicellular structures that protrude from the leaf epidermis and consist of a stalk with three pointed branches. The *distorted* class of Arabidopsis mutants display deformed trichomes due to abnormal F-actin organization [105]; all of these mutants have defect genes encoding components of the WAVE or Arp2/3 protein complexes. The *distorted* group of mutants also shows cell morphogenesis defects in cell expansion and cell adhesion, which is visible in pavement cells, hypocotyl cells and in some cases in root hair cells [106-108].

A mutant defective in SPIKE1, a plant ortholog of the Dock180 GEF domain, also displays deformed trichomes as well as an inability to form lobes in leaf pavement cells. The *spike1* mutants are defective in both microtubule and actin organization, and homozygous plants are unable to develop seeds [43]. SPIKE1 has GEF activity towards ROP and research has shown that there is a morphogenic pathway where SPIKE1-ROP regulates actin dependent morphogenesis through the WAVE and Arp2/3 complexes [44]. In addition to activation of ROP, SPIKE associates with WAVE complex components such as NAP1 and SRA1 to possibly achieve increased specificity [44]. Interaction between SPIKE1 and the WAVE complex has also been demonstrated through yeast two-hybrid and bimolecular fluorescence complementation (BiFC) experiments [91].

All the *distorted* mutant alleles as well as the *spike1* mutant alleles, are transmitted through the male gametophyte and thus demonstrates that the SPIKE1-ROP-WAVE-Arp2/3 morphogenic pathway is not essential during pollen tip growth [66].

RICs are also proposed to be downstream effectors of ROPs during pavement cell morphogenesis. ROP2 and ROP4/6 interact with RIC4 and RIC1 respectively to regulate formation of the jigsaw puzzle shape of pavement cells. ROP2 and RIC4 mediate F-actin formation during lobe expansion, whereas AtROP4/6 bind to RIC1, which associates microtubules and promotes the formation of ordered cortical microtubule arrays in non expanding areas of the cell. The spatial organization and countersignaling of these two pathways is responsible for the interdigitating (jigsaw puzzle appearance) cell growth of pavement cells [109-110].

Research in the last decade has clearly demonstrated that ROP signalling regulates F-actin dynamics during tip growth, as well as both F-actin dynamics and microtubules ordering during diffuse cell expansion. There are still unresolved questions regarding ROP mediated F-

actin nucleation. What proteins mediate F-actin dynamics during tip growth and how do these proteins link to ROP and RIC signaling? What is downstream of the RICs? During diffuse cell expansion the SPIKE1-ROP-WAVE-Arp2/3 pathway is important for F-actin formation, but parallel to this is the ROP2-RIC4 pathway which also is instrumental in diffuse cell expansion. It will be interesting to see how ROPs regulate the two pathways correlatively and understanding the relationship between them.

ROPs and membrane trafficking

In addition to binding to ROP, the RIP1/ICR1 (interactor of constitutively active ROP1) also recruits SEC3, a component of the exocyst complex whose function is to spatially localize and tether post golgi vesicles to the PM [111]. RIP1/ICR1 consists of mostly coiled coil domains and shares some similarity to ROCK1 (Rho associated coiled coil making protein kinase). RIP1/ICR1 forms homo-oligomers that can interact with both group I and group II ROPs in the PM. However PM association with ICR is dependent on ROP lipid modification. Importantly RIP1/ICR1-ROP can recruit SEC3 of the exocyst complex and co-localize to the PM. RIP1/ICR1 may therefore function as scaffold that mediates interaction between ROP and the exocyst complex. On the other hand, overexpression of CA-ROP11 led to swollen root hairs that were incapable of endocytosing FM4-64, a tracer used to visualize endocytotic membrane internalization, suggesting that ROPs also are involved in endocytosis [112]. Based on these observations, it is likely that ROPs play a role during both endocytosis and exocytosis, and that these processes are linked to maintaining cell polarity during cell growth. Notably RHO/CDC42 GTPases in yeast and animals are also involved in regulating subunits of the exocyst complex [113-115] and that this mode of regulation is evolutionary conserved process.

ROP/RACs in disease resistance

An AtROP9 ortholog in rice, OsRAC1, is a component of the innate immunity response in rice by regulating ROS production. Overexpression of CA-OsRAC1 leads to increased ROS production and increased resistance to blast fungus and bacterial blight [116]. The increase in ROS production is mediated by an NADPH oxidase that binds to OsRAC1 through its N-terminus [96]. Cinnamoyl-CoA reductase 1 (OsCCR1) is another effector regulated by

OsRAC1 during pathogen infection in rice. OsCCR1 catalyzes the first step of the monolignol pathway. Monolignols are polymerized to lignin by peroxidases using H_2O_2. Thus, OsRAC1 regulate two important elements during lignin synthesis. Lignin synthesis is induced at the pathogen infection site due to its resistance towards microbial degradation, and deposits may reinforce the cell wall [117]. GTP-bound OsRAC1 also interacts with an ortholog of human Receptor for Activated C-kinase 1 (RACK1). OsRAC1 activates RACK1, and overexpression of RACK1 results in increased ROS production and enhanced resistance to blast fungus and bacterial blight. RACK1 interacts with NADPH oxidase and several other proteins involved in OsRAC1-mediated immunity and may therefore functions as a scaffold protein for OsRAC1 signaling [118]. Interestingly, both OsRAC1 and RACK1 relocate to DRMs after chitin elicitor treatment, suggesting that OsRAC1-mediated innate immunity responses are associated with lipid rafts in the PM [119]. Recently it was also discovered that OsRAC1 binds to and is activated by PIT, a Nucleotide-binding domain and Leucine-rich Repeat (NLR) family protein that recognizes pathogen derived molecules from rice blast fungus [120]. In summary, it seems clear that OsRAC1 is a central component in mediating innate immunity responses in rice (monocots). In Arabidopsis, however, it remains to be discovered if ROPs regulate similar pathways during pathogen attack. ROP4 interacts with and possibly activates the ROP binding kinase1 (RBK1) which shows elevated expression during pathogen exposure, suggesting that ROPs may be involved in pathogen defense in some way, although the stimulating ligand for RBKs is still unknown and downstream kinase targets remains to be identified [62].

ROPs and plant hormone responses

The plant hormone abscisic acid (ABA) is involved in regulating several growth and developmental responses like seed dormancy, stomatal movement and stress responses [121]. A *rop10* T-DNA mutant showed increased seed dormancy, shorter root growth and increased stomatal closure in response to ABA treatment, suggesting that AtROP10 is a negative regulator of ABA responses. Overexpression of *CA ROP10* and *DN ROP10* lead to reduced and increased sensitivity towards ABA, respectively [122]. However, transcriptome analysis of the *rop10* mutant showed that ROP10 only affects a subset of genes at moderate levels of ABA exposure and that ROP10 does not directly regulate any major ABA pathways in Arabidopsis [123]. These results indicate that ROPs have a more indirect role in ABA signaling in Arabidopsis.

Auxin (IAA) is another plant hormone that regulates several cellular processes and developmental processes in plants [124]. Overexpression of ROP1, ROP3, ROP6 and ROP10 in tobacco protoplasts lead to auxin-responsive gene expression both in the absence and presence of exogenous auxin. Similarly overexpression of CA-NtRAC1 or DN-NtRAC1 in tobacco plants resulted in increased and decreased auxin responsive gene expression respectively. ROP GTPases therefore seem to regulate a pathway that mediates the auxin signal into a gene response [125].

The *rip1/icr1* T-DNA mutant displays phenotypes that are similar to phenotypes caused by aberrant auxin distribution [126]. The PINFORMED (PIN) proteins are localized to the PM where they act as auxin efflux carriers. They have polar PM localization which is required for the directional flow of auxin in cells and tissues [127]. RIP1/ICR1 itself is induced by auxin and in the *rip1/icr1* mutant, PIN1 and PIN2 localization was compromised, suggesting that RIP1/ICR1 together ROPs is required for PM recruitment and polar localization of PIN proteins. Therefore it is not unlikely that RIP1/ICR1 function as a scaffold protein in various ROP regulated processes [128]. It was recently demonstrated that the plant hormone auxin is an instrumental regulator of cellular interdigitation in pavement cells. Auxin-binding protein 1 (ABP1) acts upstream of ROP GTPase signaling events and in a coordinated fashion lead to the subsequent activation of both ROP2 and ROP4 countersignaling pathways [129].

The MIRO GTPases

The relatively recently discovered mitochondrial Rho GTPases or MIRO constitute a distinct family within the Ras superfamily. Research has demonstrated that these proteins regulate both mitochondrial movement and morphology in various eukaryotic cells [reviewed in 5, 130].

MIRO GTPases are not found in Entamoebidae and Parabasalia, which may not be surprising considering the fact that these organisms lack mitochondria. However, MIRO GTPases are not present in haptophyceae that contain mitochondria, which indicate that MIRO GTPases are not required in some forms of eukaryotic life (Paper II).

As previously mentioned, MIRO GTPases were discovered through a search for Rho GTPases consensus motifs. The N-terminal GTPase domain shares some similarities with other Rho GTPases and was therefore classified as such [2]. But MIRO GTPases lack the Rho defining insert helix and also differ from other Ras superfamily GTPases by containing two G domains separated by two Ca^{2+} binding EF-hand motifs (Figure 10 A). MIRO GTPases localize to the outer membrane of mitochondria with the MIRO GTPase facing towards the cytosol. Association to the mitochondrial membrane is achieved through a C-terminal transmembrane domain (TM) which does not undergo any post-translational lipid modification like most other Ras superfamily GTPases (Figure 10 B) [2, 131]. Based on both functional roles and structure, MIRO GTPases are currently considered to form their own family within the Ras superfamily [3].

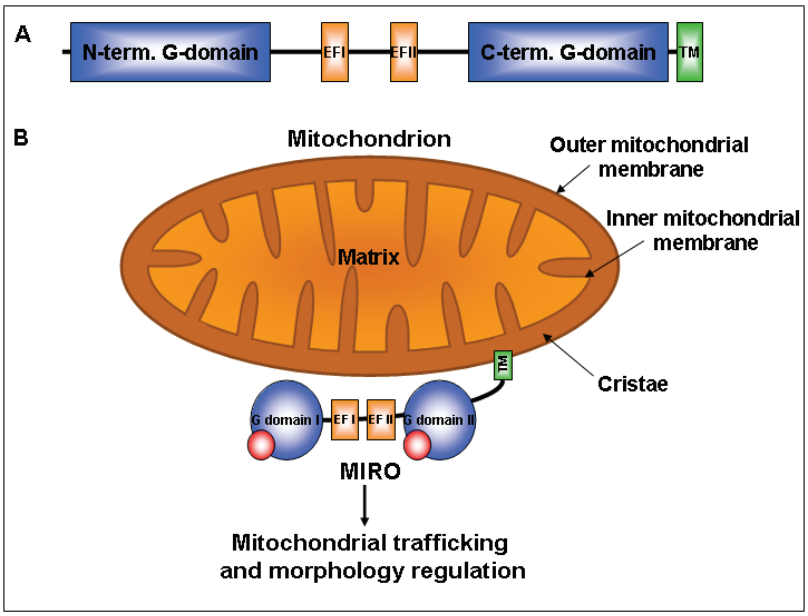

Figure 10: A) Miro GTPase domain organization. B) Miro GTPases are localized to the outer mitochondrial membrane through a trans-membrane motif with the G domains facing the cytosol.

MIRO GTPases and mitochondrial movement along microtubules

Mitochondria are a major constituent of the eukaryotic cells cytosol and are essential in energy formation through the production of ATP. In addition, mitochondria are also important for calcium buffering and during apoptosis. Mitochondria are highly motile and remarkably plastic organelles which can also fuse with one another (fusion) or divide (fission).
In nerve cells, mitochondria accumulate in axons and dendrites which correlate to the energy demands at these sites. Energy is needed to pump ions against their electrochemical gradient in order to maintain both action and synaptic potentials. These sites are often far from the central region of the neuron called soma, where the mitochondrial biogenesis occurs. Mitochondria are transported long distances from the soma through axons into dendrites and synaptic domains. For example motor neurons in the human body are up to 1 m in length. In metazoan cells long distance transport occurs via motor proteins that transport mitochondria along microtubules, whereas the actin cytoskeleton is important for short distance movement and tethering of mitochondria. Kinesin motor proteins facilitate anterograde transport towards the plus end of microtubule away from the cell body to distal parts of the cell, whereas dynein motor proteins facilitate retrograde transport in the opposite direction. Observed deficiencies in both mitochondrial transport and plasticity associated with neurological diseases such as Alzheimer, Parkinson, Huntington and Charcot-Marie-Tooth disease, underline the importance of mitochondrial dynamics in neurons [reviewed in 132, 133-134].

Homozygous EMS-induced mutations in the Drosophila MIRO (dMIRO) ortholog resulted in larvae with reduced crawling capabilities that eventually died. In muscle cells and neurons of the *dmiro* mutant, mitochondria showed abnormal distribution; mitochondria in neurons were not transported into dendrites and axons, but aggregated in neuronal soma instead. These results suggested that dMIRO is responsible for anterograde kinesin mediated transport of mitochondria and distribution in neurons [135]. In support of these findings, Glater and co-workers [136] demonstrated that dMIRO interacts with the adaptor protein MILTON, which recruits Kinesin-1 heavy chain (KHC) to mitochondria. dMIRO and MILTON most likely form a protein complex that through KHC facilitates anterograde movement of mitochondria along microtubules. These findings also suggested that MIRO may function as a regulatory switch in mitochondrial transport, based on the fact that MIRO contains two EF-hands flanked by two G domains [136]. However, co-immunoprecipitation experiments showed that

the mammalian MILTON like proteins GRIF-1 and OIP106 bind the N-terminal G domain and that these interactions are independent of both the nucleotide state of the G domain and the Ca-binding capabilities of the EF-hands. These findings suggested that the N-terminal MIRO G domain facilitates protein-protein interaction [131]. The results also indicated that the MIRO GTPases may not use the nucleotide state discriminately during protein-protein interaction, but rather that both the nucleotide state and EF-hands may be important for modulating the protein complex activity. It should also be mentioned that there so far has not been reported findings of any regulating proteins like GAPs, GEFs or GDIs associated with MIRO GTPases. How the nucleotide states of the MIRO G domains are regulated warrants further investigation.

Saotome and colleagues [137] showed that overexpression of human MIRO1, MIRO2, CA-MIRO1, CA-MIRO2 and MIRO EF hand mutants in H9c2 cardiac cell cultures lead to increased anterograde and retrograde mitochondrial movement, whereas silencing of MIRO expression had the opposite effect. The study also demonstrated that MIRO-based mitochondrial motility was Ca^{2+}-dependent and that a rise in cytoplasmic Ca^{2+} concentration above resting levels (> 100 nM) arrested mitochondrial movement in WT-MIRO1&2 and CA-MIRO1&2 overexpressing cells. On the other hand, overexpression of MIRO EF mutants and DN-MIRO1&2 partially suppressed Ca^{2+}-induced mitochondrial arrest above calcium resting levels. These findings demonstrated that MIRO functions as a Ca^{2+}-sensitive switch that facilitates mitochondrial motility along microtubules when calcium levels are maintained at resting level and that the EF-hands and the nucleotide state of the G domain are important for MIRO mediated arrest [137].

An experimental setup where hMIRO was overexpressed in hippocampal neurons led to increased mitochondrial transport into peripheral parts of the neuron. The mitochondrial transport was shown to be mediated by GRIF-1/hMIRO interaction, and disrupting the kinesin binding domain of GRIF-1 resulted in inhibition of transport. In contrast to earlier findings by Fransson and colleagues [131] it was shown that the recruitment of GRIF-1 to MIRO is dependent on the nucleotide state of the N-terminal G domain. Co-expression of Grif-1 with WT hMIRO and DN-hMIRO (N18) resulted in recruitment of GRIF-1 to mitochondria, whereas CA-hMIRO (V13) was incapable of recruiting GRIF-1 to mitochondria. In addition, expression of CA-hMIRO in neurons led to reduced transport of mitochondria into peripheral parts of the neurons and aggregation of mitochondria in the cell soma [138]. Further investigations showed that glutamate receptor-mediated calcium influx in rat hippocampal neurons recruited mobile mitochondria to and retained them at synaptic regions. However,

cells expressing MIRO EF-hand mutants did not respond to calcium influx mediated by activation of glutamate receptors. The same study showed that hMIRO is capable of binding Kinesin-1 directly in a Ca^{2+}-dependent manner, where elevated intracellular levels of calcium disrupted this interaction. In contrast, GRIF-1 was shown to bind to hMIRO in a Ca^{2+}-independent manner. The hMIRO/Kinesin-1 interaction may be partially mediated and/or modulated by GRIF-1, since all three components are necessary for mitochondrial movement. These findings suggest that during neuronal activity, hMIRO assists in localizing mitochondria close to postsynaptic membranes to provide ATP and facilitate calcium buffering. Elevated Ca^{2+} concentrations disrupts the hMIRO/GRIF-1 and Kinesin-1 interaction and uncouples mitochondria from motor proteins, thereby allowing mitochondria to accumulate at these intracellular microdomains (Figure 11 A) [139].

In contrast to these findings, it was shown that dMILTON functions as the sole link between hMIRO and Kinesin 1 ectopically expressed in rat hippocampal neurons. Ca^{2+}-mediated arrest of mitochondria in this experimental setup suggested that the Kinesin-1 motor domains interact with MIRO in a Ca^{2+}-dependent manner, rather than dissociating from the MIRO/dMILTON complex during Ca^{2+}-mediated arrest (Figure 11 B). Also in this experimental setup, mitochondrial arrest was dependent upon functional EF-hands [140]. However since MILTON shares only 35% similarity with GRIF-1, the different interaction between hMIRO/dMILTON, hMIRO/GRIF-1 towards Kinesin-1 in these separate studies may reflect a general difference between invertebrates and mammals [139].

A common denominator for the studies by Saotome et al. [137], MacAskill et al. [139] and Wang and Schwarz [140] is that MIRO functions as a Ca^{2+}-dependent switch that regulates mitochondrial arrest of both anterograde and retrograde transport through its EF-hands.

A closer investigation of bidirectional mitochondrial transport in Drosophila motor axons showed that loss of dMIRO activity reduced the effectiveness of both anterograde and retrograde mitochondrial transport, depending on the net directional transport of mitochondria. Mitochondria move in a “preprogrammed” anterograde or retrograde direction and results suggests that dMIRO executes the directional programming that favors one motor activity over the other (kinesin vs. myosin). How preprogramming of mitochondrial movement is achieved is largely unknown. However, loss of dMIRO function did not affect this directional preprogramming and the experimental results suggested that dMIRO facilitates the duration and distance of both antergrade kinesin motor movements and retrograde dynein motor movement of mitochondria. In addition, loss of dMIRO function increased the duration of short stationary phases during net movements in either direction. Overexpression of dMIRO

also had negative effects on bidirectional transport similar to loss of function in dMIRO. In contrast to *dmiro* null mutants, overexpresssion of dMIRO led to increased reversal of movements from antergrade to retrograde or *vice versa*. The duration of short stationary phase was however unchanged. These findings suggest that the observed defects in mitochondrial transport in the overexpressing mutant are due to increased rate of direction reversals, which was not the case for dMIRO null mutants. Thus, overexpression of dMIRO may reflect a titration effect that recruits factors that overrides the directional preprogramming of mitochondria. These results are in contrast to findings in mammalian cells, where overexpression of MIRO results in increased mitochondrial movement, and may also reflect a difference between invertebrates and mammals [141].

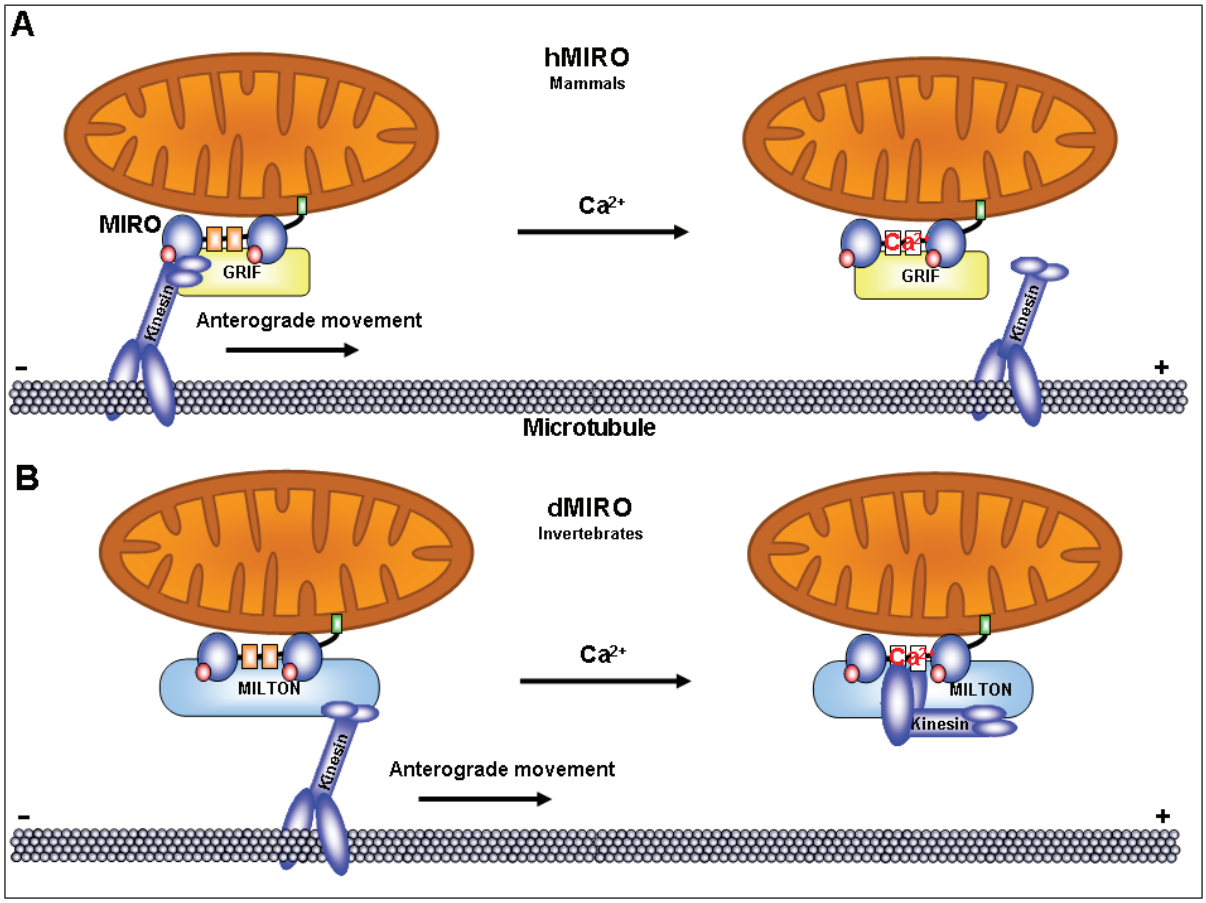

Figure 11: MIRO GTPases mediate mitochondrial transport in Metazoa. A) A model depicting Ca^{2+}-dependent mitochondrial transport in mammals. Upon increased intracellular calcium levels, Ca^{2+} ions binds to the MIRO EF-hand motifs, resulting in dissociation of Kinesin from the MIRO/GRIF complex which arrests mitochondrial movement [139]. B) In contrast to mammals, the dMIRO/MILTON/Kinesin complexes in invertebrates remain associated after Ca^{2+} binding. The binding of Ca^{2+} to dMIRO results in a shift of affinity for the Kinesin motor domains, from microtubules to the MIRO GTPase. This change in affinity leads to arrest of mitochondrial transport [140]. In both mammals and invertebrates, MIRO functions as a calcium sensor during mitochondrial transport. The figure is modified from Reis and colleagues [5].

A recent study show that Mitofusin2 (Mfn2) interacts with the hMIRO/GRIF1 complex to mediate axional transport. The Mitofusins are dynamin family GTPases that are involved in mitochondrial fusion. A dominant negative inherited point mutation in Mfn2 is known to be the cause of axonal Charcot-Marie-Tooth disease (CMT2A). Experiments show that Mfn2 in addition to playing a role in mitochondrial fusion, also is involved in mediating mitochondrial transport. Immunoprecipitation studies showed that Mfn2 binds to both hMIRO and OIP106/GRIF1, but not Kinesin. Importantly, loss of function in Mfn2 and CMT2A mutations in Mfn2 led to alterations in mitochondrial movement patterns. Both $Mfn2^{-/-}$ and CMT2A mutations resulted in slower mitochondrial movement and increased the duration of short stationary phases. These results suggests that Mfn2 together with hMIRO and OIP106/GRIF1 forms a complex that mediates mitochondrial transport through interaction with both kinesin and dynein motor proteins [142].

These findings clearly show that MIRO complexes facilitate bidirectional movement of mitochondria in both invertebrate and mammalian cells. However, little is known about how the direction is changed. Another unresolved question is whether, MIRO is in some way involved or regulated during this process or if it is the activity/availability of motor proteins that facilitates the directional change. Interestingly, a recent study reported findings of a novel hMIRO-interaction protein named HUMMR (Hypoxia Up-regulated Mitochondrial Movement Regulator), which is expressed in neurons and up-regulated during hypoxia. Loss of HUMMR function during hypoxia reduces the number of mitochondria in axons and reduces the number of mitochondria moving in the antergrade direction, whilst the number of mitochondria moving in the retrograde direction increases. Thus, HUMMR biases the net mitochondrial movement in an anterograde direction during hypoxia. HUMMR was also shown to recruit GRIF-1 to mitochondria, which may enhance kinesin motor recruitment, subsequently resulting in increased anterograde transport [143]. These results suggest that the MIRO/GRIF1 complex may be modulated by other factors to enhance or decrease both kinesin and dynein binding to change the direction of mitochondrial movement.

Miro and mitochondrial morphology

Mitochondria are highly plastic organelles that are able to change their morphology depending on intracellular conditions and needs. Mitochondria in many cell types fuse and divide by fission and fusion mechanisms in a highly controlled manner. The tightly regulated

balance between fission and fusion events does not only regulate size and number of mitochondria, but also their function and distribution. Mitochondria can form tubular networks through fusion to facilitate exchange of mitochondrial content; mitochondria divide through fission events to facilitate transport of mitochondria into small dendrites. Alterations in mitochondrial fission and fusion mechanics have impact on neuronal function, and defects in the mitochondrial fusion/fission machinery are often associated with several neurological diseases [Reviewed in 144, 145].

Overexpression of WT-hMIRO1 but not WT-hMIRO2 in mammalian cell lines resulted in formation of long threadlike mitochondria which was not formed when DN-hMIRO1 or EF-hand mutants were overexpressed. In contrast, cells overexpressing CA-hMIRO1+/-,MIRO2+/+ displayed perinuclear aggregation of mitochondria. Furthermore DN-hMIRO1 showed a higher degree of collapsed mitochondrial network compared to WT-hMIRO overexpressing cells. CA or DN mutations in the C-terminal G domain however, showed no effect on mitochondrial morphology. These findings suggested that hMIRO have a role in regulating mitochondrial morphology. The observed aggregation or formation of threadlike mitochondria may be a result of increased fusion and/or decrease of fission events. There may also be some functional difference between hMIRO1 and hMIRO2 since hMIRO2 does not induce the formation of threadlike mitochondria. Results also demonstrated that the nucleotide state of the N-terminal G domain and the functionality of the EF-hands of hMIRO affects mitochondrial morphology [2, 131].
Similar results were obtained in H9c2 cells; hMIRO overexpression lead to the formation of long threadlike mitochondria and aggregation, whereas overexpression of DN-hMIRO or silencing of hMIRO lead to fragmentation and aggregation. Therefore, there seems to be a link between the availability and nucleotide state of hMIRO and the length of mitochondria. Further investigation indicated that under resting levels of Ca^{2+}, hMIRO promoted elongation of mitochondria by possibly suppressing Dynamin-related protein1 (Drp1)-mediated fission events and/or recruiting fusion mediating proteins. This elongation was dependent on the nucleotide state of the N-terminal G domain and not the EF-hands. In resting cortical neurons, overexpression of hMIRO1&2 resulted in elongated mitochondria in dendritic regions, whereas overexpression of hMIRO1&2 EF mutants further increased mitochondrial length in the same regions of the neuron. These finding suggests that dendritic Ca^{2+} levels has an inhibitory effect on hMIRO mediated mitochondrial fusion events. Repeated depolarization of neurons (transient increase of cytosolic Ca^{2+}) in WT-hMIRO overexpressing neurons resulted

in shorter mitochondria; importantly, overexpression of hMIRO-EF hand mutants repressed this shortening of mitochondria. These findings demonstrate that hMIRO mediates Ca^{2+}-dependent fragmentation of dendritic mitochondria and that this process is dependent on functional EF-hands. Thus, hMIRO functions as a Ca^{2+} sensitive switch that mediates fragmentation of mitochondria under cellular conditions where Ca^{2+} levels are above the resting level [137].

In Drosophila, loss of function and overexpression of dMIRO also affected mitochondrial length in motor axons. Both anterograde and retrograde moving mitochondria were significantly shorter in the *dmiro* null mutant. In contrast, overexpression of dMIRO on the other hand significantly increased the length of anterograde moving mitochondria, whereas retrograde moving mitochondria were not significantly longer. These findings are indicative of dMIRO facilitating fusion or suppressing fission events of antergrade moving mitochondria. This observation may be caused by "new" anterograde moving mitochondria that fuse with "old" mitochondria to possibly ensure mitochondrial health. The velocity and kinetics of normally sized and elongated mitochondria were similar, suggesting that dMIRO-regulated morphology is independent from dMIRO-mediated transport [141].

In conclusion, it has become evident that MIRO GTPases also are modulators of mitochondrial morphology besides being instrumental facilitators of mitochondrial transport in mammals and invertebrates. Thus, it is correct to state that MIRO GTPases have evolved to be an essential component in mitochondrial function.

However, little is known about which components of the fusion/fission machinery MIRO modulates to alter mitochondrial morphology and the nature of this modulation. Interestingly, it was recently discovered that MIRO and OIP106 interact with mitochondria localized PTEN-induced putative kinase 1 (PINK1) and that loss of function of this gene is associated with inheritable Parkinson disease. In Drosophila and mammalian cells, it has been shown that dPINK genetically interacts with known components of the mitochondrial fusion/fission machinery. Overexpression of dPINK leads to increased fission and silencing results in increased fusion of mitochondria [146]. Notably, overexpression of both MIRO and OIP106 restores mitochondrial morphology after PINK1 silencing, making MIRO and OIP106 a potential drug target for Parkinson treatment [147].

Thus, it seems likely that MIRO GTPases in mammals and invertebrates interacts with several proteins/pathways to modulate mitochondrial morphology by mechanisms that have not yet been fully investigated.

Loss of function in Gem1p, a yeast MIRO ortholog resulted in distorted mitochondrial distribution and morphology. The normal tubular network of yeast mitochondria was lost and over half the cells contained larger globular mitochondria that were not collapsed tubular mitochondria. The remainder of the *gem1p* cells displayed either collapsed tubular networks or clusters of smaller mitochondria. In contrast to hMIRO, not only the N-terminal G domain and EF-hands but also the nucleotide state of the C-terminal G domain of Gem1p is functional. Interestingly, the number of mitochondrial fusion and fission events was unchanged in mutant strains lacking known fission/fusion genes in addition to loss of function in Gem1p. These results suggest that Gem1p regulate mitochondrial morphology via a novel pathway in yeast [148]. Mitochondria in yeast are transported and organized along the actin cytoskeleton. This suggests that Gem1p function most likely is different compared to mammalian cells. Further studies showed that Gem1p is important for proper mitochondrial inheritance. However, Gem1p seem to act independently from other known components that are important for mitochondrial inheritance in yeast [149]. To date, no Gem1p interacting partner has been discovered, and a mechanism by which Gem1p regulates mitochondrial morphology is yet to be described.

MIRO in plants

Compared to metazoan MIRO GTPases, relatively little functional information is published on plant MIRO GTPase orthologs. Arabidopsis MIRO was initially reported by Fransson and colleagues in 2003 [2], but since then only three publications have described functional roles for MIRO in Arabidopsis. Jayasekaran and colleagues reported in 2006 [150] the finding of a novel calcium-binding GTPase (AtCBG) that in fact is a MIRO GTPase (AtMIRO2). AtCBG/AtMIRO2 expression was found to be up-regulated by salt and ABA stress and AtCBG/AtMIRO2 knockout mutants were reported to be sensitive towards both salt and ABA treatment. To date, there have not been any publications that have further explored a possible link between AtMIRO and ABA stress.

Yamaoka and Leaver described the three Arabidopsis MIRO orthologs in 2008 [151] and showed that two of the genes, MIRO1 and MIRO2, are ubiquitously expressed in all tissues and stages. A third MIRO GTPase, MIRO3, showed negligible expression rates in comparison. Like their counterparts in other species, plant MIRO GTPases attach to the outer mitochondrial membrane through a C-terminal transmembrane domain. Further investigations by Yamaoka and Leaver showed that embryos homozygous for T-DNA insertion in the *MIRO1* gene arrested during early stages of development. *miro1* pollen also showed reduced germination rates and impaired pollen tube growth, which resulted in reduced male genetic transmission of the *miro1* allele. In addition genetic transmission through the female gamete was slightly reduced. During sporophytic stages of the lifecycle, *miro1* T-DNA mutants showed no apparent defects. T-DNA inserion in the *MIRO2* gene showed no apparent phenotypes in neither sporophytic nor gametophytic stages of the life cycle, leading the authors to suggest that MIRO2 plays no major role in plant development.

A closer look into the evolution of MIRO GTPases in embryophyta shows that in dicots, MIRO1 and MIRO2 paralogs cluster into two distinct subgroups which probably originated due to a gene or genome duplication after the diversification of monocots and eudicots (Paper II).

Since MIRO2, the paralog to MIRO1 seems to be conserved in dicots, the possibility that there may be some form of functional redundancy between the two genes arises. Investigations of Arabidopsis plants that were heterozygous for a T-DNA insertion in the MIRO1 gene and homozygous for T-DNA insertions in the MIRO2 gene showed that an additional loss of function in MIRO2 enhanced *miro1* phenotypes (Paper II).

The *miro1*$^{+/-}$,*miro2*$^{-/-}$ double mutant showed increased segregation distortion compared to the sole miro1 mutant (16.8 % vs. 57.1 %). The *miro1*$^{+/-}$,*miro2*$^{-/-}$ mutant also showed increased gametophytic defects with siliques containing a larger number of unfertilized ovules (34.5 % vs. 7.2 %) and less aborted embryos (3.4 % vs. 17.4 %). Reciprocal crosses showed that the co-transmission of the mutant alleles through the male gametophyte was nearly absent (0.12 %) and severely reduced through the female gametes (34.7 %). The absent male co-transmission of both T-DNA insertions shows that the formation of homozygous *miro1* embryos does not occur in the double mutant and therefore explains the absence of aborted embryos in the double *miro* mutant. Further investigations showed that male gametes carrying both T-DNA insertions (*miro1/miro2*) displayed reduced pollen tube growth compared to *miro1* mutant pollen and were most likely unable to fertilize ovules. The presence of unfertilized ovules is therefore most likely a result of impaired female gametogenesis caused by *miro1/miro2* alleles. Indeed, a larger number of embryo sacs from the double *miro* mutant showed delayed or defects in polar nuclei fusion compared to *miro1* mutation alone. Interestingly, defects in polar nuclei fusion have been associated with knock-out of mitochondria targeted proteins [152-155].
Taken together, the observations from the *miro1/miro2* double mutant show that Arabidopsis *MIRO1* and *MIRO2* are unequally functionally redundant genes. The *MIRO1* gene is most likely the “ancestral gene” and has retained most of the function, whereas the *MIRO2* paralog originated from a gene or genome duplication and has retained a fraction of the ancestral function. Therefore the true effect of loss of function in *MIRO2* is only visible in a *miro1* background (Paper II).

Naturally, one would suspect that plant MIRO GTPases have similar functional roles in mitochondrial transport and morphology as their yeast and metazoan counterparts. Yamaoka and Leaver [151] investigated mitochondrial morphology in pollen and discovered that *miro1* pollen contained enlarged mitochondria with an abnormal intracellular distribution.
In addition to having abnormal size, a large portion of these mitochondria showed a tubular morphology. These abnormally sized and shaped mitochondria were motile and dependent on the actin cytoskeleton, suggesting that plant MIRO does not affect actin-dependent mitochondrial transport. However, the overall streaming of mitochondria during pollen tube growth in *miro1* was disrupted compared to wild type tubes. Based on these observations the authors hypothesized that MIRO is an important regulator of mitochondrial morphology and distribution [151]. In a recent publication, Yamaoka and colleagues show that the cause for

the lethality in homozygous *miro1* embryos is due to abnormal distribution of mitochondria during embryonic cell division. The mitochondria in the developing embryos were also enlarged in size. These findings further strengthens the role of MIRO as a regulator of mitochondrial morphology and distribution [156]. Further studies should be undertaken to discover MIRO-interacting proteins to elucidate the mechanisms by which MIRO regulate mitochondrial distribution and morphology.

In tobacco protoplasts it was observed that mitochondrial transport is dependent on myosin/actin interactions, whereas cortical positioning is based on both F-actin structures and microtubule structures [157]. A closer investigation of organelle movement in lily pollen tubes showed that the density of mitochondria increases towards the tip; the highest density of mitochondria is found in the sub-apical region, whereas relatively few mitochondria reside in the apex of the pollen tube. Mitochondria stream towards the apex along the cortical cytoplasm, move inward toward the core of the tube in the sub-apical region and return from the apex in a reverse fountain-like pattern. In pollen tubes treated with an actin depolymerizing drug (latrunculin B), mitochondrial enrichment in the sub-apical region was lost and mitochondria relocated into the apex, emphasizing the role of the actin cytoskeleton in mitochondrial transport in plants. Drug-mediated disruption of microtubules on the other hand, did not alter mitochondrial streaming [158].
These observations indicate that microtubules may be dispensable with regard to mitochondrial movement during pollen tube growth. However, both actin and microtubule dependent motor proteins are present on pollen tube mitochondria, suggesting that microtubule/kinesin interactions have functional roles. *In vitro* motility assays of mitochondria isolated from tobacco pollen showed that movement was slow and continuous along microtubules, but fast and irregular along actin filaments. Motility assays using both actin and microtubules resulted in net lower velocities, suggesting that microtubule/kinesin interactions affects the overall velocity of mitochondria [159]. In slight contrast to these findings, a recent *in vivo* study of mitochondrial movement in *P. wilsonii* pollen tubes using actin or microtubule drugs showed that mitochondrial movement mainly is driven by myosin/actin interactions and actin filament dynamics (tread milling). In contrast, microtubules were shown to have a more indirect role in mitochondrial velocities, trajectory and positioning by functioning as a scaffold directing the arrangement of actin filaments [160]. These differences may as well reflect differences between angiosperm and gymnosperm

pollen. The role of microtubule motors and organelle transport in pollen is not clear and warrants further investigation [reviewed in 161].

The observation that functional kinesin motor proteins are present on plant mitochondria clearly suggests some sort of link to microtubules. It remains to be investigated whether plant MIRO interacts with kinesin to form a protein complex similar to what is found in Drosophila and mammals. Clear orthologs to MILTON and GRIF-1/OIP106 proteins are not present in plants [151], suggesting that if an interaction with kinesin occurs, it is either direct or possibly through a novel plant protein.

Finally, one cannot rule out the possibility that plant MIRO GTPases have evolved to facilitate other mitochondrial events/functions than transport. The disruption of mitochondrial morphology and streaming in *miro1* pollen tubes suggest that MIRO is involved in both fusion/fission and transport. However, this disruption of movement may be an indirect result of an imbalance between fusion and fission events rather than disrupted transport mechanics. If plant MIRO is involved in regulation of fission/fusion events, the observed reduced movement of mitochondria in the *miro1* mutant may be a result of the transport machinery being incapable of transporting enlarged mitochondria. Similar observations have been reported for mitochondria in lymphocytes during chemotaxis. Lymphocytes relocate mitochondria to the uropod (posterior of the cell) during migration. In cells where a profission protein (DRP1) was down-regulated or pro-fusion proteins (Opa1, MFN1) were up-regulated, mitochondria were unable to relocate in the uropod. These observations indicate that the redistribution of mitochondria is dependent on a balance between fusion and fission events and that a shift in the balance towards fusion and enlargement of mitochondria interferes with mitochondrial transport [162].

Mitochondrial fusion and fission in plants

The chondriome (defined as all the mitochondria within a cell) in higher plants usually consists of numerous discrete organelles that continuously go through fusion and subsequent fission events. The mtDNA in plant mitochondria exists as a discontinuous whole, necessitating regular fusion and fission events to provide mtDNA, mtDNA encoded mRNA or proteins to mitochondria that are not able to do so by themselves. It is therefore hypothesized that the maintenance of a partial genome in individual mitochondria forces fusion to occur and by doing so, the chondriome health is maintained. Fusion of plant mitochondria has been

documented by several research groups, but the molecular mechanisms that underlie fusion events to date are largely unknown. In contrast, plant mitochondrial fission events are regulated by components that are conserved in eukaryotes, as well as novel plant components [reviewed in 163, 164].

The plant genes *BIGYIN/FISSION1B* and *FISSION1A* are orthologs to the human and yeast *FISSION1* (*FIS1*) genes and are targeted to both mitochondria and peroxisomes. Mitochondria and peroxisomes in *fis1A/B* knockout plants show an increase in size and a reduction in numbers per cell [165-167]. FIS1 orthologs bind to the outer mitochondrial membrane and facilitates the recruitment of Dynamin related proteins (DRPs), another evolutionary conserved component of the fission machinery. DRPs belong to the dynamin family of GTPases, and presumably form oligomers that are recruited to the outer mitochondrial membrane by FIS1. Once associated with FIS1 and the OMM, DRPs fully oligomerize and form a necklace-like structure around the mitochondria, and subsequent GTPase activity most likely constricts the DRP structure and causes fission of the mitochondria [reviewed in 134]. The Arabidopsis DRP3 orthologs (DRP3A and DRP3B) are targeted to both mitochondria and peroxisomes. In *drp3A* and *drp3B* knockout mutants mitochondria are only slightly enlarged and fewer in number, whereas in the *drp3A/drp3B* double mutant the mitochondria are elongated and forms networks. Overexpression of either DRP3A or DRP3B in the double mutant restored the WT morphology, suggesting that DRP3A and DRP3B are genetically partially redundant in function [168].

Thus, it is not unlikely that the evolutionary conserved role for MIRO in eukaryotes is regulation of mitochondrial morphology (Figure 12) rather than transport, which may be a function/role that MIRO has acquired in metazoan lineages. This notion is supported by the fact that silencing, overexpression or mutations in MIRO GTPases result in altered mitochondrial morphology in all model organisms investigated to date [reviewed in130]. This is further supported by Saotome and colleagues [137] who demonstrated that MIRO functions as a calcium sensitive switch that regulates mitochondrial fusion and fission dynamics. This process seems to involve regulation of the fission mediating dynamin-related protein 1 in humans.

In some contrast to these observations and speculations, it was recently demonstrated that disruption of a *D. discoideum* (slime mold) MIRO ortholog gemA, resulted in no alteration of mitochondrial morphology. It was also shown that gemA is not involved in mitochondrial transport, but that disruption gemA function resulted in considerable growth defects due to a cellular decrease of mitochondrial mass and ATP levels [169].

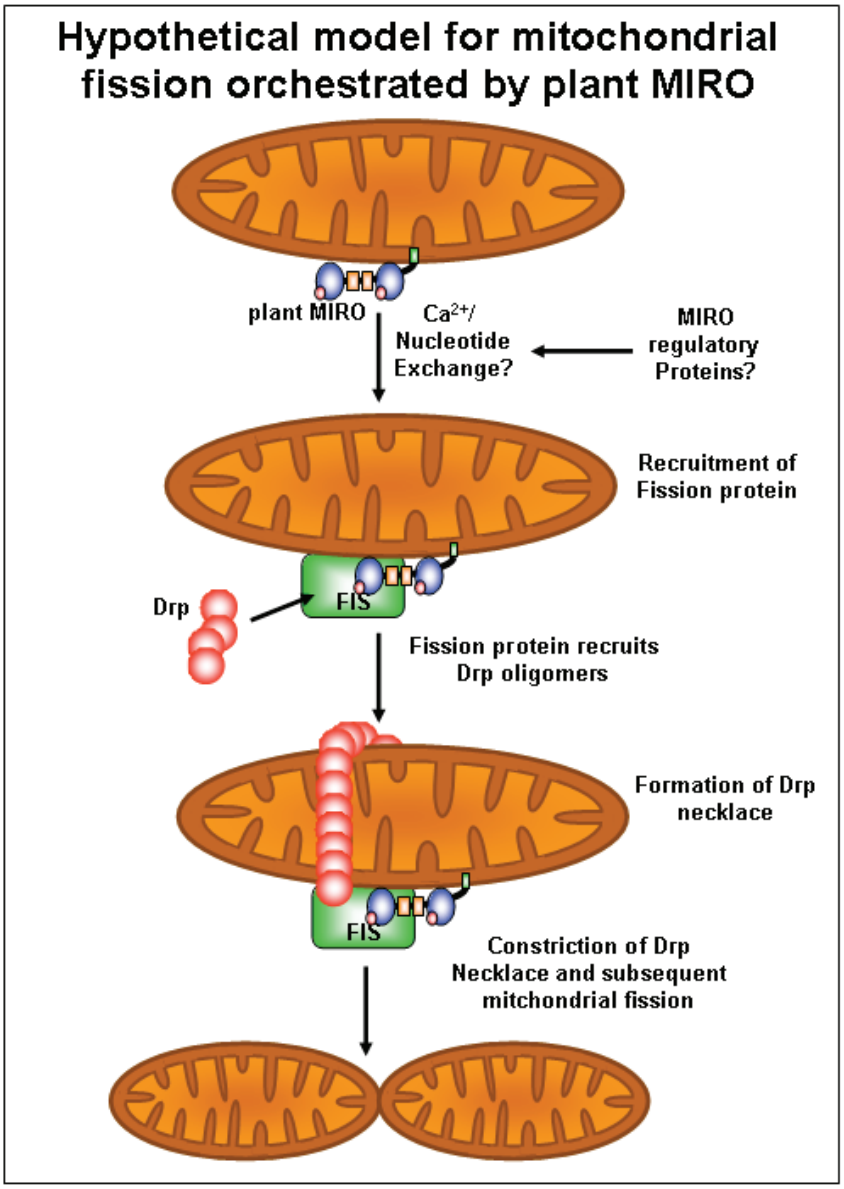

Figure 12: A hypothetical model for putative MIRO-mediated mitochondrial fission in plants. Possibly, changes in intracellular calcium levels or activation of MIRO G domains may facilitate interaction with fission promoting proteins like the conserved FISSION1 (FIS) protein. After MIRO associates with FIS in the mitochondrial outer membrane the fission of mitochondria in plants most likely follows a more conserved pathway. Drp oligomers are recruited to the MIRO/FIS complex and a Drp necklace-like structure forms around the mitochondria. Constriction of the mitochondrion by the Drp necklace results in subsequent fission. The figure is modified from MacAskill and Kittler [134].

Concluding remarks

Since their discovery in the early/mid nineties, research has revealed that the Rho GTPases in plants are involved as key regulators in several aspects of plant cellular processes. Having originated from an ancestral Rac GTPase, the Rho of plants as well as their regulators and possibly effectors, have steadily evolved in the kingdom of plants. The ROP structure is based on a conserved Rho G domain architecture but also contain structural elements that are most likely specific and of functional importance for ROPs. The PRONE domain-containing ROPGEF family, are proteins that are only found in plants and may also have evolved other functional aspects that remains to be discovered. Crystal structures of various forms of ROP interacting with the PRONE domain have revealed a universal mechanism for GEF nucleotide exchange that is relevant for all small GTPases.

The role of ROPs during polar growth (pollen) and diffuse growth (pavement cells) have been studied extensively in the last decade. Results from these studies show that ROPs, like their metazoan counterparts, are instrumental regulators of the cytoskeleton during these cellular events. Plant-specific ROP regulators and effectors have been discovered, whose function is not yet fully understood, and co-crystals of ROP in complex with these proteins would further enlighten our understanding of ROP-mediated signaling.

Recent research on post-translational modification of ROPs shows that S-acetylation is important for ROP activity and localization to DRMs or lipid rafts in the PM. These findings have added a new dimension to “ROP science” and will certainly be the basis for important discoveries in the future. The prospect of ROPs being a part of and possibly being instrumental in forming lipid rafts from which signaling events are mediated, is rather interesting and would certainly not only be of interest to the plant molecular biologist. Recent research have also identified upstream RLKs that activate a ROP signaling cascade and hopefully in the near future, a ROP signaling pathway will be indentified with all the components from receptor to intracellular response. The field of “ROP-science” is not by far completed, and there are several unsolved topics within the field to keep scientists occupied for years to come.

The MIRO GTPases are relatively speaking the “new kids on the block”, and since the initial characterization of human MIRO GTPases by Fransson and colleagues in 2003, “only” 19 scientific articles on MIRO function in Arabidopsis, Drosophila, yeast, slime molds and humans have been published. However, regardless of the number, these publications firmly

place MIRO as a central actor in mitochondrial transport and morphology in eukaryotic organisms. Most of the studies have focused on MIRO function in neuronal cells, demonstrating the important role of MIRO in mitochondrial transport and morphology in these cell types. The discoveries that hMIRO interacts with proteins that in mutated forms are associated with neurological diseases, has broadened the basic knowledge of the cellular mechanisms behind these diseases.

In plants, on the other hand, only three MIRO papers have been published to date. In contrast to research on hMIRO and dMIRO, no plant MIRO interacting proteins have yet been discovered. However, it is evident that plant MIRO in some way affects fusion and/or fission events of mitochondria in plant cells as well. One possibility is therefore that the evolutionary conserved role for MIRO is regulation of mitochondrial morphology. In metazoan lineages, transport may have been acquired as an additional function for MIRO, or it may have been lost or plays a minor role in plants as a result of predominant actin/myosin based organelle transport. To shed light on plant MIRO functions, future investigations should therefore focus on isolating potential MIRO interacting proteins. When considering possible MIRO interacting proteins, it is worth noting that for each GTPase family within the Ras superfamily there is a conserved set of G domain regulatory proteins. Both guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) are reported for all families except MIRO GTPases. In this respect it would not be surprising if GEF- and GAP-like regulatory protein also exist for MIRO GTPases. The prospect of putative MIRO regulatory proteins in eukaryotes is certainly interesting and should warrant further investigation. Similarly to the ROP PRONE domain, the putative MIRO GEF and GAP proteins may have evolved domains that share little or no homology with known Ras superfamily regulatory domains. This may be a part of the reason for not having discovered such regulatory proteins yet, if any exists. However, it should also be mentioned that some of the mammalian Rho GTPases, like the Rnd, RhoH and RhoBTB GTPases are not regulated by GAPs and GEFs. They are GTPase deficient and are most likely regulated at the transcriptional and proteasome level rather than by GTP/GDP cycling. Recent MIRO/Gem1p research however, shows that MIRO GTPases have not adopted a similar mode of regulation. Instead, both G domains seem capable of binding and hydrolyzing GTP, but similarly to other RAS G domain this intrinsic activity is slow [170]. Consequently, the possibility that MIRO regulatory proteins may have evolved should be investigated. Much is still to be discovered about the role of MIRO GTPases in eukaryotes, and future research will enhance our understanding of the mechanisms and importance of mitochondrial dynamics in cellular life.

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Paper I

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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

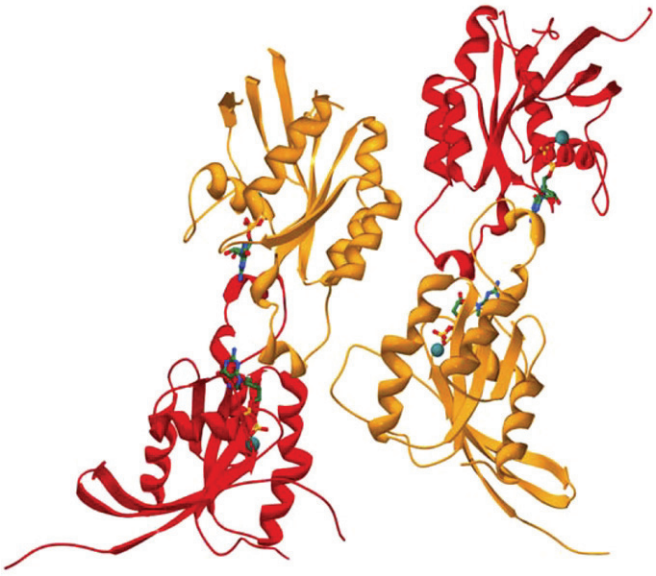

Fig. 1. The four AtRAC7/ROP9 molecules in the crystallographic asymmetric unit organized into two dimers. GDP is shown in stick representation and Mg is depicted as a green sphere. The figure was made using GlaxoSmithKline Swiss-PdbViewer v3.7 (http://ca.expasy.org/spdbv/), and visualized using the ray 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.)

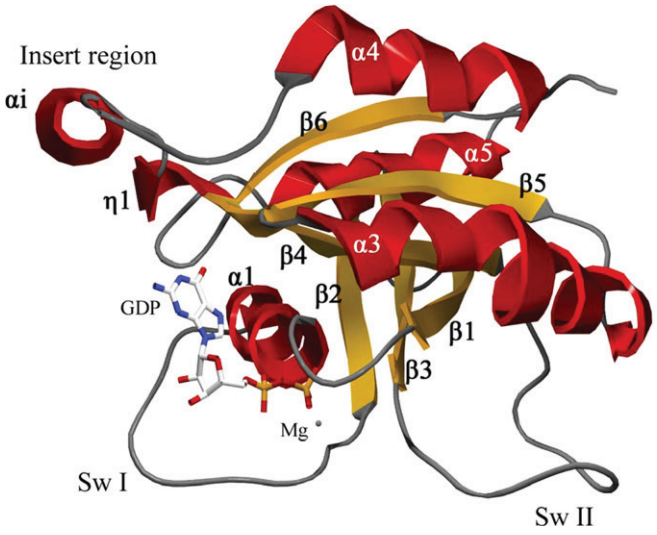

Fig. 2. The overall structure (ribbon representation) of AtRAC7/ROP9 (residues 1–181, molecule B) bound to GDP (stick representation) and Mg (grey sphere). The α-helices are in red and β-strands are shown in yellow. Secondary elements, are labelled as insert and switch (Sw) regions. αi denotes the insert 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

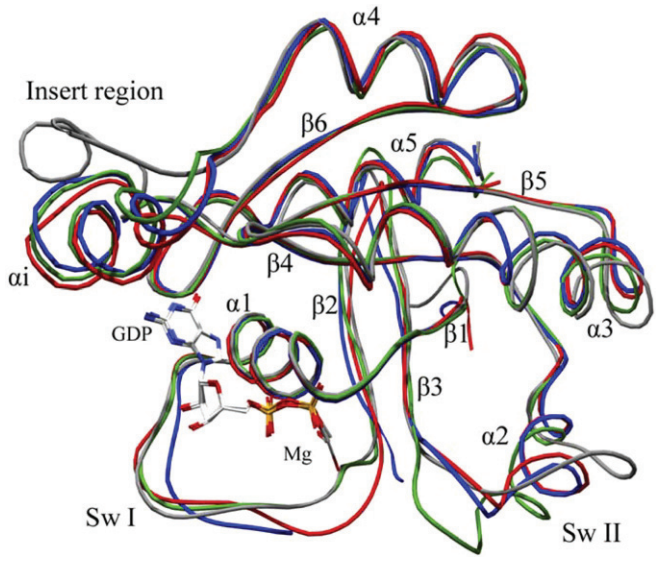

Fig. 3. The superposition of C_α-atom tracing of AtRAC7/ROP9 is shown in gray, HsCDC42 (pdb: 1A4R) in red, HsRHOA (pdb: 1FTN) in green and HsRAC3 (pdb: 2C2H) in blue. Visible secondary elements have been labeled, as well as insert and switch (Sw) regions. The figure was made using GlaxoSmithKline Swiss-PdbViewer 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.

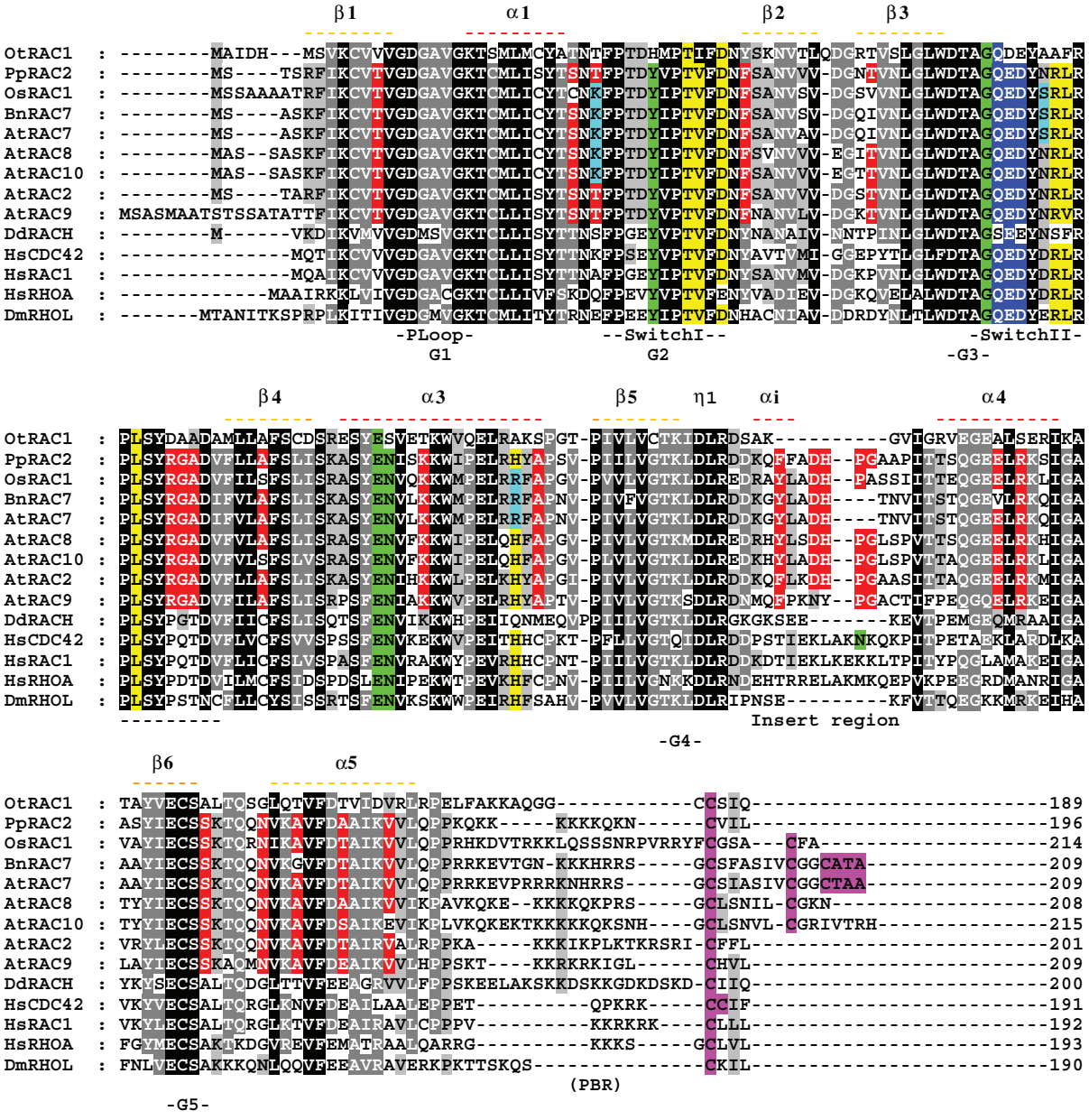

Fig. 4. An alignment of selected AtRAC/ROP proteins with homologues from different species: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum* (slime mold); Hs, *Homo sapiens*; Os, *Oryza sativa* (rice); Ot, *Ostreococcus tauri* (green algae); 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-

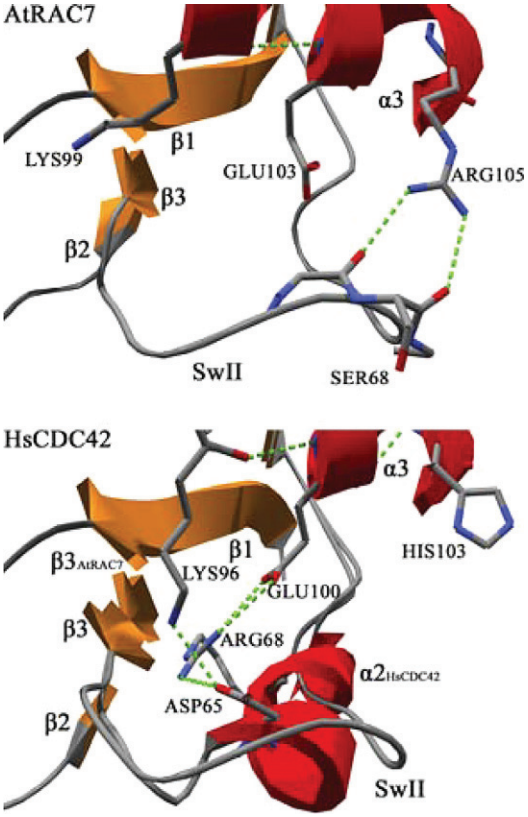

Fig. 5. The switch II region of AtRAC7/ROP9 (molecule B) and HsCDC42 (PDB: 1A4R) are shown, respectively. In addition to the ribbon presentation of secondary structures within and adjacent to the switch II region of each GTPase, side chains of key residues are also shown. Hydrogen bonds are shown with 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$^{134}_{\mathrm{AtRAC2}}$. 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$_{\mathrm{AtRAC7}}$) 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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Paper II

Arabidopsis thaliana MIRO1 and MIRO2 GTPases are unequally redundant in pollen tube growth and fusion of polar nuclei during female gametogenesis

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Abstract

MIRO GTPases have evolved to regulate mitochondrial trafficking and morphology in eukaryotic organisms. A previous study showed that T-DNA insertion in the Arabidopsis *MIRO1* gene is lethal during embryogenesis and affects pollen tube growth and mitochondrial morphology in pollen, whereas T-DNA insertion in *MIRO2* does not affect plant development visibly. Phylogenetic analysis of MIRO from plants revealed that MIRO 1 and 2 orthologs in dicots cluster in two separate groups due to a duplication event, suggesting that functional redundancy may exists between the two *MIRO* genes. To investigate this possibility, we generated a *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ double mutant. Compared to the *miro1*$^{(+/-)}$ single mutant, the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant showed increased segregation distortion. Siliques from the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant contained less aborted seeds, but more than 3 times the number of undeveloped ovules. In addition, reciprocal crosses showed that co-transmission through the male gametes was nearly absent, whereas co-transmission through the female gametes was severely reduced in the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant. Further investigations revealed that loss of *MIRO2* (*miro2*$^{(-/-)}$) function in the *miro1*$^{(+/-)}$ background enhanced pollen tube growth defects. In developing *miro1*$^{(+/-)}$/*miro2*$^{(-/-)}$ mutant embryo sacs, fusion of polar nuclei was further delayed or impaired compared to the *miro1* mutant. A defective polar nuclei fusion phenotype has previously not been reported for any *miro* mutants. Such defects have been associated with mutations in mitochondria-targeted genes. Our observations show that loss of function in *MIRO2* in a *miro1*$^{(+/-)}$ background enhances the *miro1*$^{(+/-)}$ phenotype significantly, even though the *miro2*$^{(-/-)}$ mutant alone does not display any phenotypes. Based on these findings, we conclude that MIRO1 and MIRO2 are unequally redundant and that a proportion of the *miro1*$^{(+/-)}$/*miro2*$^{(-/-)}$ plants haploid gametes displays the complete null phenotype of MIRO GTPase function at key developmental stages.

Introduction

Mitochondria are main cellular source for energy in eukaryotic cells. Additionally, mitochondria are important for calcium homeostasis, oxidative stress processes and production of metabolic intermediates and programmed cell death (PCD). Mitochondria are highly dynamic organelles that are transported on microtubule/actin structures within the cell. Their dynamic behavior is also reflected in fusion and fission events that change the number and morphology of mitochondria. In plants, research has elucidated how mitochondria move along cytoskeletal tracks and how mitochondrial fission takes place in plant cell. Still, the molecular events behind mitochondrial fusion are largely unknown in plants [1,2,3]. Studies of mitochondrial dynamics in cultured tobacco cells showed that movement mainly is dependent on cytoplasmic actin strands, whereas immobilization is dependent on both actin and microtubules [4]. In contrast to plants, the movement of mitochondria in animal cells mainly occurs along microtubules and is facilitated by kinesins. In neurons, transport along axons is necessary for accumulation of mitochondria in regions with high energy demands. The main players involved in linking kinesin to mitochondria are the MIRO GTPases and Milton [5].

Human MIRO GTPases were discovered through a genome search for RHO consensus domains by Fransson and colleagues [6], and were classified as mitochondrial RHO GTPases. They are atypical to conventional Rho GTPases in possessing two G-domains separated by two calcium binding EF-hand motifs. MIRO GTPases are exposed towards the cytosol, and are connected to the outer membrane of mitochondria through a C-terminal transmembrane domain [6,7]. The two GTPase domains of Miro lack the typical Rho-specific insert region and have an overall sequence divergence from other Rho GTPases. Thus, MIRO GTPases may be considered to constitute a new subfamily of the Ras superfamily of small GTPases [8]. Orthologs of MIRO GTPases have been discovered in yeast (Gem1p) and Drosophila (dMIRO). Common for these orthologs is their importance in mitochondrial trafficking and morphology [9,10]. In Drosophila, the adaptor protein Milton binds to MIRO and recruits kinesin heavy chain to form a microtubule transport complex in axons [11]. In humans, two Milton-related proteins (GRIF-1 and TRAK1/OIP106) have been shown to interact with hMIRO through the N-terminal

GTPase domain and mediate mitochondrial transport by modulating kinesin activity [12,13].

The Arabidopsis genome encodes three MIRO GTPases that are predicted to have the same domain organization as MIRO GTPases described in other species. Localization experiments showed that MIRO1 (At5g27540) and MIRO2 (At3g63150) localize to mitochondria through a C-terminal trans-membrane domain [14]. *MIRO1* and *MIRO2* are ubiquitously expressed in all plant tissues, whereas *MIRO3* (At3g05310) shows very low expression in comparison [14]. Further observations revealed that developing embryos homozygous for a T-DNA insertion in *MIRO1* arrests during early stages of development [14]. A recent study shows that aberrant mitochondrial morphology and distribution in *miro1*$^{(-/-)}$ embryonic cells significantly contributes to the observed developmental arrest. Apical cells in arrested two-celled *miro1*$^{(-/-)}$ embryos contain significantly less mitochondria compared with wild type cells [15]. Mutation in *MIRO1* also influence pollen germination as well as mitochondrial morphology and streaming during pollen tube growth, which in turn resulted in reduced male genetic transmission of the mutant allele [14]. In the same study two mutant lines with T-DNA insertions in the *MIRO2* gene were studied. Homozygous *miro2* mutant plants showed no apparent mutant phenotypes, suggesting that MIRO2 plays no important role during plant development and that MIRO2 apparently is not functionally redundant to MIRO1.

An Arabidopsis Calcium Binding GTPase (AtCBG) discovered in a screen for EF hands and GTPase domain reported by Jayasekaran and colleagues [16] is actually MIRO2. According to the study, MIRO2 shows calcium dependent GTPase activity and two MIRO2 T-DNA mutants investigated were reported to be sensitive to both NaCl and ABA stress.

Here we show, by generating a *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant plant, that MIRO2 is unequally redundant to MIRO1 during specific stages of gametophyte development and function. Unequal genetic redundancy is defined as a phenomenon where loss of function in one gene produces mutant phenotypes, whereas a mutant with loss of function in the paralogous gene does not display any phenotypes. Importantly, loss of function in both paralogous genes results in strong enhancement of the initial phenotype [reviewed in 17]. Our results show that crossing of *miro1* and *miro2* produces mutant plants with enhanced *miro1* mutant phenotypes and that a proportion

of the developing haploid male and female gametes display the full null phenotype of MIRO GTPase function.

Methods

Gene expression and phylogenetic analysis

For gene expression analysis, transcriptome data were obtained from the Arabidopsis eFP browser [18] and visualized using Microsoft Excel 2003.

Plant MIRO protein sequences were downloaded from the NCBI database. Predicted protein sequences were imported into the ClustalX program [19] and a pairwise alignment was made using the Gonnet 250 score matrix. The resulting protein alignment was exported as a MSF file and imported into the GeneDoc program [20] for manual editing. The edited alignment was re-imported into ClustalX and a bootstrapped neighbor joining (NJ) tree was made running 1000 bootstrap trials. A rooted phylogenetic tree was constructed with the TreeView program [21], where the *Physcomitrella patens* PpMIRO2 was used as an outgroup. Accession numbers for the various Miro GTPases are listed in supplemental file S1.

Plant growth conditions

Seeds were surface-sterilized using vapor phase chlorine gas for 3-4 hours and plated onto half strength Murashige-Skoog medium, pH 5.8, 0.6% (w/v) agar. The growth media was supplemented with 25 μg/ml Kanamycin (*miro2-2*) and/or 10 μg/ml BASTA (*miro1*). Seeds were vernalized for 48 hours before germination at 22^{o}C, 16-h light and 18^{o}C, 8-h dark conditions. 7 DAG selection resistant seedlings were transferred to soil and grown under the same conditions as above.

miro T-DNA mutants; identification and crosses

The *miro2-2* (SALK_157090) mutant was backcrossed into Col-WT background before it was crossed with the *miro1* (*emb2473*) mutant; thus *miro2-2* was backcrossed twice and *miro1* once. Genomic DNA was isolated using SP Plant Mini Kit (Omega) and REDExtract-N-AMP Plant PCR Kit (Sigma) was used for the segregation analysis.

The various mutant T-DNA insertions were verified using PCR with T-DNA specific primers and gene specific primers (Figure 1B and C); *miro1*: (WT) 5'-CAGGAATCAACTACTGATGAGC3' and 5'-CCAGTTGCTTGTAGAAGTTGCA-3', (T-DNA) 5'-CCAGTTGCTTGTAGAAGTTGCA-3' and 5'-GCATCTGAATTTCATAACCAATC-3'; *miro2-2*:(WT) 5'-

GTTAGTAGCAAAAGTCTGAACT-3' and 5'-GGGTTCTCTGCTGTACTCACGA-3', (T-DNA) 5'-GTTAGTAGCAAAAGTCTGAACT-3' and 5'-CGGAACCACCATCAAACAGGAT-3'.

Phenotypical analysis

Mature siliques from the same positions along the main inflorescence were measured for length and dissected to identify aborted ovules and embryo lethality. The 5 first siliques on the main inflorescence were avoided for this analysis. Pollen viability test using Alexander stain was performed as described in [22]. Mature pollen nuclei were stained using 1 μg/ml DAPI in extraction buffer (0.1% Nonidet P40, 10% DMSO, 50 mM PIPES pH 6.9, 5 mM EGTA pH 7.5). Pollen germination assays were performed as described in [22] and germinated over night. Germinated pollen was stained over night at 37^{o}C with 1 mg/ml X-Gluc solution containing 50 mM Na_3PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM EDTA, 0.01% Triton X-100 and 10% (w/v) sucrose. For embryo sac analysis, siliques were cleared over night in Hoyer's solution. Images were acquired with a Nikon E800 microscope/Nikon DsRi1 camera using NisElements F software. Pollen tube lengths were measured using ImageJ [23] software. Images were processed using Adobe Photoshop Elements 4.0.

Results

Evolution of MIRO GTPases within Embryophyta

Database searches indicates that MIRO GTPases exist in Metazoa, Fungi, Rhodophyta, Stramenopiles, Alvoelata, Heterolobosea, Euglenozoa, Mycetozoa and Viridiplantae, whereas they are missing from the anaerobic Entamoebidae and Parabasalia that lack mitochondria all together, suggesting that MIRO GTPases are only found organisms that contain mitochondria. However, MIRO GTPases are not present in Haptophyceae that contain mitochondria, which indicate that MIRO GTPases are not required in some forms of eukaryotic life [24]. A phylogenetic analysis of MIRO proteins in Embryophyta was performed based on protein primary structure alignments, and the phylogentic relationship between 35 MIRO proteins was visualized as a phylogram rooted with a *Physcomitrella patens* MIRO ortholog as an outgroup (Figure 1). In Embryophyta, Miro GTPases are found in mosses, Coniferales, monocots and dicots. In dicots, the paralog MIRO genes (MIRO1 and MIRO2) cluster into two distinct MIRO subgroups (I & II) with bootstrap confidence levels above 99%.

The origin of the MIRO paralogs in dicots is due to a gene/genome duplication event that occurred after the diversification of monocots and eudicots. Additionally, sometime during evolution of the Brassicaceae family an additional duplication event within MIRO subgroup I resulted in development of the MIRO3 paralogs that show a rapid divergent evolution compared to other subgroups.

Since paralogous genes often have the same or similar function, it is likely that MIRO paralogs may display some degree of functional redundancy during plant development.

Yamaoka and Leaver reports that the two paralogs *MIRO1* and *MIRO2* are expressed in all plant tissues investigated, implying functional roles during plant growth and all developmental stages. However, neither *miro1* nor *miro2* T-DNA mutants shows developmental defects during sporophytic growth [14].

To investigate quantitative expression differences between *MIRO1* and *MIRO2* during gametophyte development closer, we used the Arabidopsis eFP browser [18]. The *in silico* analysis revealed that both *MIRO1* and *MIRO2* are expressed in all tissues and stages (figure 2). Comparing these expression profiles with the *miro1*$^{(+/-)}$ phenotypes reported by Yamaoka and Leaver [14], it is striking that *MIRO2* shows higher

expression at the globular stage and the following stages during embryo development compared to *MIRO1*. The *miro1*$^{(-/-)}$ embryos abort early during embryo development, between the zygote and the four-terminal-cell stage. However, data from the Arabidopsis eFP browser does not contain any expression data from these stages. Still, these findings indicate that MIRO2 may be functionally redundant to MIRO1 during embryo development. Yamaoka and Leaver [14] also reports that *miro1* pollen show reduced germination rate and pollen tube growth compared to wild type pollen. The expression data presented here shows that during pollen development and germination, *MIRO2* has higher expression levels compared to *MIRO1* and clearly suggests that MIRO2 could be functionally redundant to MIRO1.

Interestingly, *MIRO3* shows very high expression in both chalazal and peripheral endosperm during seed development (from pre-globular to heart stage) with up to 110 and 80 fold higher expression levels compared to *MIRO1* and *MIRO2*, respectively [Data from Arabidopsis Seed eFP browser, 25]. This expression pattern suggests that within Brassicaceae, MIRO3 orthologs may have evolved to function mainly in endosperm development.

Considering the evolution of eudicot MIRO GTPases, the expression pattern divergence during gametophyte development and the absence of phenotype in the *miro2* T-DNA mutants, we wanted to investigate if unequal genetic redundancy exits between the MIRO1 and MIRO2 paralogs in Arabidopsis. By generating *miro1*$^{(+/-)}$/*miro2*$^{(-/-)}$ plants it should be possible to discern if genetic redundancy between the MIRO1 and MIRO2 paralogs exists. Importantly, if genetic redundancy exists this should be manifested as novel or enhanced *miro1*$^{(+/-)}$ phenotypes.

miro T-DNA mutants

In order to study the functional relationship between MIRO GTPases in Arabidopsis, we obtained independent mutant lines from publicly available seed collections. *miro1*/*emb2473* was obtained from the Seed Genes Project [26] and *miro2*/SALK_157090 was obtained from the SALK collection [27]. These two mutant lines are the same as those studied by Yamaoka and Leaver. Both lines are in the Columbia background (Col-0) and are henceforth designated as *miro1* and *miro2-2* respectively [in accordance with 14]. The *miro1* and *miro2-2* mutants harbor T-DNA insertions in the beginning and the end of the 12th exon of *MIRO1* and *MIRO2*, respectively (Figure 3A). To investigate whether genetic redundancy between the

MIRO1 and *MIRO2* genes exits, we crossed the heterozygous *miro1* mutant with *miro2-2* in order to possibly obtain a *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ double mutant.

Segregation analysis of self-pollinated *miro1*$^{(+/-)}$ plants showed that 57.1% (Table 1) of the progeny were viable on MS media supplemented with BASTA, which concurs with Yamaoka and Leavers observations [14]. For self-pollinated *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants from the crossings we expected 37.5% (10:6) viable progeny on MS media supplemented with BASTA (*miro1*) and kanamycin *(miro2-2*). Since *MIRO1* and *MIRO2* are located on two separate chromosomes, one would expect that if the T-DNA insertions in the *MIRO2* locus do not contribute to gametophyte development and function, they will segregate independently from the *miro1* allele.

If so, expected segregation of *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ alleles from self-pollinated *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants would be 33.3% (2:1) within all progeny resistant to selection agents. Notably, no *miro1*$^{\underline{(-/-)}}$/*miro2-2*$^{(-/-)}$ progeny will be formed during self-fertilization of *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants. However, segregation analysis (Table 1) showed that 29.9% of the progeny from self-pollinated *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants were resistant to both selection agents. This is significantly lower than the expected 37.5% (*P* value = 0.0007) and suggested that additional loss of function in MIRO2 has an additional effect on gametophyte development or function. To validate this finding further, we genotyped the progeny from the self-fertilized *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants. PCR analysis (Supplemental file S2) of 80 individual plants grown on selective media showed that 17 plants (21.3%) were *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutants. This result deviates significantly from the 2:1 hypothesis (*P* value = 0.0218) and clearly indicates that the two alleles do not segregate independently.

From self-pollinated *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants however, only 16.8% of the germinating progeny were resistant to both selection agents and viable on MS media. In comparison, 57.1% of the *miro1*$^{(+/-)}$ plants were resistant to BASTA. Taken together, the segregation analysis of the *miro1* and *miro2-2* alleles clearly indicates that a T-DNA insertion in the *MIRO2* locus does not segregate independently of the *miro1* locus, but rather that there is some level of functional redundancy between the *MIRO1* and *MIRO2* genes.

The *miro1*/*miro2-2* double mutant show increased gametophytic defects

During sporophyte development, no visible phenotypes were observed in *miro1*/*miro2-2* heterozygous plants or the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants. A closer

investigation of siliques from the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants showed that the siliques are significantly shorter compared to both wild type plants and the individual *miro* plants (Figure 4A). The length of siliques collected from the same positions of the main inflorescence of wild type and *miro* plants was measured and an unpaired Student's T-tests analysis was performed. T-tests showed significant differences ($P < 0.0001$) in silique length between WT-Col (1.33 cm, SD = 0.056 cm, n = 10), *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ (1.11 cm, SD = 0.04 cm, n = 10) and *miro1* (1.22 cm, SD = 0.038 cm, n = 10) (results shown are representative data from one of three separate experiments and each experiment showed significant differences in comparison of silique length). We believe that this phenotype is not of sporophytic origin but that it may be a result of a lower degree of fertilization in mutant plants.

Yamaoka and Leaver reported 10% unfertilized ovules and 13% aborted seeds within *miro1* siliques [14]. During our experiments we observed similar numbers, with 7.4% unfertilized ovules and 17.2% aborted seeds (n = 1318) in *miro1* siliques (Table 2). In contrast, the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants (Figure 4B) produced siliques with 34.5% unfertilized ovules and 3.4% aborted seeds (n = 1165) randomly dispersed inside the silique, which indicate that the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant plant has an increased impact on male and/or female gametogenesis and/or gamete function compared to *miro1*$^{(+/-)}$ mutant.

Furthermore, *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ siliques contained less aborted seeds than *miro1*$^{(+/-)}$ siliques. The background of this phenotype was further studied by co-transmission efficiency (TE) analysis of the mutant alleles. Reciprocal crosses showed that co-transmission (TE: selection^{R}/selection^{S}) of both *miro* alleles through the male gametes was 0.12% (n = 796); through the female gametes the co-transmission efficiency was 34.7% (n = 625, % of total seedlings: 25.8%). These co-transmission efficiencies are significantly lower than what was reported for the transmission *miro1* allele alone (12.8% and 75.2%, respectively) [14].

The severe impact of *miro2-2* allele on male genetic transmission in the *miro1* background means that formation of homozygous *miro1* embryos rarely occurs in the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ siliques, thereby explaining the reduction of aborted seeds in the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants. This also implies that most of the observed unfertilized ovules may be a result of impaired female gametophyte development caused by maternally inherited *miro1/miro2-2* alleles. However, the penetrance of the female gametophyte defect is not complete since 16.8% of the offspring carry both

miro1/miro2-2 alleles. Incomplete penetrance is not an uncommon phenomenon and has been reported for other mutants affected in female gametophyte development as well [28].

Loss of function in MIRO2 enhances pollen tube growth defects in the *miro1*$^{(+/-)}$ background

The low co-transmission efficiency through the male gamete suggests aberrant pollen development, germination and/or tube growth. Previous studies showed that pollen from *miro1*$^{(+/-)}$ plants matured normally, but that both pollen germination and tube growth was impaired [14].In the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant, half of the developing male gametes carry the *miro1 and miro2* T-DNA alleles, which could possibly lead to defects in pollen development. This notion is supported by the fact that *MIRO2* shows higher expression levels compared to *MIRO1* during male gametophyte development and tube growth.

A pollen viability test using Alexander´s stain was performed and showed that all of the mature pollen from *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutants were viable (Figure 5A). Mutant pollen was morphologically undistinguishable from wild type pollen (Figure 5B). Nuclear staining with DAPI showed that the pollen developed normally and reached maturity with two sperm cell nuclei and a vegetative nucleus (Figure 5C). We therefore conclude that homozygous loss of MIRO2 function in *miro1*$^{(+/-)}$ background does not give an additional effect on pollen development and viability.

The pCSA110 T-DNA insertion in *miro1*$^{(+/-)}$ mutants contains the *GUS* reporter gene regulated by the pollen-specific LAT52 promoter, making distinction between mutant and wild type pollen possible [29]. Pollen from *miro1*$^{(+/-)}$ and *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant plants were collected and germinated on solid pollen media and stained with X-Gluc solution to assess if loss of MIRO2 function in the *miro1*$^{(+/-)}$ background affects pollen tube growth. GUS negative pollen from both *miro1*$^{(+/-)}$ and *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ appeared to grow normally . As expected from previous results, GUS positive pollen in *miro1*$^{(+/-)}$ showed reduced germination and tube growth [14]. The *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants showed highly significant ($P < 0.0001$) additional impairment of pollen tube growth compared to the *miro1*$^{(+/-)}$ alone (Figure 6). GUS positive pollen tubes from *miro1*$^{(+/-)}$ grew to an average of 436.2 μm (SD = 136.0 μm, n = 133) whereas GUS positive pollen tubes from *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants grew to an average of 178.3 μm (SD = 84.8 μm, n = 209) after 17 hours of growth (results

shown are data from 4 separate experiments). All in all, these observations clearly indicate that loss of MIRO2 function in a *miro1*$^{(+/-)}$ background does not affect pollen development but has an additional strong negative effect on pollen tube growth.

miro1$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutants are affected in embryo sac development

Since co-transmission of both *miro* mutant alleles through the male gametophyte is nearly absent, the observed undeveloped ovules must be due to a combined effect of the *miro1/miro2-2* alleles during female gametophyte development. To investigate closer at what stage the undeveloped ovules are affected, both *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ and *miro1*$^{(+/-)}$ flowers were emasculated, and the siliques were cleared and observed with DIC-microscopy after 48 hours. In ovules from the *miro1*$^{(+/-)}$ plant, 19.0% (n = 327) of the embryo sacs displayed two slightly larger nuclei localized adjacent to each other in addition to both egg cell nuclei and synergid cell nuclei. This phenotype was interpreted as a defect or delay during fusion of the polar nuclei (karyogamy) (Figure 7B). In the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant we observed that 43.1% (n = 418) of the ovules displayed embryo sacs with defects in fusion of polar nuclei. The remainder of the ovules from *miro1*$^{(+/-)}$ and *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutants and all ovules from WT plants (n = 228), had an embryo sac with a normal cellular constitution (one enlarged central cell nucleus, one egg cell nucleus and synergid cell nuclei) (Figure 7A). This defect or delay in fusion of polar nuclei indicates that both MIRO1 and MIRO2 play a role during karyogamy. Karyogamy occurs three times during the lifecycle of angiosperms: once during embryo sac development when the two polar nuclei fuse to form the central cell nucleus and twice during fertilization, where the two sperm cell nuclei fuse with the egg cell and central cell nuclei [28].

Crosses of *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant (female) with wild type pollen showed a co-transmission efficiency of 34.7%, approximately twice of what is observed with self-fertilized mutant plants. This result strongly indicates that pollen carrying wild type MIRO1 and MIRO2 are able to fertilize and thereby "salvage" some mutant ovules during fertilization. In this case, where male co-transmission is close to zero, it is therefore reasonable to assume that some of the structures that are observed as undeveloped ovules in *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ siliques are fertilized ovules that are arrested during or shortly after fertilization. In self-fertilized *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants, ovules are mainly fertilized by MIRO1/*miro2-2* pollen. Homozygous *miro1* embryos rarely forms and the defects in fertilization/early embryo development may

be an additional effect of the paternally inherited *miro2-2* allele. This is also in line with the increase in aborted embryos in *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant siliques (3.4% versus 0.8% in Col-wild type).

Discussion

Our results show that MIRO1 and MIRO2 are unequally redundant in function and that both genes affect pollen tube growth, fusion of polar nuclei during embryo sac development and possibly also nuclei fusion during fertilization. A total loss of MIRO2 function in heterozygous *miro1*$^{(+/-)}$ background results in enhanced *miro1* phenotypes. Even though MIRO2 initially appeared to be dispensable in gametophyte function, ovule development and embryo development compared to MIRO1, it has retained a significant functional role. In an evolutionary context, this fact may be the reason for maintaining a genomic copy of *MIRO2*, which is manifested as unequal genetic redundancy.

Unequal genetic redundancy is in part attributed to differences in expression patterns and/or expression levels between paralogous genes [17,30]. In the case of *MIRO1* and *MIRO2*, expression levels are both overlapping and quantitatively different in key developmental stages where phenotypes are present in both *miro1*$^{(+/-)}$ and *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants (Figure 2).

Contradictory to the observed lack of phenotype in *miro2*$^{(-/-)}$ plants, *MIRO2* shows higher expression compared to *MIRO1* in male gametophytic tissues and several of the embryonic stages (Figure 2). One would expect that loss of function in MIRO2 alone would result in deleterious phenotypes at these developmental stages.

The fact that *MIRO1* and *MIRO2* shows quantitative divergence in expression is indicative of the following fates of the paralogous genes after the duplication; A) neofunctionalization, where the duplicated genes gain a novel function, or B) subfunctionalization, where the function is sub-divided between the two paralogs. Notably, in the latter scenario, both of the paralogous genes represent the total function of the two genes [30,31]. After duplication, both the regulatory and coding sequences of the paralogous genes may acquire mutations or be subjected to epigenetic effects that affect both the functions and expression patterns of the genes. In support of this assumption, statistical analysis of the expression pattern of 280 phylogenetically identified paralogous pairs in Arabidopsis revealed that 85% of the pairs showed differential expression levels depending on the organ investigated. These findings suggest that mutations in *cis*-acting elements in the promoter regions of the gene pairs contribute to the observed expression pattern shifts. Therefore it is

believed that regulatory subfunctionalization and/or neofunctionalization will in part be responsible for the maintenance of the paralogous pair over time [30].

The expression pattern shifts between *MIRO1* and *MIRO2* (Figure 2) supports a hypothesis where a genomic copy of *MIRO2* is retained since it may have undergone regulatory subfunctionalization and/or neofunctionalization after duplication. However, one can not rule out the possibility that MIRO2 also have accumulated mutations in coding regions, resulting in functional subfunctionalization and/or neofunctionalization. Thus, MIRO2 may not have the same level of protein activity as MIRO1, which could explain why *miro2*$^{(-/-)}$ plants do not display any phenotype. In a *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ setting, however, the cumulative protein activity of the gene pair is below a certain threshold that results in enhanced *miro1* phenotypes [17].

Finally, it should be noted that plants grown under optimal condition in the laboratory does not reflect the various environmental conditions that the plants have been subjected to throughout its evolutionary history. Under certain natural conditions these expression shifts may provide a fitness advantage and therefore result in maintenance of the paralogous pair [32]. This may also be the case for Arabidopsis MIRO2 since it has been implicated in ABA and salt stress [16], which could indicate that MIRO2 have other functional roles compared to MIRO1 during certain environmental conditions. If this is the case, it could explain the difference in the phenotypes between *miro1*$^{(+/-)}$ and *miro2*$^{(-/-)}$ plants during regular growth.

The *miro1/miro2-2* alleles showed very low co-transmission through the male gametes, suggesting aberrant pollen development and/or function. However, our microscopic studies show that there is no additive or new aberrant effect of loss of function of MIRO2 in the *miro1*$^{(+/-)}$ background, suggesting that loss of function in MIRO1 and MIRO2 does not affect pollen development. This observation is intriguing when taking into consideration that MIRO1 affects mitochondrial morphology in pollen, possibly leading to changes in the intracellular distribution of mitochondria [14]. Furthermore, the fact that metabolic rates in developing pollen are higher compared to sporophyte tissue [33] should warrant the necessity for proper intracellular distribution and morphology of mitochondria during pollen development. Alternatively, male gametophyte development may not be affected due to initial transcription of wild type *MIRO1* in the diploid parental microsporocytes, resulting in sufficient amounts functional protein to rescue developing mutant male gametes in

miro1$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants. Such a hypothesis has been put forth by Berg and colleagues [34], in connection with loss of function in aminoacyl-tRNA synthetases predicted to function in mitochondria. As a consequence, mitochondria with wild type MIRO1 are inherited in the daughter cells during meiotic division and therefore proper mitochondrial distribution is sustained during development. However, an additional loss of MIRO2 function in a *miro1*$^{(+/-)}$ background enhanced pollen tube growth defects compared to single *miro1*$^{(+/-)}$ mutants. All GUS positive pollen tubes from the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ plants had significantly reduced tube growth compared to GUS positive *miro1*$^{(-)}$ pollen tubes (Figure 6). Our hypothesis is that these *miro1*$^{(-)}$/*miro2-2*$^{(-)}$ male gametophytes are not capable of fertilizing ovules due to impaired tube growth, which is confirmed by the reciprocal crosses where co-transmission through the male gametes was nearly absent.

Our data indicate that loss of function in both MIRO1 and MIRO2 affects female gametophyte development during fusion of the polar nuclei. Notably, this phenotype has not been reported earlier for loss of function in plant MIRO GTPases. A fraction of the mutant ovules mature normally, become fertilized and produce viable offspring. Similar to developing pollen, this observation may in part be explained by inheritance of wild type mitochondria with functional MIRO1 from the diploid megasporocyte. Furthermore, the surrounding sporophytic cells could provide sufficient amounts of metabolites to salvage the developing gametophytes. Nonetheless, our results show that both MIRO1 and MIRO2 affect mitochondrial function during female gametophyte development, and could also play a role in fertilization and early embryo development. Several knock-out studies of genes that encode mitochondria-targeted proteins show defects in gametogenesis. A particularly interesting mutant embryo sac phenotype observed in some of these mutants is the defect in fusion of polar nuclei (karyogamy) [35,36,37,38], which we also observe in the *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ mutant. In a recent publication by Kägi and colleagues [39] it was demonstrated that a deleterious point mutation in mitochondria localized cysteinyl-tRNA synthetase (SYCO) and an ATP/ADP translocator AAC2 results in defects of polar nuclei fusion. Central cell mitochondria in *syco* and *aac2* plants lack cristae, indicating that SYCO and AAC2 is important for the structural integrity of the central cell mitochondria [39]. These results confirm that polar nuclei fusion in the central cell is a mitochondria dependant process. Investigations further showed that, the antipodal

cells of the developing *syco* and *aac2* female gametophytes do not undergo PCD, suggesting that antipodal cell PCD is regulated by the adjacent central cell [39]. Our results are therefore in line with these findings where polar nuclei fusion is affected as a consequence of defects in mitochondrial function. The presence of EF-hands in the MIRO GTPases suggests a role for calcium ions in regulation of MIRO activity. Interestingly, during a large scale screen of mutants with impaired female gametophyte development, calmodulin binding proteins and Ca^{2+}-binding proteins were reported and linked to defects in fusion of polar nuclei [40].

Research on MIRO orthologs in other model organisms (Drosophila, mammalian and human cell lines) have shown that MIRO GTPases facilitates mitochondrial movement and distribution along microtubuli in a Ca^{2+}-dependent manner [reviewed in 41,42]. It is therefore not unlikely that plant MIRO GTPases perform a similar role, despite the fact that mitochondria in plants mainly move along actin filaments. The observation that mitochondrial streaming in growing pollen tubes is disrupted in *miro1*$^{(+/-)}$ mutants [14] supports this hypothesis. However, mitochondria in both *miro1*$^{(+/-)}$ pollen and embryos are enlarged, possibly due to increased fusion or the absence of fission events [14,15]. It is therefore tempting to speculate that the observed defects in mitochondrial streaming may be a secondary effect due to inability of the transport machinery to shuttle enlarged mitochondria along actin strands. Furthermore, this suggests that plant MIRO GTPases play a significant role in mitochondrial fusion/fission events rather than movement. Saotome and colleagues showed that overexpression of human MIRO promoted the formation of elongated mitochondria seemingly by suppression of Dynamin-related protein1 (Drp1) mediated fission of mitochondria [43]. The Arabidopsis orthologs of human Drp1; DRP3A and DRP3B, have also been shown to regulate mitochondrial fission in a functionally redundant manner [44] and therefore a similar link between plant MIRO GTPases and plant DRPs may exists as well. Future investigations should therefore focus on identifying plant MIRO-interacting proteins to elucidate how MIRO GTPases regulate mitochondrial morphology and possibly mitochondrial movement in plants.

The fact that *MIRO1* and *MIRO2* are unequally redundant should be taken into consideration in future functional investigations. This especially applies to studying gamete development and function since the *miro1*/*miro2-2* haploid gametes display the full null phenotype of MIRO GTPase function.

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Supporting information files

Supplemental file S1: Accession numbers for protein sequences used for phylogenetic analysis.

Supplemental file S2: PCR genotyping analysis.

Figures:

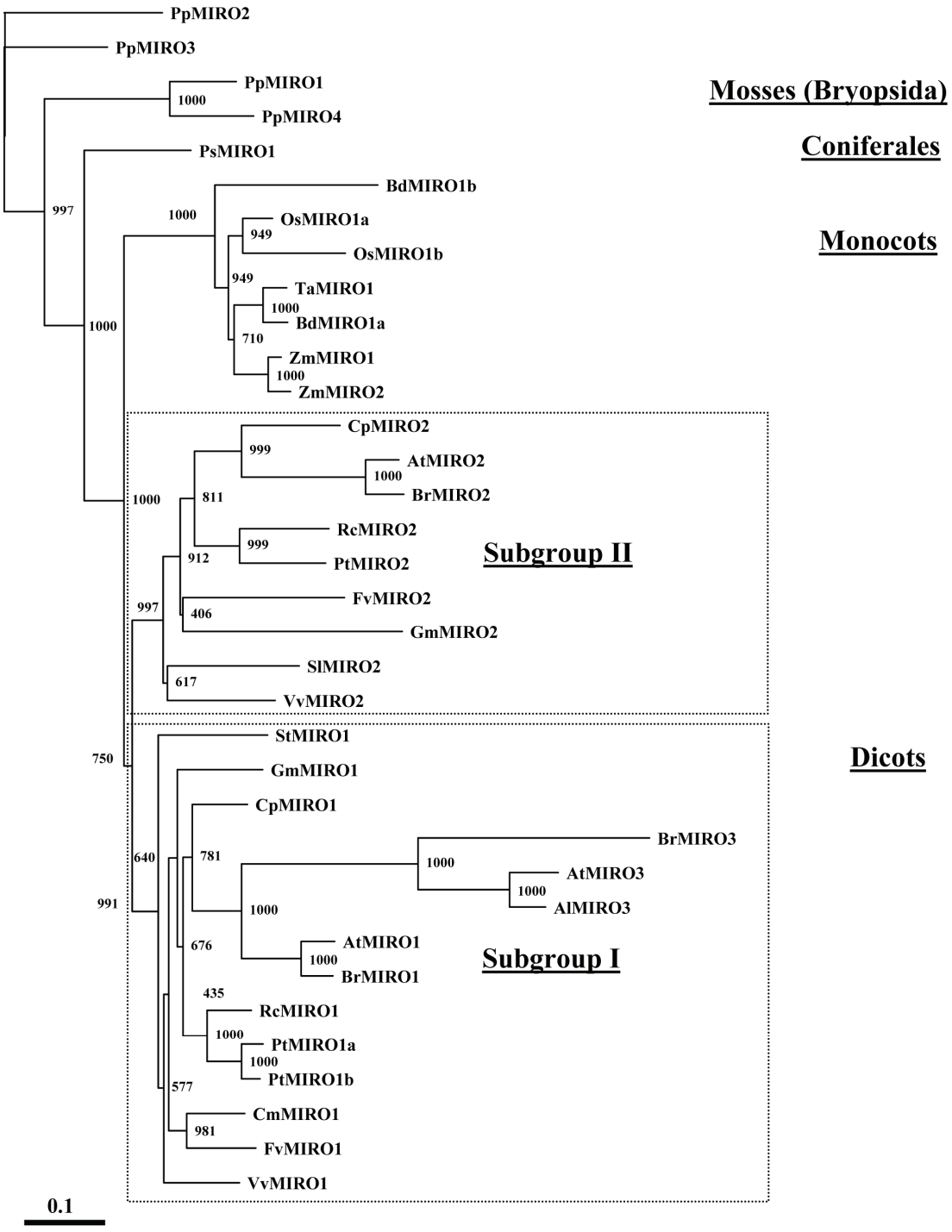
PpMIRO2
PpMIRO3
PpMIRO1
1000
PpMIRO4
Mosses (Bryopsida)
PsMIRO1
Coniferales
BdMIRO1b
997
1000
OsMIRO1a
949
OsMIRO1b
Monocots
949
TaMIRO1
1000
1000
BdMIRO1a
710
ZmMIRO1
1000
ZmMIRO2
CpMIRO2
999
AtMIRO2
1000
1000
811
BrMIRO2
RcMIRO2
999
912
PtMIRO2
Subgroup II
997
FvMIRO2
406
GmMIRO2
SlMIRO2
617
VvMIRO2
StMIRO1
750
GmMIRO1
CpMIRO1
BrMIRO3
640
781
1000
AtMIRO3
1000
991
AlMIRO3
1000
676
AtMIRO1
1000
Subgroup I
BrMIRO1
435
RcMIRO1
1000
PtMIRO1a
1000
PtMIRO1b
577
CmMIRO1
981
FvMIRO1
VvMIRO1
Dicots
0.1

Figure 1 - Phylogenetic tree of MIRO GTPases in Embryophyta

Phylogenetic tree based on protein sequence alignment of MIRO GTPases from plants. The tree is rooted with a *Physcomitrella patens* MIRO ortholog as an outgroup. Numbers indicate bootstrap values. Dashed line boxes enclose the two MIRO ortholog subgroups in dicots. Abbreviations: At- *Arabidopsis thaliana*, Al- *Arabidopsis lyrata*, Bd- *Brachypodium distachyon*, Br- *Brassica rapa*, Cm- *Cucumis melo*, Cp- *Carica papaya*, Fv- *Fragaria vesca*, Gm- *Glycine max*, Os- *Oryza sativa* (Japonica), Pp- *Physcomitrella patens*, Ps- *Picea sitchensis*, Pt- *Populus trichocarpa*, Rc- *Ricinus communis*, Sl- *Solanum lycopersicum*, St- *Solanum tuberosum*, Ta- *Triticum aestivum*, Vv- *Vitis vinifera*, Zm- *Zea mays*

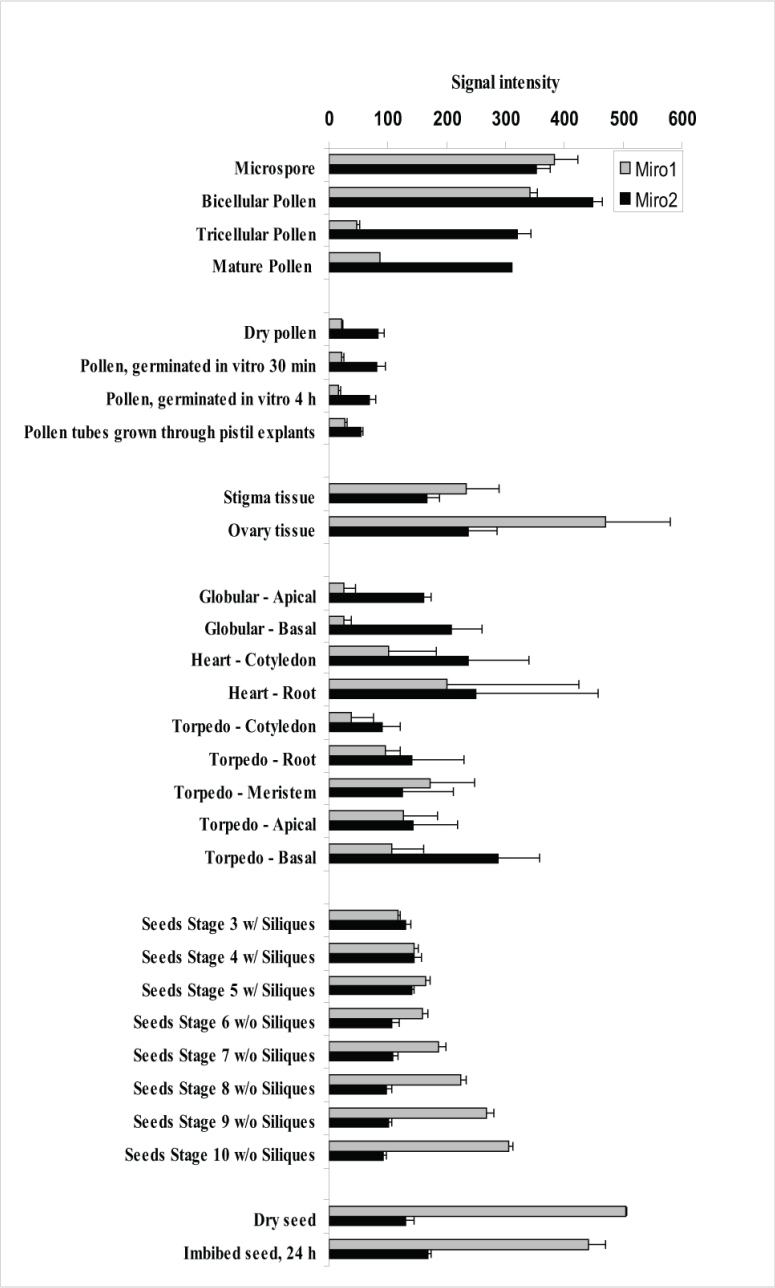

Figure 2 - Gene expression of Arabidopsis MIRO1 and MIRO2 in different plant tissues

Note the difference in expression levels between MIRO1 and MIRO2 during pollen development, especially in mature pollen and during pollen germination. During embryo development there are also both overlapping and quantitative differences in between *MIRO1* and *MIRO2* gene expression. Data used were retrieved from the Arabidopsis eFP browser [18]. Values are means, +SD.

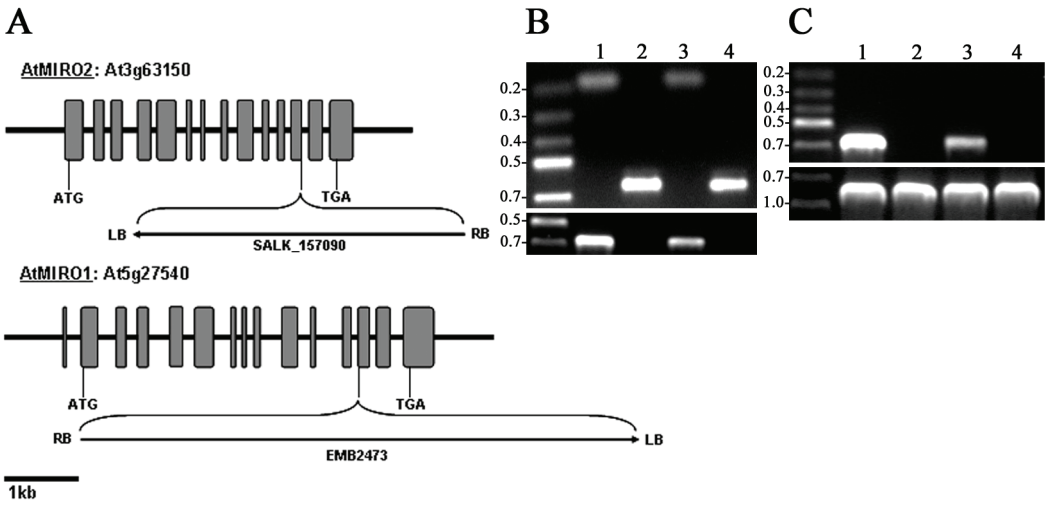

Figure 3 - Characterization of MIRO T-DNA mutants

(A) A schematic overview of the *MIRO1* and *MIRO2* gene structures and the position and orientation of the T-DNA insertion sites within the genes. Closed gray boxes indicate exons. (**B**) Genotyping of *MIRO* T-DNA mutants.

1: *miro1*$^{(+/-)}$, **2**: *miro2-2*$^{(-/-)}$, **3**: *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$, **4**: *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$.

Top panel: Verification of T-DNA insertions using gene and T-DNA specific primers

Bottom panel: Verification of WT allele. Underline: allele investigated

(**C**) Genotyping primer control using Col-WT gDNA. Top panel: **1**: *MIRO1* WT allele primers, **2**: *miro1* T-DNA primers, **3**: *MIRO2* WT allele primers, **4**: *miro2-2* T-DNA primers. Bottom panel: 18s ribosomal RNA PCR control

1: *miro1*$^{(+/-)}$, **2**: *miro2-2*$^{(-/-)}$, **3**: *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$, **4**:Col

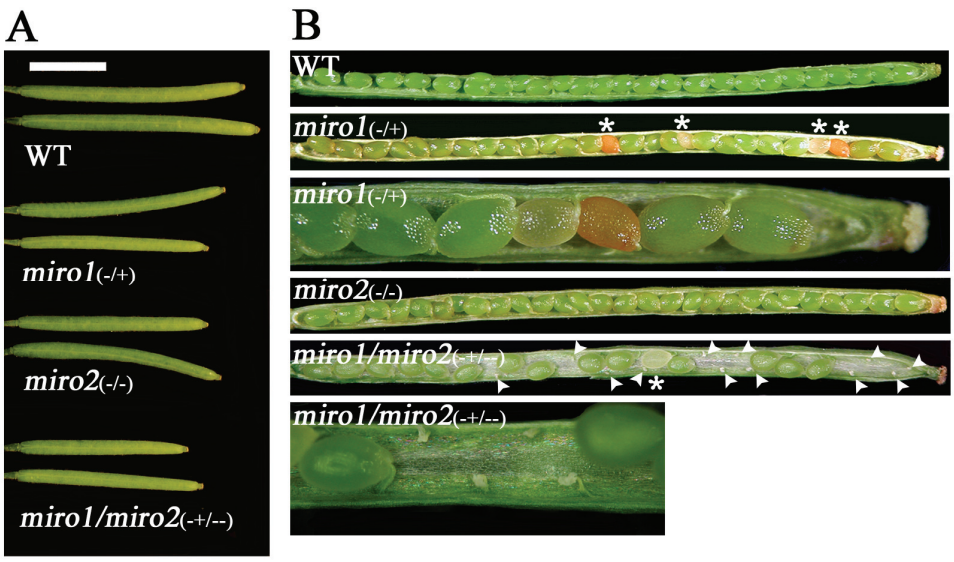

Figure 4 - Silique size and embryo development in *miro* mutants

A: Siliques from wild type and *miro* mutant plants grown simultaneously and under equal conditions. Siliques are from the same positions along the main inflorescence. Scale bar: 0.5 cm

B: Open *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ siliques contain a larger number of undeveloped ovules and fewer terminated embryos compared to the *miro1*$^{(+/-)}$ siliques. Asterisks indicate terminated embryos and arrowheads indicate undeveloped ovules.

Picture 3 and 6 from the top are higher magnification of the siliques from *miro1*$^{(+/-)}$ and *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$,respectively.

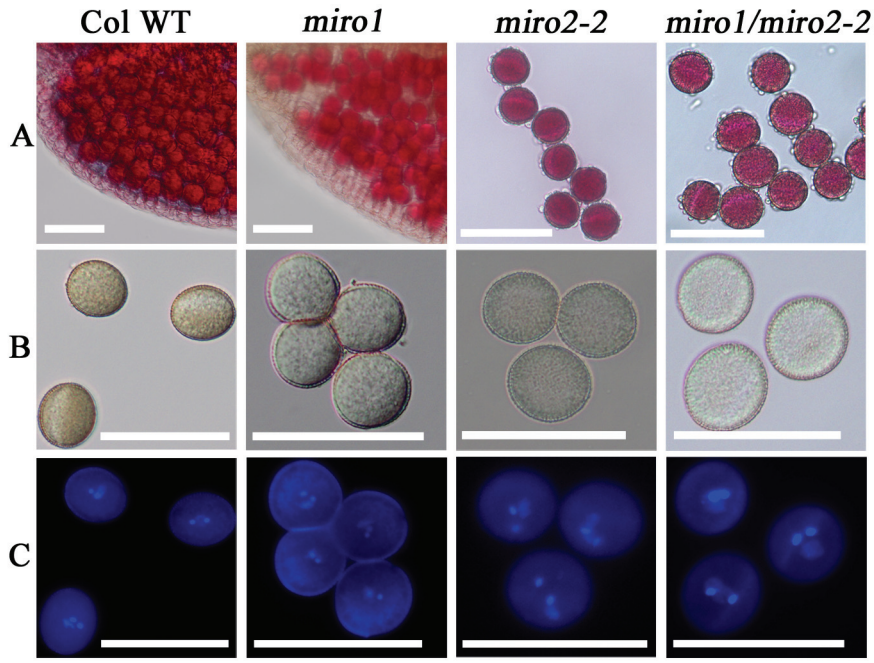

Figure 5 - Pollen viability and development

A: Viability test using Alexander's stain. For Col-WT and *miro1*, anthers were fixed and stained. B: DIC images. Note that the *miro1* mutant is in the *quartet* background (*quartet1*$^{(-/-)}$: At5g55590), which is outcrossed in the *miro1*/*miro2-2* pollen. C: DAPI staining (same as B) shows that mature *miro1/miro2-2* pollen are correctly differentiated with two brightly stained sperm nuclei and one diffusely stained vegetative nucleus. Scale bar: 50 µm

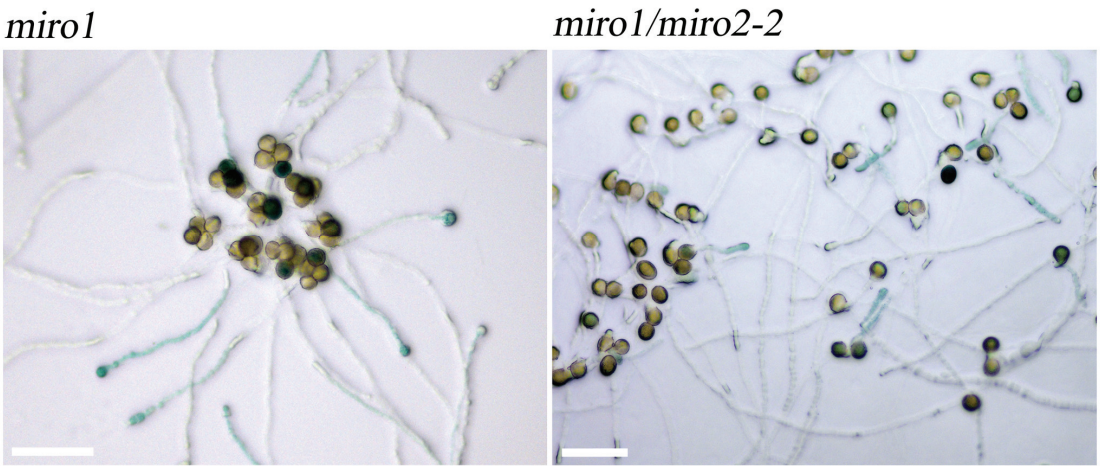

Figure 6 - Additional loss of function in MIRO2 enhances pollen tube growth defects in the *miro1* background

Pollen germinated on solid medium for 17 hours and stained with X-Gluc. Scale bar: 100 µm

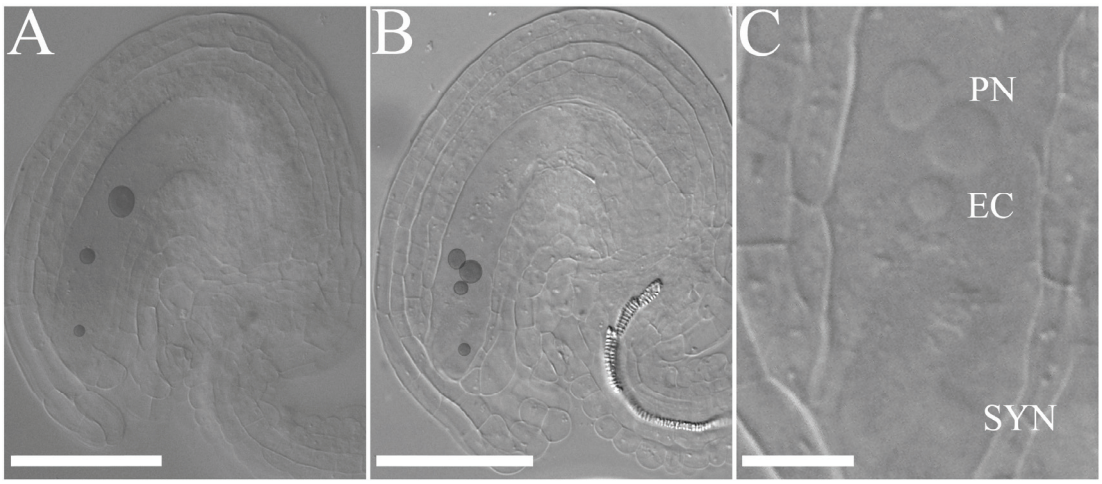

Figure 7 - *miro1*/*miro2-2* female gametophytes are affected during fusion of polar nuclei

Phenotypes of *miro1*$^{(+/-)}$/*miro2-2*$^{(-/-)}$ female gametophytes 48 hours after emasculation. A: Normal mature embryo sac. B: The polar nuclei have failed to fuse. C: Higher magnification of B (PN; Polar nuclei, EC; Egg cell, SYN; Synergid). Contrast of nuclei (except in C) has been artificially enhanced. Scale bar: 50 µm except in C: 10 µm

Table 1: Segregation analysis of the *miro1* and *miro2-2* mutant alleles.

Parental genotype		Seed germ. (%)	Total seeds	Selection^{R}	Selection^{S}	Selection^{R} (%)	Hypothesis	χ^2	*P*(*P*<0.05)
miro1$^{(+/-)}$/***miro2-2***$^{(+/-)}$ ***miro1***$^{(+/-)}$/***miro2-2***$^{(+/-)}$	x	93.2	502	140	328	29.9	3:5	11.489	0.0007
miro1$^{(+/-)}$/***miro2-2***$^{(-/-)}$ ***miro1***$^{(+/-)}$/ ***miro2-2***$^{(-/-)}$	x	94,4	1026	163	805	16.8			
miro1$^{(+/-)}$ x ***miro1***$^{(+/-)}$		89.9	614	315	237	57.1			
miro2$^{(-/-)}$ x ***miro2***$^{(-/-)}$		97.1	593						
Colombia WT		97.1	414						

Selection^{R} (Seedlings with resistance to selection agent): *miro1*$^{(+/-)}$/ *miro2-2*$^{(-/-)}$; BASTA/Kanamycin, miro1/MIRO1; BASTA, *miro2-2*; Kanamycin. Selection^{S}: Seedlings with sensitivity to selection agent

Table 2: Silique analysis of *miro* mutants.

	Wild Type	***Miro2-2*$^{(-/-)}$**	***miro1*$^{(+/-)}$**	***miro1*$^{(+/-)}$/ *miro2-2*$^{(-/-)}$**
Total # of embryos	642	1135	1318	1165
Unfertilized ovules (n)	**1.7%** (11)	**1.4%** (16)	**7.4%** (98)	**34.5 %** (402)
Embryo lethal (n)	**0.8%** (5)	**0.5%** (6)	**17.2%** (226)	**3.4 %** (40)
Total lethality	**2.5 %**	**1.9 %**	**24.6 %**	**37.9 %**
Seed set/silique (n)	**57** (11)	**53** (21)	**43.2** (23)	**36.1** (20)

Supplemental data 1: Accession numbers

Genes	Accession numbers	Organism
AtMIRO3	AT3G05310	*Arabidopsis thaliana*
AlMIRO3	ADBK01000443.1, lyrata ARALYscaffold_3_Cont443	*Arabidopsis lyrata subsp*
BrMIRO3	AC189329.1, pekinensis genomic clone	*Brassica rapa subsp*
AtMIRO1	AT5G27540	*Arabidopsis thaliana*
BrMIRO1	AC189657.1, pekinensis clone KBrS016J18	*Brassica rapa subsp*
CpMIRO1	ABIM01011531.1, chromosome LG6 contig_11546	*Carica papaya*
PtMIRO1a	XP_002319545.1	*Populus trichocarpa*
PtMIRO1b	XP_002328439.1	*Populus trichocarpa*
RcMIRO1	XM_002512327.1 and AASG02000413.1	*Ricinus communis*
CmMIRO1	ABR67417.1	*Cucumis melo*
GmMIRO1	AK286579	*Glycine max.*
FvMIRO1	From EST contig.	*Fragaria vesca*
VvMIRO1	XP_002284757.1	*Vitis vinifera*
StMIRO1	From various EST from Solanum lycopersicum and Solanum tuberosum	*Solanum lycopersicum and Solanum tuberosum.*
AtMIRO2	At3g63150	*Arabidopsis thaliana*
BrMIRO2	AC189426.1, pekinensis clone KBrB065N19	*Brassica rapa subsp*
CpMIRO2	ABIM01012020.1	*Carica papaya*
RcMIRO2	XP_002520752.1	*Ricinus communis*
PtMIRO2	XP_002306771.1	*Populus trichocarpa*
FvMIRO2	EST (DY670610) and EST-contig.	*Fragaria vesca*
GmMIRO2	ACUP01002127.1, chromosome 3 GLYMAchr_03_Cont2127	*Glycine max*

SlMIRO2	BABP01012491.1, DNA, contig: SlSBM_S02784_01	*Solanum lycopersicum*
VvMIRO2	XP_002275434.1	*Vitis vinifera*
OsMIRO1a	NP_001051665.1 Os03g0810600	*Oryza sativa (japonica cultivar-group)*
OsMIRO1b	AACV01002660.1, chromosome 1. Edited, pseudo or seq. errors	*Oryza sativa (japonica cultivar-group)*
TaMIRO1	AK332629.1	*Triticum aestivum*
BdMIRO1a	ADDN01000017.1, strain Bd21 chromosome 1	*Brachypodium distachyon*
BdMIRO1b	ADDN01000858.1, strain Bd21 chromosome 3	*Brachypodium distachyon*
ZmMIRO1	ACG44216.1	*Zea mays*
ZmMIRO2	BT018890	*Zea mays.*
PsMIRO1	From several ESTs from Picea sitchensis	*Picea sitchensis*
PpMIRO1	XP_001779282.1	*Physcomitrella patens subsp. patens*
PpMIRO2	XP_001778992.1	*Physcomitrella patens subsp. patens*
PpMIRO3	XP_001775852.1	*Physcomitrella patens subsp. patens*
PpMIRO4	XP_001767645.1	*Physcomitrella patens subsp. patens*

Supplemental data S2: PCR-genotyping of viable offspring on selection MS-media from self fertilized *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ plants.

Parental genotype	**Total seedlings**	***miro1*$^{(+/-)}$/ *miro2-2*$^{(+/-)}$**	***miro1*$^{(+/-)}$/ *miro2-2*$^{(-/-)}$**	***miro1*$^{(+/-)}$/ *miro2-2*$^{(-/-)}$ (%)**	**Hypothesis**	**χ^2**	***P*(*P*<0.05)**
***miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$ x *miro1*$^{(+/-)}$/*miro2-2*$^{(+/-)}$**	**80**	**63**	**17**	**21.3**	**1:2**	**5.262**	**0.0218**

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos Zoology	Breeding events of birds in relation to spring temperature and environmental phenology
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation
1984	Eivin Røskaft	Dr. philos Zoology	Sociobiological studies of the rook *Corvus frugilegus*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos Zoology	Biochemical genetic studies in fish
1985	John Solem	Dr. philos Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains
1985	Randi E. Reinertsen	Dr. philos Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds
1986	Bernt-Erik Sæther	Dr. philos Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach
1986	Torleif Holthe	Dr. philos Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna
1987	Helene Lampe	Dr. scient Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires
1987	Olav Hogstad	Dr. philos Zoology	Winter survival strategies of the Willow tit *Parus montanus*
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoolog	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction
1988	Hans Christian Pedersen	Dr. philos Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care
1988	Tor G. Heggberget	Dr. philos Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure
1988	Marianne V. Nielsen	Dr. scient Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*)
1988	Ole Kristian Berg	Dr. scient Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.)
1989	John W. Jensen	Dr. philos Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth
1989	Helga J. Vivås	Dr. scient Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*
1989	Reidar Andersen	Dr. scient Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture
1990	Bengt Finstad	Dr. scient Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season
1990	Hege Johannesen	Dr. scient Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams
1990	Tor Jørgen Almaas	Dr. scient Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues
1990	Magne Husby	Dr. scient Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*
1991	Tor Kvam	Dr. scient Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway
1991	Jan Henning L'Abêe Lund	Dr. philos Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods

1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway
1991	Odd Terje Sandlund	Dr. philos Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism
1991	Nina Jonsson	Dr. philos	Aspects of migration and spawning in salmonids
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks
1992	Arne Vollan Aarset	Dr. philos Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
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1994 Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994 Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1994 Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
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1996 Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996 Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
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1997 Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997 Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming
1997 Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture

1997 Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997 Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors
1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997 Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997 Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997 Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997 Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998 Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998 Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998 Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
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1999 Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999 Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)

1999	Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999	Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999	Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis
1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway

2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective

2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge

2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes

2007	Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Bilogy	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes

2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease
2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments -Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity

2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics