Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*.

by

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2004

Submitted for the degree of Doctor Scientiarum

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ACKNOWLEDGMENTS

The work presented in this thesis was carried out at UNIGEN Center for Molecular Biology and the Department of Biology at the Norwegian University of Science and Technology (NTNU), with financial support from NTNU, the Norwegian Research Council and Norwegian Hydro.

My first thanks go to Professor Atle Bones, who has been my supervisor through both my Master studies and my Ph.D. studies. Naturally, he has been central for my scientific education. Thank you for giving me the opportunity to become a researcher!

I specially want to thank Per Winge, who was my scientific supervisor during my Master studies, and has continued to be an inexhaustible source of help and knowledge. He put down the groundwork that this thesis is built upon.

I also want to thank my "room-mates", Birgit Borgen and Ishita Ahuja, who have endured sharing office with me during these years. You have been a great support!

Big thanks go to Ralf Kristensen, Tove Klungervik and Linda Ripel, who have taken part in the RAC project as Master students; and Christopher Sørmo and Anita Johnsen, who are currently working as Master students. I will also thank the other present and former members of the Bones lab for providing an enthusiastic and stimulating environment.

I am grateful to the members of the PLA2 group, with whom we share the lab, the co-workers at UNIGEN and the technical staff at the Department of Biology for helping me with any problem that might occur (and believe me - problems did occur!).

Last, but not least, I want to thank my family for supporting my education throughout the years. Special thanks go to my sister Kirsti and my brother-in-law Geir, who have pulled me out of the lab for Sunday dinners and other activities the last year.

SUMMARY

Small GTP-binding proteins are molecular switches that serve as important regulators of numerous cellular processes. In animal and plant cells, the Rho family of small GTPases participate in e.g. organisation of the actin cytoskeleton, production of reactive oxygen species through the NADPH oxidase complex, regulation of gene expression. The three most extensively studied subgroups of the Rho GTPase family are Cdc42, Rho and Rac. One of the mechanisms by which animal Rac and Cdc42 GTPases regulate actin filament organisation is through activation of the ARP2/3 complex, a multimeric protein complex which induces branching and nucleation/elongation/polymerisation of actin filaments. Activation of the ARP2/3 complex by Rac and Cdc42 is mediated through the proteins WAVE and WASP, respectively.

In a search for Ras-like GTPases in *Arabidopsis*, we identified a family of genes with similarity to Rac GTPases. Screens of cDNA and genomic libraries resulted in the finding of 11 genes named *ARACs/AtRACs*. Genes encoding Rho, Cdc42 or Ras homologues were not identified. Expression analysis of *AtRAC1* to *AtRAC5* indicated that *AtRAC1*, *AtRAC3*, *AtRAC4* and *AtRAC5* are expressed in all parts of the plant, whereas *AtRAC2* is preferentially expressed in root, hypocotyl and stem.

The *AtRAC* gene family can be divided into two main groups based on sequence similarity, gene structure and post-translational modification. *AtRAC* group II genes contain an additional exon, caused by the insertion of an intron which disrupts the C-terminal geranylgeranylation motif. Instead, group II AtRACs contain a putative motif for palmitoylation. Phylogenetic analyses indicated that the division of plant RACs into group I and group II occurred before the split of monocotyledonous and dicotyledonous plants. Analyses of the genes neighbouring *AtRAC* genes revealed that several of the plant *RAC* genes have been created through duplications.

The restricted/tissue-specific expression pattern of *AtRAC2* led us to do a more detailed expression analysis of this gene. A 1.3 kb fragment of the upstream (regulatory) sequence of AtRAC2 directed expression of *GUS* or *GFP* to developing primary xylem in root, hypocotyl, leaves and stem. In root tips, the onset GUS staining or GFP fluorescence regulated by the *AtRAC2* promoter slighty preceded the appearance of secondary cell walls. In stems, GUS staining coincided with thickening

of xylem cell walls. Transgenic plants expressing constitutively active AtRAC2 displayed defects in the polar growth of leaf epidermal cells, indicating that AtRAC2 may be able to regulate the actin cytoskeleton. Surprisingly, an AtRAC2 T-DNA insertion mutant did not show any observable phenotypes. GFP fusion proteins of wild type and constitutively active AtRAC2 were both localised to the plasma membrane. The data suggest that AtRAC2 is involved in development of xylem vessels, likely through regulation of the actin cytoskeleton or NADPH oxidase.

The role of RAC GTPases in regulation of the actin cytoskeleton in plants is well documented. However, although the ARP2/3 complex had been identified in plants/*Arabidopsis*, the mechanisms regulating this complex were unknown. Through database searches, we identified three *Arabidopsis* genes, *AtBRK1*, *AtNAP* and *AtPIR*, which encoded proteins with similarity to subunits of a protein complex shown to regulate the activity of WAVE1 in mammalian cells. T-DNA inactivation mutants of AtNAP and AtPIR displayed morphological defects on epidermal cells undergoing polar expansion, such as trichomes and leaf pavement cells. The phenotypes were similar to those observed for ARP2/3 complex mutants, suggesting that AtNAP and AtPIR act in the same pathway as the ARP2/3 complex in plants. The actin cytoskeleton in *atnap* and *atpir* mutants was less branched than in wild type plants; instead, actin filaments aggregated in thick actin bundles.

Finally, we have recently discovered a small gene family encoding putative WAVE homologues. In mammalian cells, Rac activates WAVE1 through binding to PIR121 or Sra1 (the mammalian homologues of AtPIR). The discovery of a putative WAVE regulatory complex as well as putative WAVE homologues in *Arabidopsis* suggests that plant RAC GTPases regulate organisation of the actin cytoskeleton during polar growth at least partly through the ARP2/3 complex, using an evolutionarily conserved mechanism.

LIST OF PAPERS

1. Winge, P., Brembu, T. and Bones, A.M. (1997). Cloning and characterization of rac-like cDNAs from *Arabidopsis thaliana*. *Plant Mol. Biol.* **35** (4), 483-495.

2. Winge, P., Brembu, T., Kristensen, R. and Bones, A.M. (2000). Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. *Genetics*, **156** (4), 1959-1971.

3. Brembu, T., Winge, P. and Bones, A.M., The small GTPase AtRAC2 is specifically expressed during late stages of xylem differentiation in *Arabidopsis*, submitted manuscript.

4. Brembu, T., Winge, P., Seem, M. and Bones, A.M., *AtNAP*, *AtPIR* and *AtBRK1* encode subunits of a WAVE-like regulatory protein complex involved in plant cell morphogenesis, submitted manuscript.

ABBREVIATIONS

ABA	abscisic acid
Abi2	Abelson interactor 2
ABP	actin binding protein
ARP	actin-related protein
Cdc42	cell division cycle 42
CRIB	Cdc42/Rac-interactive binding
EVH1	Ena/VASP homology 1
GAP	GTPase-activating protein
GBD	GTPase-binding domain
GDI	GDP dissociation inhibitor
GEF	GDP/GTP exchange factor
IAA	indole acetic acid
IP3	Inositol-3-phosphate
JNK	jun N-terminal kinase
MAPK	mitogen activated protein kinase
Nap	Nck-associated protein
NPF	nucleation promoting factor
PIR121	121F-specific p53 inducible RNA
PtdIns(3,4)P2 = PIP2	phosphatidylinositol-3,4-diphosphate
PtdIns P-K	phosphatidylinositol monophosphate kinase
Rac	Ras-related C3 botulinum toxin substrate
Ras	rat sarcoma viral oncogene homologue
Rboh	respiratory burst oxidase homologue
Rho	Ras homologous
RIC	RAC/ROP-interactive CRIB motif-containing protein
Rop	Rho of plants
ROS	reactive oxygen species
Scar	suppressor of cAMP
SH3	Src homology 3
SHD	Scar homology domain
Sra-1	specifically Rac1-associated protein

WASP	Wiskott-Aldrich syndrome proteins (WASP)
WAVE	WASP family verprolin-homologous protein

INTRODUCTION

Structure and function of Rho GTPases in animal and plant cells

The Ras superfamily of small GTPases

Proteins using guanosine triphosphate (GTP) as a co-factor constitute a large and diverse group of proteins found in all organisms studied to date. GTP-binding proteins are central components in several aspects of cellular functions, such as translation (EF-Tu), cytoskeletal structure (tubulin) and signal transduction (heterotrimeric G proteins and small GTPases).

A subgroup of the GTP-binding proteins are the Ras superfamily of small GTPases, which are small, monomeric GTP-binding proteins with molecular masses of 20-40 kDa. About 25 years ago, the Ras genes Ha-Ras and Ki-Ras were the first small GTPases to be identified, first in sarcoma virus (Chien et al., 1979; Shih et al., 1978) and subsequently in human carcinomas (Parada et al., 1982; Santos et al., 1982). Other similar, but distinct gene families were discovered throughout the 1980s, leading to the classification of the Ras superfamily into five families; the Ras, Rho, Rab, Arf/Sar and Ran families (reviewed by Takai et al., 2001). The subgroups of small GTPases have important and, to some extent, overlapping functions in the cell; Ras GTPases regulate gene expression and transduce signals from cell membrane receptors; Rho GTPases regulate both cytoskeletal reorganisation and gene expression; members of the Rab and Arf/Sar family GTPases function in intracellular vesicle trafficking, whereas Ran GTPases have been implicated in transport of proteins and mRNA across the nuclear envelope.

Rho GTPases

The first Rho (short for ras homologous) family gene was isolated from the mollusc *Aplysia* (Madaule and Axel, 1985). Subsequently, Madaule and co-workers (1987) cloned the *S. cerevisiae* genes RHO1 and RHO2, and found RHO1 to be and RHO2

not be necessary for cell viability. At the same time, other research groups identified a class of proteins with a molecular weight of 21-24 kDa as substrates for ADP-ribosylation by type D botulinum toxin from the bacterium *Clostridium botulinum* (Banga et al., 1988; Ohashi and Narumiya, 1987) and showed that these proteins were able to bind GTP (Bokoch et al., 1988; Morii et al., 1988). The protein substrate was identified as *rho* (Narumiya et al., 1988; Quilliam et al., 1989) and the site for ADP-ribosylation was found to be an asparagine residue (Sekine et al., 1989). Members of the Rac subfamily were first identified from human cells, and shown to be substrates for ADP-ribosylation by type C3 botulinum toxin (Didsbury et al., 1989). This property led the authors to name the proteins *rac* (short for ras-related C3 botulinum toxin substrate). A third member of the Rho protein family, Cdc42, was discovered in *Saccharomyces cerevisiae* as the target of mutation in *cdc42* mutant cells, which are unable to form buds during cell division, resulting in large, multinucleate cells (Johnson and Pringle, 1990). Since then, a total of twenty mammalian Rho proteins have been described; Rac (three isoforms), Rho (three isoforms) and Cdc42 (one isoform) are the best characterised members of the family in mammalian cells (Etienne-Manneville and Hall, 2002). Some of the best known functions of Rho GTPases and the mechanisms regulating their activity in metazoan cells are summarised below.

Mechanism and regulation of Rho GTPase activity

Similar to other Ras superfamily GTPases, Rho GTPases are molecular switches that cycle between two conformational states: a GTP-bound, "active" state and a GDP-bound, "inactive" state. An intrinsic GTPase activity hydrolyse GTP to GDP (Bourne et al., 1990)(Fig. 1).

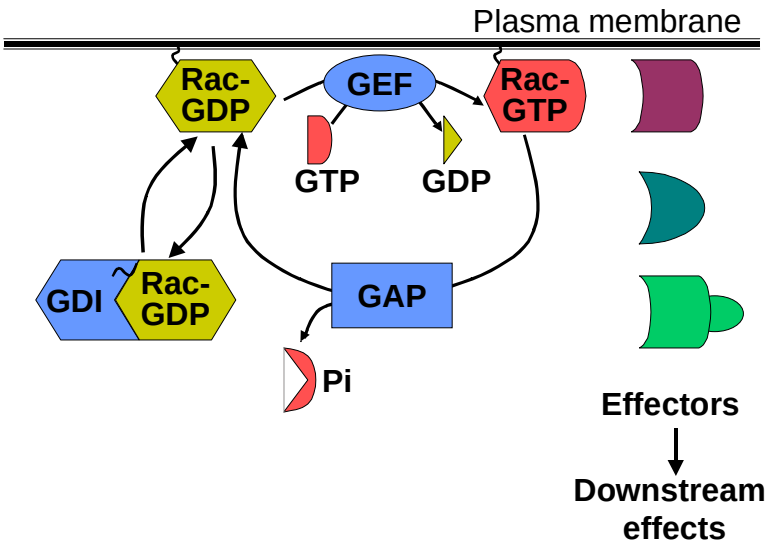

Figure 1. Regulation of Rac GTPase activity. GAP, GTPase-activating protein; GEF, GDP exchange factor; GDI, GDP dissociation inhibitor; Pi, inorganic phosphate. Modified from Takai et al. (2001).

When a cell receives an appropriate stimulus, it creates a signal that stimulates dissociation of GDP from the inactive GTPase. Due to much higher concentration of GTP as compared to GDP in the cell, GTP quickly occupies the nucleotide-binding site of the GTPase. The binding of GTP induces a structural change in the GTPase, enabling it to interact with downstream effectors (Bishop and Hall, 2000). Rho proteins are prenylated at their C-terminus; upon activation the prenyl group is exposed and inserted into a target cell membrane (Adamson et al., 1992). Two classes of Rho protein mutants have been instrumental in functional studies of the Rho GTPases. Constitutively activated mutants (usually G12V or Q61L for mammalian Rho GTPases) are GTPase deficient, whereas dominant negative mutants (usually T17N) are unable to exchange GDP with GTP.

Dissociation of GDP from the GDP-bound GTPase is stimulated by a group of regulators called guanine exchange factors (GEFs) (Kjøller and Hall, 1999). The main class of metazoan Rho GEFs contains a Dbl-homology (DH) domain and a pleckstrin homology (PH) domain. Proteins containing DH domains appears to be absent in plants (Yang, 2002). Recently, a new family of proteins showing Rho GEF activity is represented by Dock180 in mammalian cells, Myoblast City (MBC) in *Drosophila* and CED-5 in *C. elegans*. Dock180 proteins promote nucleotide exchange on Rac and

Cdc42 as a complex with another protein, ELMO (Braga, 2002). The *Arabidopsis* genome contains a single Dock180 homologue, *SPIKE* (*SPK1*), which will be discussed later.

The intrinsic GTP-hydrolysing activity of Rho GTPases is enhanced by GTPase-activating proteins (GAPs) (Moon and Zheng, 2003). Thus, RhoGAPs acts as negative regulators, shutting down the signal to downstream effectors. All known RhoGAPs contain a conserved protein fold where an arginine residue, the 'arginine finger', is central for GAP activity. Upon binding a Rho GTPase, the arginine finger of the RhoGAP enters the GTPase active site and stabilises the transition state of GTP hydrolysis (Rittinger et al.,1997a, Rittinger et al. 1997b) .

In their unactivated state, Rho GTPases are bound by a third class of regulatory proteins, Rho GDP dissociation inhibitors (RhoGDIs) (Olofsson,1999). RhoGDIs appear to extract GDP-bound Rho GTPases from cell membranes and inhibit their spontaneous GDP↔GTP exchange activity, maintaining a cytosolic pool of inactive Rho GTPases. The precise role of RhoGDIs is still poorly understood.

Biological functions of (metazoan) Rho GTPases

For Rho, Rac and Cdc42, over 60 effector proteins have so far been identified experimentally in metazoa (Etienne-Manneville and Hall, 2002). The large number of target proteins participating in a number of signal transduction pathways places the Rho GTPases as central regulators of several cellular processes.

The best characterised and a major function of Rho GTPases is to regulate the assembly and organisation of the actin cytoskeleton (Hall, 1998). In 1992, Ridley and Hall reported that injection of constitutively activated Rho into fibroblasts led to the assembly of contractile actin-myosin filaments (stress fibers) and associated focal adhesion. Injection of constitutively activated Rac and Cdc42 induced actin-rich surface protrusion called lamellipodia and finger-like protrusions called filopodia, respectively (Nobes and Hall, 1995; Prigmore et al., 1995; Ridley et al., 1992). There seem to be substantial cross-talk between the different Rho GTPases; in fibroblasts, Cdc42 can activate Rac, and Rac can activate Rho (Nobes and Hall, 1995). In other cell types, however, Rho has opposing effects compared with Cdc42 or Rac. During neuronal extension of neurites, Cdc42 and Rac promote neurite outgrowth, whereas

Rho inhibits neurite extension (Luo et al., 1996). Similarly, in macrophages, Cdc42 and Rac promote membrane protrusions and anchoring of the cell to its substrate, and Rho is responsible for loosening of cell adhesions and membrane retraction at the rear end of the moving cell (Aepfelbacher et al., 1996; Allen et al., 1997). An important class of Rac and Cdc42 effectors in these processes is the p21-activated kinases (PAKs), which are serine/threonine kinases whose activity is stimulated by the binding of active Rac and Cdc42 to their Cdc42/Rac-interactive binding (CRIB) domain (reviewed by Bokoch, 2003). Rho GTPase-regulated organisation of the actin cytoskeleton is important in a number of cell type specific processes, such as selection of bud site in yeast cells, cellular polarity during embryogenesis, establishment of cell asymmetry in epithelial cells and phagocytosis in macrophages (Etienne-Manneville and Hall, 2002). There is also increasing evidence that Rho GTPases are involved in regulation of microtubules (Cau et al., 2001; Palazzo et al., 2001).

Besides regulating the actin cytoskeleton, Rho GTPases also contribute to a number of other processes. Studies on cell cycle progression have shown that Rho, Rac and Cdc42 each contribute to G1 progression in fibroblasts (Olson et al., 1995). Rac stimulates G1 progression in T cells through activation of the JNK mitogen-activated protein (MAP) kinase cascade after antigen stimulation of T cells (Cantrell, 1998). Rac proteins regulate production of reactive oxygen species (ROS) in leukocytes such as neutrophils through activation of the NADPH oxidase complex (Bokoch and Knaus, 2003). The NADPH oxidase complex consists of a catalytic, membrane-associated flavocytochrome b_{558}, constituted of the subunits $gp91^{phox}$ and $p22^{phox}$, and the cytosolic components $p47^{phox}$ and $p67^{phox}$. The generation of ROS contributes to killing of phagocytosed bacteria. Rac interacts with at least one of the subunits, $p67^{phox}$, but the mechanism for Rac-mediated activation of the NADPH oxidase complex is not fully understood (Bokoch and Diebold, 2002). In addition, ROS produced by NADPH oxidase are believed to act as signal molecules, regulating redox-sensitive signalling proteins (Nimnual et al., 2003; Sulciner et al., 1996).

Rho GTPases are also involved in many stages of vesicular trafficking (Symons and Rusk, 2003). Some of these processes, such as macropinocytosis and phagocytosis, involve regulation of the actin cytoskeleton, whereas other processes, such as

clathrin-mediated endocytosis and certain exocytotic pathways are at least partly regulated through other mechanisms.

The RAC/ROP GTPase family in plants

With the completion of the *Arabidopsis* genome sequencing project (The Arabidopsis genome initiative, 2000), we now have a fairly good overview of the protein families that are present in plants. A search for small GTP-binding proteins in the *Arabidopsis* genome sequence resulted in identification of 93 proteins, classified within four of the five small GTPase families known in metazoa: 57 Rab GTPases; 21 Arf GTPases; 11 Rho GTPases; and 4 Ran GTPases (Vernoud et al., 2003). A discussion of the Rab, Arf/Sar and Ran GTPase families is beyond the scope of this thesis (for general reviews on GTP-binding proteins in plants, see Ma, 1994; Bischoff et al., 1999; Yang, 2002; Vernoud et al., 2003. For recent reviews on *Arabidopsis* Rab GTPases, see Rutherford and Moore, 2002; on Ran GTPases, see Rose et al., 2004).

Research on RAC/ROP GTPases in plants date back to 1993, when Zhenbiao Yang and John Watson reported the cloning of a RAC-like GTPase from garden pea which they called Rho1Ps (Yang and Watson, 1993). Since then, several research groups have turned their attention to this exciting protein family, and more than 50 research papers and reviews on plant RAC/ROP GTPases have been published to date. As a result, a considerable body of evidence points towards a role for RAC/ROP GTPases as central regulators of a number of important processes in plant cells, such as organisation of the actin cytoskeleton, pathogen responses and hormone responses (Fig. 2). Some of the suggested functions of RAC/ROP GTPases are described (below).

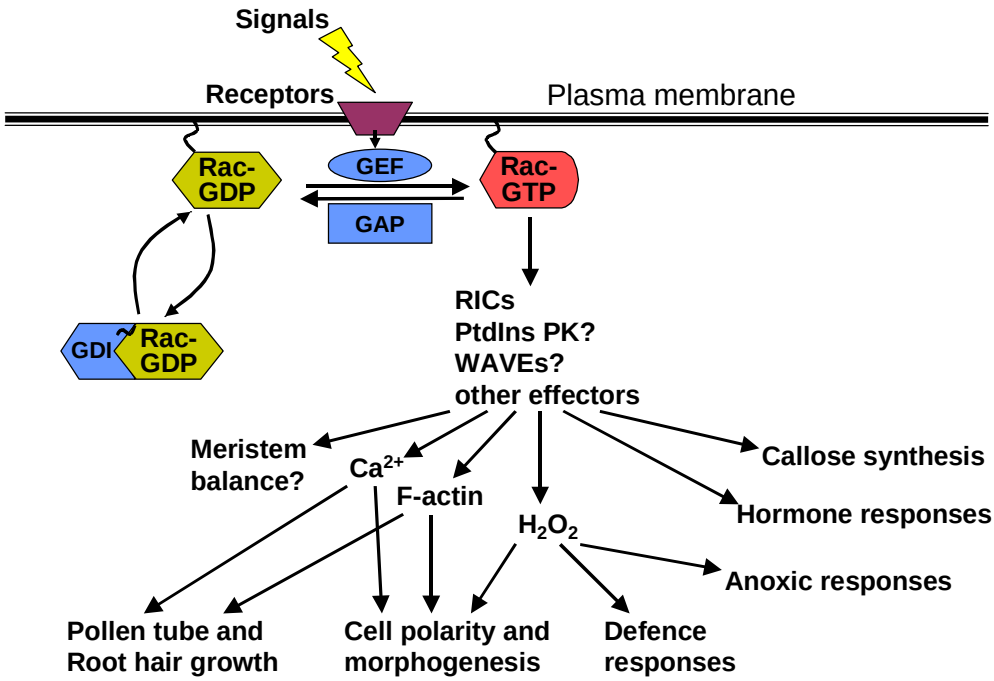

Figure 2. Overview of known functions of RAC/ROP GTPases in plants. GAP, GTPase-activating protein; GEF, GDP exchange factor; GDI, GDP dissociation inhibitor; PtdIns PK, phosphatidylinositol monophosphate kinase; RIC, RAC/ROP interactive CRIB motif-containing protein; WAVE, WASP family verprolin-homologous protein. Modified from Yang (2002).

A majority of the studies on RAC/ROP GTPases in plants have been done on *Arabidopsis thaliana*. The RAC/ROP family in *Arabidopsis* is constituted by eleven members (Winge et al., 1997; Winge et al., 2000). The strucure and evolution of the *Arabidopsis* RAC/ROP family is discussed in Paper I and II. Different research groups have given different names to the same genes, leading to confusion in the nomenclature of the gene family. Table 1 summarises some of the names that have been used on *Arabidopsis* RAC/ROP GTPases.

Table 1. Nomenclature of the *Arabidopsis* RAC/ROP GTPase family, modified from Yang, 2002.

[a]**AtRAC/Arac**	AtRAC1	AtRAC2	AtRAC3	AtRAC4	AtRAC5	AtRAC6	AtRAC7	AtRAC8	AtRAC9	AtRAC10	AtRAC11
[b]**ROP**	ROP3	ROP7	ROP6	ROP2	ROP4	ROP5	ROP9	ROP10	ROP8	ROP11	ROP1
[c]**At-Rac/AtRac**			AtRac1			AtRac2					
[d]**AtROP**			AtROP4		AtROP6						

[a]Winge et al., 1997; Winge et al., 2000

[b]Li et al., 1997; Yang, 2002

[c]Kost et al., 1999; Lemichez et al., 2001

[d]Bischoff et al., 2000; Molendijk et al., 2001

Proteins regulating plant RAC/ROP activity

GTPase activating proteins (GAPs)

The *Arabidopsis* genome appears to encode three distinct families of RAC/ROP-specific GTPase activating proteins (GAPs), based on sequence similarity and domain composition (personal observations).

The first Rac/RopGAPs were identified in yeast two-hybrid assays as proteins binding a constitutively active mutant of LjRac2, a *Lotus japonicus* homologue of AtRAC2 (Borg et al., 1999). *In vitro* GAP activity assays performed with one of the three GAPs, LjRacGAP1, induced GTPase activity in LjRac2. The Rac/RopGAP family in *Arabidopsis* has six members (Wu et al., 2000). Rac/RopGAPs contain a conserved CRIB domain immediately N-terminal to the GTPase-activating domain,. This domain combination is unique for plants. The CRIB domain is believed to facilitate the formation of or stabilise the transitional state between RAC/ROP-GTP and RAC/ROP-GDP (Wu et al., 2000).

An uncharacterised family of putative plant RAC/ROP-specific GAPs contain an N-terminal pleckstrin homology (PH) domain (Winge, 2002). PH domains are 100-120 amino acid protein modules with the ability to bind phosphoinositides, and are found in various proteins, amongst others Rac/Rho GEFs (Lemmon et al., 2002). In *Arabidopsis*, three genes (At4g24580, At5g12150 and At5g19390) encode proteins with an N-terminal PH domain, followed by a Rac/RopGAP domain (Brembu, personal observations). Presently, no functional data exist on these proteins.

A single gene (At5g61530) in *Arabidopsis* represents the third type of RAC/ROP GAP-like proteins. The protein is smaller than the two other types of plant RAC/ROP GAPs, and contains no conserved domains or motifs besides the putative GAP domain.

Guanine exchange factors (GEFs)

As mentioned earlier, the most common type of metazoan RhoGEFs, which contains PH and Dbl domains, does not exist in plants. However, an *Arabidopsis* protein called SPIKE1 has similarity to the other known class of RhoGEFS, Dock180 (Qiu et al., 2002). The *spike1* mutant dies at seedling stage and has severe morphological

defects, probably due to altered organisation of the microtubule cytoskeleton. This is somewhat surprising, since RAC/ROP GTPases in plants mainly have been implicated in regulation of actin cytoskeleton, as discussed below. SPIKE may be a specific regulator of RAC/ROP coupled to regulation of microtubules.

GDP dissociation inhibitors (GDIs)

RhoGDIs are well conserved between animals and plants. Three RhoGDIs are present in *Arabidopsis*. Physical interaction between RhoGDI and RAC/ROP has been shown in *Arabidopsis* (Bischoff et al., 2000) and tobacco (Kieffer et al., 2001) using yeast two-hybrid assays and co-immunoprecipitation. No functional data has been published on plant RhoGDIs.

RAC/ROP regulation of polar cell growth

The first functional data published on plant RAC/ROP GTPases suggested a role in cell polarity. Expression of AtRAC3 in *Schizosaccharomyces pombe* resulted in small, round cells compared with the elongated appearance of wild type cells, the AtRAC3 expressing cells also showed diffuse actin staining (Xia et al., 1996). Since then, plant RAC/ROP proteins has been shown to take part in regulation of polar growth in a number of cell types.

Polar tip growth in pollen

Polar tip growth in pollen has become an attractive model system for the study of RAC/ROP-dependent regulation of the actin cytoskeleton, as well as other cellular processes controlled by RAC/ROPs (Gu et al., 2003; Zheng and Yang, 2000). In the course of fertilisation, pollen tubes elongate by highly polarised growth termed tip growth in order to deliver the sperm to an ovule, in a process with similarity to axon guidance in animal cells (Palanivelu and Preuss, 2000). Tip growth is characterised by directed delivery of Golgi vesicles to a defined site in the cell membrane where the vesicles fuse with the plasma membrane. Lin et al. (1996) showed that the pea RAC/ROP GTPase was mainly expressed in pea pollen, and that Rop1Ps was localised to the apex of the pollen. Inhibition of Rop1Ps activity by injection of anti-Rop1Ps antibodies into pea pollen blocked growth of the pollen tube, suggesting that RAC/ROP GTPases play a crucial role in pollen tube growth (Lin and Yang, 1997). In *Arabidopsis*, at least three closely related RAC/ROP GTPases, AtRAC11/ROP1,

AtRAC6/ROP5 and AtRAC1/ROP3, are expressed in pollen (Li et al., 1998). Expression of dominant negative (T20N) mutants of AtRAC11/ROP1 and AtRAC6/ROP5 inhibited pollen growth, in accordance with the antibody experiments from pea (Kost et al., 1999; Li et al., 1999). In contrast, overexpression of wild type AtRAC11/ROP1 and AtRAC6/ROP5 induced depolarised growth of pollen tubes. Expression of constitutively active AtRAC11/ROP1 and AtRAC6/ROP5 mutants resulted in even more severe depolarised phenotypes (Kost et al., 1999; Li et al., 1999). These results suggest that RAC/ROP GTPases regulate both cell expansion and polarity in pollen. Filamentous actin (F-actin) in pollen tubes can be divided into two populations: cortical, longitudinally oriented actin cables extending along the pollen tube towards the tip, and short, dynamic actin filaments immediately behind the actin-free zone of the tip apex (Fu et al., 2001). Expression of dominant negative ROP1/AtRAC11 in tobacco pollen reduced the short tip actin, whereas overexpression of wild-type ROP11/AtRAC11 led to formation of a network of actin filaments at the tip and abnormal transverse actin bands behind the tip apex, probably as a consequence of stabilisation of tip actin filaments (Fu et al., 2001).

The upstream regulators of RAC/ROP in this process are not known, but at least some of the downstream effectors have been identified. At-Rac2/AtRAC6 interacts with phosphatidylinositol monophosphate kinase (PtdIns P-K). Phosphatidyl inositol 4,5 diphosphate (PIP2), the product of PtdIns P-K, also localises to the apical plasma membrane. Furthermore, removing PIP2 inhibits pollen tube elongation (Kost et al., 1999), suggesting that this lipid is an important messenger in pollen tip growth. A Ca^{2+} gradient exists in pollen tubes with highest concentrations at the tip; this gradient is believed to mediate exocytosis. Inositol-3-phosphate (IP3), a hydrolysis product of PIP2, induces the release of Ca^{2+} from intracellular stores (Hay et al., 1995). Thus, PIP2 may play a role in creating a Ca^{2+} gradient in pollen tips.

Another group of RAC/ROP effectors likely to be involved in pollen tip growth are the RAC/ROP-interactive CRIB motif-containing proteins (RICs) (Wu et al., 2001). RICs are small proteins characterised by a conserved CRIB domain, but share little sequence homology outside of this domain. Overexpression of *Arabidopsis* RICs in pollen produced distinct phenotypes, implying distinct roles for various RICs; both elongation, radial growth and polarisation were differentially affected (Wu et al., 2001). RICs will likely be explored as putative RAC/ROP effectors in cellular

processes in the years to come. The *Arabidopsis* genome does not encode any PAK-like, CRIB domain-containing kinase (Brembu, personal observation).

Root hair development

Root hairs are epidermal root cells that develop into exceptionally polarised structures extending laterally from the root. The initial growth phase is characterised by diffuse growth, where expansion is driven by turgor pressure and occurs across the entire cell surface. The root hair elongates further via tip growth (Carol and Dolan, 2002). RAC/ROP proteins are localised to the initial site of root hair formation, hence they are thought to participate in initiation of root hair outgrowth (Molendijk et al., 2001; Jones et al., 2002). Brefeldin A (BFA), an ARF1 GEF inhibitor, inhibits early localisation of RAC/ROP, indicating that ARF GTPase(s) regulates this process, through a yet unknown mechanism (Molendijk et al., 2001). During elongation, RAC/ROP proteins localise to the root hair tip. Furthermore, expression of constitutively active mutants of AtRop4/AtRAC5, AtRop5/AtRAC3 and ROP2/AtRAC4 all resulted in depolarised growth of root hairs, whereas expression of dominant negative ROP2/AtRAC4 inhibited root hair tip growth (Molendijk et al., 2001; Jones et al., 2002). Ca^{2+} gradients and organisation of actin cytoskeleton are both affected in root hairs expressing constitutively active mutants of AtRop4/AtRAC5, AtRop5/AtRAC3 and ROP2/AtRAC4. Thus, RAC/ROP proteins appear to control tip growth in root hairs through regulation of actin cytoskeleton and Ca^{2+} gradients in root hair tips, similar to what has been observed in pollen. A recent publication proposes a ($gp91^{phox}$) NADPH oxidase homologue as a putative RAC/ROP effector responsible for the creation of Ca^{2+} gradients in root hairs (Foreman et al., 2003). In *Arabidopsis*, the NADPH oxidase AtRbohC is mutated in the *root hair defective2* (*rhd2*) mutant, which has stunted roots and root hairs, and shows defective uptake of Ca^{2+}. Reactive oxygen species produced by AtRbohC/RHD2 is believed to activate hyperpolarisation-activated Ca^{2+} channels in order to facilitate Ca^{2+} influx. NADPH oxidases in plants are further discussed in a later section.

Polar growth in developing tissues

In addition to pollen and root hairs, other cell types also show polar growth during certain phases of development. Polar cell expansion during organ development

occurs in two phases: an early phase in which cell expansion can occur in both longitudinal and radial or lateral directions, and a late phase involving only elongation (Fu et al., 2002). Expression of AtRop4/AtRAC5 and AtRop5/AtRAC3 lead to isotropic growth of epidermal cells (Molendijk et al., 2001). Expression of ROP2/AtRAC4 results in altered morphology of leaf epidermal cells called pavement cells (Fu et al., 2002). In wild type plants, pavement cells expand by both polar tip growth and diffuse growth to produce mature cells with extended lobes. Enrichments of cortical F-actin is observed at the lobe tips during elongation in the early phase of polar expansion, suggesting a role for actin in this process. In constitutively active ROP2/AtRAC4 expressing pavement cells, cortical F-actin is delocalised during the early expansion phase, and expansion is rather uniform over the cell surface, producing cells without lobes (Fu et al., 2002). The results suggest that ROP2/AtRAC4 and possibly other RAC/ROP GTPases regulate polar lobe expansion by activating the assembly of cortical F-actin in discrete regions of the cell cortex.

RAC/ROP and production of ROS through NADPH oxidases

When challenged by a pathogen, plants rapidly produce reactive oxygen species (ROS), reminiscent of the oxidative burst in neutrophils (Levine et al., 1994). With the cloning of a family of genes in *Arabidopsis* that encoded homologues of the mammalian $gp91^{phox}$ (Keller et al., 1998; Torres et al., 1998), a link between RAC/ROP GTPases and ROS production was quickly suspected. However, homologues of the other, cytosolic components of the complex have not been found in plants. The *Arabidopsis thaliana* respiratory burst oxidase homologues (AtRbohs) contain an additional N-terminal domain containing two Ca^{2+}-binding EF-hand motifs, so Ca^{2+} is probably important in the regulatory mechanism of Rbohs. Several reports from the last few years show that RAC/ROP is an important activator of NADPH oxidases in plants, and that RAC/ROP-induced ROS production is central in defence responses, as well as root hair growth (Foreman et al., 2003) and transcriptional activation (Baxter-Burrell et al., 2002). The mechanism by which RAC/ROP GTPases regulate NADPH oxidases is, however, unclear.

RAC/ROP, NADPH oxidases and plant defence responses

The role for RAC/ROP GTPases as activators of ROS production through NADPH oxidase during defence responses is well documented (Agrawal et al., 2003). Expression of constitutively active mutants of the rice RAC protein OsRac1 (an AtRAC7 homologue) or human Rac in rice soybean suspension cultures resulted in elevated levels of ROS production, whereas expression of dominant negative mutants reduced ROS production (Kawasaki et al., 1999; Park et al., 2000). Moreover, addition of the NAPDH oxidase inhibitor diphenylene iodonium (DPI) to constitutively active OsRac1-expressing rice cells abolished ROS production, indicating that NADPH oxidase is indeed the source of ROS generation (Kawasaki et al., 1999). Transgenic rice plants expressing constitutively active OsRac1 show increased resistance against virulent races of rice blast fungus (*Magnaporthe grisea*) and bacterial blight (*Xanthomonas oryzae*), whereas dominant negative OsRac1 expressing plants delays and reduces the defence responses to avirulent pathogen races (Ono et al., 2001). These results support a role for OsRac1 in pathogen defence responses. Expression of Gα, a subunit of the heterotrimeric G protein, is induced by pathogen infection. The Gα defective *dwarf1* (*d1*) mutant has a strongly impaired defence response. Interestingly, expression of constitutively active OsRac1 in *d1* mutants restored pathogen resistance (Suharsono et al., 2002). Thus, a signal pathway from heterotrimeric G protein via OsRac1 to NADPH oxidase appears to mediate pathogen-induced ROS production in rice. Curiously, transforming single barley cells with GFP-tagged, constitutively active mutants of three barley RAC/ROP genes resulted in increased susceptibility of the transformed cells to the fungal pathogen powdery mildew (*Blumeria graminis*)(Schultheiss et al., 2003). The contradiction between these results may be caused by different functional properties of the investigated RAC/ROP proteins or differences in the responses against the pathogens used in the studies.

H_2O_2-mediated regulation of gene expression

Work by Baxter-Burrell and colleagues (2002) revealed a role for RAC/ROP proteins in regulation of gene expression, with hydrogen peroxide (H_2O_2) as a second messenger. A mutant *Arabidopsis* line disrupted in the RAC/ROP GTPase activating protein RopGAP4 showed increased response to oxygen deprivation, but reduced

tolerance to anoxic stress. Furthermore, RopGAP4 expression was induced by O_2 deprivation. After a series of experiments, the following model was proposed. Upon oxygen deprivation, i.e. during flooding, one or several unidentified RAC/ROP GTPases are activated, and induce NADPH oxidase activity. H_2O_2 is produced and operates as a second messenger, inducing expression of alcohol dehydrogenase (ADH), which switches metabolism to anaerobic ethanolic fermentation. RAC/ROP signalling is attenuated through negative feedback by H_2O_2-stimulated transcription of RopGAP4.

In rice, RAC-mediated production of H_2O_2 during defence responses appears to activate transcription of defence-related genes (Ono et al., 2001; Suharsono et al., 2002).

Synthesis of secondary cell walls

Expression of the cotton RAC GTPase *GhRac13* (an AtRAC2 homologue) is highly induced during transition from primary to secondary cell wall synthesis in cotton fibers (Delmer et al., 1995). At the same time, H_2O_2 production increases strongly. Expression of constitutively active or dominant negative GhRac13 in soybean or *Arabidopsis* cells resulted in activation or suppression of ROS production, respectively (Potikha et al., 1999). These results imply that GhRac13 may be involved in develpomental control of cotton fibers via H_2O_2 production. Recently, Kurek and co-workers (2002) showed that the cotton cellulose synthases GhCesA1 and GhCesA2 are activated by dimerisation via a redox-regulated zinc-finger domain. Considering the *GhRac13* expression pattern, it is tempting to speculate that secondary cell wall synthesis is induced through RAC/ROP-mediated activation of an NADPH oxidase.

RAC/ROP GTPases and plant hormone responses

Results are emerging which links RAC/ROPs to hormone responses.

Abscisic acid (ABA)

Plant water homeostasis is maintained by ABA, which trigger closure of stomatal pores in response to drought stress (Himmelbach et al., 2003). *AtRac1/AtRAC3* is highly expressed in guard cells (Lemichez et al., 2001). Expression of constitutively

active AtRac1/AtRAC3 inhibited ABA-induced stomatal closure in wild type *Arabidopsis*, whereas expression of dominant negative AtRac1/AtRAC3 caused stomatal closure both in wild type and the *abscisic acid insensitive1* (*abi1*) mutant (Lemichez et al., 2001). Thus, AtRac1/AtRAC3 activity is down-regulated by ABA, probably leading to disruption of the actin cytoskeleton in guard cells, which causes stomatal closure. Another negative regulator of ABA responses in *Arabidopsis* is ROP10/AtRAC8 (Zheng et al., 2002). ABA downregulates *ROP10/AtRAC8* expression in root tips. Disruption of ROP10/AtRAC8 leads to enhanced ABA responses. Similar to AtRAC3, expression of constitutively active and dominant negative ROP10/AtRAC8 results in reduced and enhanced ABA responses, respectively. The mutant phenotype is observed in a number of tissues, suggesting that ROP10/AtRAC8 may be a more general negative regulator of ABA-induced responses compared to AtRAC3.

Auxin

The phytohormone indole acetic acid (IAA) regulates a number of cellular processes, and contributes to many aspects of plant development (Leyser, 2002). Using tobacco protoplasts, Tao and co-workers (2002) showed that overexpression of *AtRAC1*, *AtRAC3*, *AtRAC10* and *AtRAC11* stimulated auxin-responsive expression. Protein assays also indicated that RAC/ROP proteins were activated by auxin. Furthermore, expressing constitutively active or dominant negative mutants of the tobacco NtRac1 (highest similarity to AtRAC1/AtRAC6/AtRAC11) activated or suppressed auxin-induced gene expression, respectively. RAC/ROP GTPases therefore seems to participate in a signal pathway from auxin leading to expression of auxin-responsive genes.

Involvement of RAC/ROP in cell proliferation

The shoot meristem is responsible for all of the aboveground organs that are formed during the lifespan of a plant (Clark, 2001). The CLAVATA protein complex is required to maintain the balance between cell proliferation and organ formation at shoot and flower meristems in *Arabidopsis*. CLAVATA1, a receptor kinase, was used to isolate other components of the protein complex. One of the subunits was a 25 kDa protein that was recognised by an anti-RAC/ROP antibody, suggesting that a

RAC/ROP protein is part of the CLAVATA signalling complex (Trotochaud et al., 1999). The role of RAC/ROP may be to relay a signal from the CLAVATA complex to regulate gene expression, possibly through a MAP kinase cascade. Immunolocalisation using an anti-RAC/ROP antibody detected high amounts of RAC/ROP proteins in meristems, consistent with a role for RAC/ROP GTPases in regulation of meristem activity (Li et al., 2001).

Regulation and dynamics of the actin cytoskeleton

Actin

Actin microfilaments constitute a fundamental element of the cytoskeleton in eukaryotic cells, the other two main components being microtubuli and intermediate filaments. Actin microfilaments are polymers of actin, an evolutionarily conserved protein with a size of about 42 kDa. Actin exists either as monomers (G-actin) or as a polymeric filament (F-actin). When F-actin decorated with myosin subfragment 1 is viewed in an electron microscope, it appears arrowhead-shaped (Moore et al., 1970). Accordingly, the ends of the filament have been named the "barbed" and the "pointed" ends. Furthermore, actin binds ATP in complex with Mg^{2+}. G-actin is almost exclusively associated with ATP (Rosenblatt et al., 1995). Upon actin polymerisation, ATP becomes hydrolysed; however, this process is not directly coupled to the addition of the actin monomer to the filament, but takes place after a time lag (Carlier, 1990).

Actin filaments are highly dynamic structures, capable of elongation and rapid shortening through regulation of polymerisation and depolymerisation. Association and dissociation of actin monomers can take place at either end of the filament, but the former predominantly occurs at the barbed end and the latter at the pointed end. This property results in polarity of actin filament elongation, the barbed end being the rapidly growing end. As a consequence, actin monomers added to the barbed end move progressively towards the pointed end. This phenomenon is called "treadmilling" (Wegner, 1976).

The actin protein family in *Arabidopsis* consists of eight expressed members, and can be further subdivided into a vegetative and a reproductive class, based on their sequence and expression patterns (McDowell et al., 1996; Meagher et al., 1999).

Actin-binding proteins

Actin binds a large number of diverse protein classes collectively called actin binding proteins (ABPs). These proteins have varying properties; some classes bind G-actin, others bind F-filaments, and still others bind both G-actin and F-actin. ABPs can also be divided into classes that promote filament assembly or disassembly. Below is a brief review of the most common ABPs that have been found in plants (Meagher and Fechheimer, 2003).

Profilin

Profilins are small globular proteins that bind monomeric actin. Profilin promotes the exchange of ADP for ATP (Goldschmidt-Clermont et al., 1991), and also inhibits the hydrolysis of ATP bound to actin (Ampe et al., 1988). The effect of profilin on actin polymerisation is somewhat complex: on one hand, profilin can inhibit actin nucleation by keeping the free monomer concentration low, but on the other hand, it can promote polymerisation by transporting monomers to the fast-growing barbed ends of filaments. The *Arabidopsis* genome contains five profilin-encoding genes, designated PRF1 to PRF5 (Christensen et al., 1996; Huang et al., 1996). Similar to actins, the *Arabidopsis* profilin family can be divided into a vegetative and a reproductive class based on sequence and expression patterns. Antisense suppression of PRF1 and closely related members of the vegetative profilin resulted in dwarfed plants with reduced cell elongation compared to wild type. In contrast, overexpression of PRF1 produced plants with longer roots and root hairs, supporting a role for profilins in cell elongation (Ramachandran et al., 2000).

Actin depolymerising factor (ADF)/cofilin

ADFs accelerate the rate of actin filament turnover by increasing the rate of depolymerisation at the pointed ends of filaments (Carlier et al., 1997). In presence of

both ADF and profilin, the rate of filament turnover is further enhanced because the two proteins act at each end of the filament; profilin adding ATP-actin to the barbed end and ADF dissociating ADP-actin from the pointed end (Didry et al., 1998). The 12 members of the *Arabidopsis* ADF family vary in length from 130 to 140 residues. Expression analysis of three maize ADFs revealed that the genes seems to have expression either in vegetative or reproductive tissues (Jiang et al., 1997; Lopez et al., 1996). In metazoa, ADF is negatively regulated by phosphorylation of an N-terminal serine by LIM kinase. LIM kinase is in turn activated by Rac (Arber et al., 1998; Yang et al., 1998). No plant homologues of LIM kinase have been identified, but calmodulin-like domain protein kinase(s) (CDPK) have been found to phosphorylate Ser-6 in maize ADF3 (Allwood et al., 2002). Expression of constitutively active NtRac1 in tobacco pollen increases the ratio of phosphorylated to unphosphorylated ADF, indicating that RAC/ROP GTPases negatively regulates ADF activity in both plants and metazoa, although the ADF phosphorylation enzyme is different (Chen et al., 2003). Since the calmodulin-like domain of CDPKs is believed to bind Ca^{2+}, RAC/ROP regulation of CDPKs probably involves release of Ca^{2+}.

Capping protein

Capping protein (CP) binds with high affinity to the barbed ends of filaments and prevents loss and addition of actin monomers (Casella et al., 1986; Cooper and Schafer, 2000). CP is a heterodimeric protein consisting of an α and a β subunit, both subunits appear to exist as single copy genes in the *Arabidopsis* genome. Biochemical analyses of AtCP suggest that it has comparable properties with its metazoan homologues (Huang et al., 2003).

Formin

Formins are actin-nucleating proteins with the ability to stimulate *de novo* polymerisation of actin filaments (Wallar and Alberts, 2003; Zigmond, 2004b). They are conserved throughout the eukaryota, and are recognised by the presence of the highly conserved formin homology 2 (FH2) domain. *In vitro*, FH2-domains form dimers which bind G-actin and induce nucleation at the barbed end (Pruyne et al., 2002). The FH2 is usually coupled on its N-terminal side by a proline-rich region

called the formin homology domain 1 (FH1)(Wasserman, 1998). FH1 binds profilin, SH3 domains and WW domains (Macias et al., 2002), and is necessary for FH2 activity *in vivo*; actually, the FH1-FH2 region functions as a constitutively active formin when expressed in a cell (Evangelista et al., 2002). Profilin has been shown to be essential in *vivo* for a number of formin-dependent processes in metazoan cells (Severson et al. 2002), probably by increasing the local concentration of G-actin. A subgroup of metazoan formins also contain a GTPase binding domain (GBD), which binds Rho family GTPases (Evangelista et al., 1997; Watanabe et al., 1997). Binding of a Rho GTPase to the GBD releases an autoinhibitory interaction, thereby activating the formin (Li and Higgs, 2003). Formin stays associated during filament elongation, and thus protects the filament from capping (Zigmond et al., 2004b). Since formins induce *de novo* actin polymerisation, they produce unbranched actin filaments which assemble into actin bundles.

The formin family appears to be expanded in plants compared to animals; the *Arabidopsis* genome encodes 21 putative formins (Deeks et al., 2002d), whereas only 9 genes encoding formins so far have been identified in mammalian genomes (Li and Higgs, 2003). The plant formins all contain an FH2 domain and most of them also carry a FH1 domain. However, none of these proteins have a GBD, indicating that plant RAC GTPases do not participate in formin regulation, at least not directly (Deeks et al., 2002). Based on amino sequence similarity and domain composition, plant formins can be divided into two subfamilies (Deeks et al., 2002). Type I plant formins (in *Arabidopsis* constituted by AtFH1 to AtFH11) generally contain an N-terminal signal peptide or membrane anchor followed by a hydrophobic region that probably serves as a transmembrane domain (Cvrckova, 2000; Deeks et al., 2002). The N-terminal part of type II plant formins (in *Arabidopsis* constituted by AtFH12 to AtFH21) contains no known protein domains. Overexpression of the *Arabidopsis* type I formin AtFH1 in tobacco pollen tubes induces the formation of actin cables throughout the cytoplasm (Cheung and Wu, 2004). Moreover, overexpression of AtFH1 leads to growth depolarisation and growth arrest of pollen tubes, as well as deformation of the plasma membrane at the pollen tip apex. These results suggest that formins are important regulators of actin polymerisation in plants.

The Arp2/3 complex

During the last ten years, the Arp2/3 complex has emerged as an important initiator of actin polymerisation. The ARP2/3 complex was first identified in *Acanthamoeba* as a protein complex binding to profilin (Machesky et al., 1994), and appears to be conserved in all eukaryotes (Machesky and Gould, 1999). The complex consists of seven different subunits (Machesky et al., 1994; Mullins et al., 1997). Two of the subunits, Arp2 and Arp3, belong to the family of actin-related proteins (Arps) and are predicted to share the same protein fold as actin, although the amino acid sequence is divergent from that of conventional actins (Kelleher et al., 1995). ARPC1 contains seven putative WD40 motifs that may be involved in protein-protein interaction (Welch et al., 1997). The other four subunits, ARPC2, ARPC3, ARPC4 and ARPC5, are novel proteins without any described protein domains.

An important feature of the Arp2/3 complex is the ability to nucleate actin filaments and promote polymerisation at the filament's barbed end (Mullins et al., 1998). The Arp2/3 complex binds both to the sides and pointed ends of actin filaments, but kinetic and microscopic experiments indicate that activated Arp2/3 complex preferentially nucleates branches along the sides of pre-existing filaments (Amann and Pollard, 2001; Carlsson et al., 2004). At the side of a "mother" filament, the Arp2/3 complex will nucleate a new "daughter" filament at an angle of 70° (Mullins et al., 1998). In motile eukaryotic cells, the ARP2/3 complex is central in the formation of the highly branched actin filament structures at the leading edge of lamellipodia (Svitkina and Borisy,1999). Cryo-EM reconstruction of Arp2/3 complex at the branch-point between the mother and daughter filament (Volkmann et al., 2001) and an X-ray crystal structure of bovine Arp2/3 complex (Robinson et al., 2001) has provided more details on the mechanism by which the Arp2/3 complex initiate branching of actin filaments. The Arp2/3 complex appears to become attached to the side of the mother filament via bridges between the subunits ARPC1, ARPC2 and ARPC5 and three actin subunits. Arp2 and Arp3 constitute the two first subunits of the daughter filament. In the inactive crystal structure, the Arp2 and Arp3 subunits do not bind ATP. Furthermore, Arp2 and Arp3 are oriented in such a way that filament nucleation is impossible. Activation of the Arp2/3 complex by binding of ATP and one of several nucleation promoting factors (NPFs, discussed below) is believed to induce a

structural change which brings Arp2 and Arp3 into a conformation resembling the barbed end of an actin filament.

A common feature of the Arp2/3 complex in all eukaryotic cells is that most of the subunits are encoded by genes present in only one copy in the genome (Higgs and Pollard, 2001). In humans, only the Arp3 and the ARPC1 subunits are encoded by two genes, whereas in yeast, there is only one gene for each subunit. Similarly, in *Arabidopsis*, the ARPC1 is the only subunit encoded by two genes (Le et al., 2003; Li et al., 2003; Mathur et al., 2003).

The Arp2/3 complex and formins both have actin nucleating activity. However, there are important differences between these two actin nucleation activators (Zigmond, 2004a). First, formins produce actin bundles, whereas Arp2/3 produces a highly branched network of filaments. Second, formins are able to nucleate actin filaments at a new site. In contrast, Arp2/3 requires an existing filament on which to nucleate a new branch. Third, formins bind the barbed end of actin filaments, thereby protecting them from capping proteins. Arp2/3, on the other hand, binds the pointed end of filaments, leaving the barbed ends susceptible to capping. These differences imply that formins and the arp2/3 complex have distinct, though probably connected roles in regulation of actin dynamics.

Mutational analyses of Arp2/3 complex subunits have been performed in yeast, *Drosophila*, *C. elegans*, mouse and *Arabidopsis* (reviewed by Vartiainen and Machesky, 2004). Disruption of Arp2/3 subunits in budding yeast showed that ARPC1 and Arp2 are essential for viability. Mutants of the other subunits show varying degrees of growth defects (Winter et al., 1999; Morrell et al., 1999). Loss-of-function mutations of two Arp2/3 subunits in *Drosophila*, Arp3 and ARPC1, produce identical phenotypes. Although both subunits appear to be essential for viability, only a subset of actin structures is affected in the mutants. Among the observed phenotypes are defects in the early embryo and in the central nervous system (Hudson and Cooley, 2002). In *Arabidopsis*, Arp2, Arp3 and ARPC2 and ARPC5 mutants have recently been characterised by several groups (Mathur et al., 2003a; Mathur et al., 2003b; Le et al., 2003; Li et al., 2003; El-Din El-Assal et al., 2004). All mutants show similar phenotypes, characterised by aberrant cell shapes caused by misdirected cell expansion. The most striking phenotype is the development of trichomes with reduced size and irregular shape. A previous screen for trichome mutants identified eight complementation groups showing this phenotype, collectively

called "distorted" mutants (Hülskamp et al., 1994). Arp2, Arp3 ARPC2 and ARPC5 are defect in the "distorted" mutants *wurm* (*wrm*), *distorted1* (*dis1*) , *distorted2* (*dis2*) and *crooked* (*crk*), respectively (Mathur et al., 2003a; Mathur, et al. 2003b; Le et al., 2003; El-Din El-Assal et al., 2004). The mutants display defects in the organisation of actin cytoskeleton, such as aggregation of filaments.

Nucleation promoting factors (NPFs): activators of the Arp2/3 complex

During the last few years, a number of different activators of the Arp2/3 complex has been discovered (reviewed by Higgs and Pollard, 2001; Weaver et al., 2003). These include the WASP/WAVE family proteins (Machesky et al., 1999; Rohatgi et al., 1999); ActA from the intracellular pathogenic bacterium *Listeria monocytogenes* (Welch et al., 1998); the cortactin family proteins (Uruno et al., 2001; Weaver et al., 2001); and the yeast type I myosins, Abp1p and Pan1p (Duncan et al., 2001; Evangelista et al., 2000; Goode et al., 2001; Lechler et al., 2000). To date, NPFs have not been reported in plants. Blast searches did not lead to the discovery of any plant proteins with similarity to the known NPFs, with the possible exception of a small family of putative WAVE proteins (Brembu et al., unpublished results; see also results and discussion). Since the WAVE/WASP family is the most extensively studied group of NPFs, I will focus on these proteins.

Identification of WASP/WAVE proteins

As the name implies, The WASP/WAVE protein family can be divided into two groups: the Wiskott-Aldrich Syndrome proteins (WASP) and the WASP family Verprolin-homologous protein (WAVE). WASP was identified as the target of mutation in Wiskott-Aldrich Syndrome, a rare, X-linked disease which is characterised by eczema, bleeding and recurrent infections (Derry et al., 1994). Expression analysis show that WASP is expressed exclusively in hemapoietic cells, hence the restricted defects in the syndrome. The other member of the WASP group in mammals, neural WASP (N-WASP), is expressed more widely than WASP and is especially enriched in brain and nerve cells (Miki et al., 1996). The first WAVE group protein was identified in a database search for WASP-like proteins (Bear et al., 1998). In mammals, the WAVE group appears to consist of three members, WAVE1 to WAVE3.

Domain composition and function of WASP/WAVE

As shown in Fig. 3, WASP and WAVE proteins share two main regions of homology: a central, proline-rich region, and a C-terminal module containing a verprolin-homology (V), a central (C) and an acidic (A) region, collectively called the VCA region (see also Fig. 7)(Miki et al., 1996). The proline-rich region binds several SH3 domain-containing proteins as well as profilin (Caron, 2002; Finan et al., 1996). The VCA region is essential for actin binding and activation of the Arp2/3 complex (Machesky et al., 1999; Rohatgi et al., 1999; Yarar et al., 1999). The V region binds G-actin, whereas the A region appears to be the main site of Arp2/3 complex binding (Marchand et al., 2001). Upon binding the Arp2/3 complex, the C region forms an amphipathic helix that is required for activation (Panchal et al., 2003). Binding of the VCA region enhances the affinity of Arp2/3 complex for the side of an actin filament and promotes the formation of a quaternary complex of VCA, an actin monomer, Arp2/3 complex and an actin filament. A subsequent activation step promotes the nucleation of a daughter filament from the side of the mother filament (Higgs and Pollard, 2001). Furthermore, actin filaments increase the affinity of the VCA region for the Arp2/3 complex, implying that nucleation by filament-bound Arp2/3 is favoured over that by free Arp2/3 (Marchand et al., 2001).

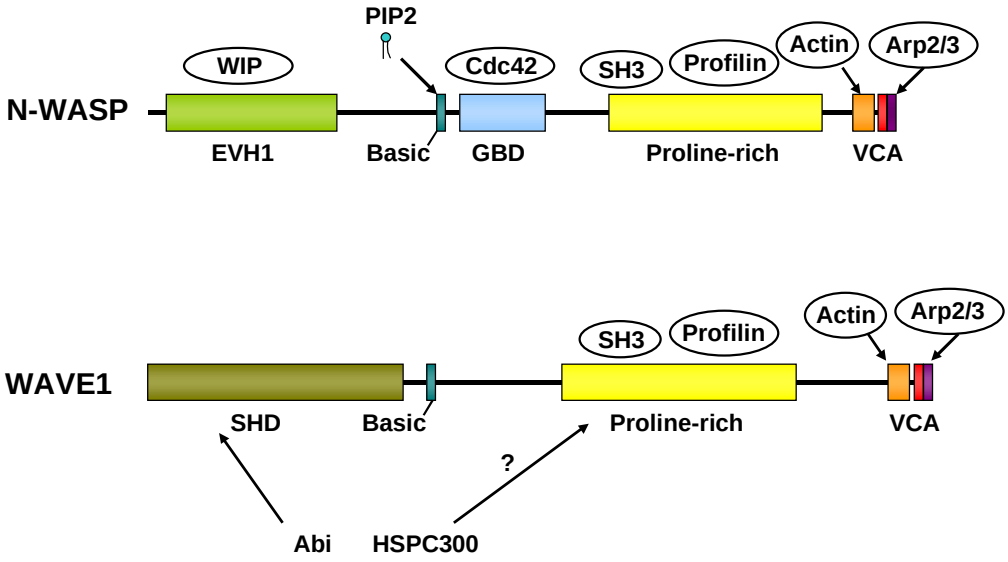

Figure 3. Domain structure of mammalian WASP and WAVE proteins, and interacting proteins and molecules. HSPC300 and Abi are components of a WAVE regulatory protein complex (Eden et al., 2002) and interacts with WAVE (Gautreau et al., 2004; Innocenti et al.,

2004). The HSPC300-binding site in WAVE is unknown. Abbreviations: EVH1, Ena/VASP homology 1; GBD, GTPase-binding domain; PIP2, phosphatidylinositol-3,4-diphosphate; SH3, Src homology 3; SHD, Scar homology domain; WIP, WASP interacting protein. Modified from Takenawa and Miki (2001).

The N-terminal parts of WASP and WAVE proteins are quite divergent (Fig. 3, see also Fig. 8). At the N-terminal end, WASP proteins contain a domain similar to the Ena/VASP homology 1 (EVH1) domain. EVH1 domains generally recognise and bind specific proline-rich sequences, and are found in many proteins involved in re-organisation of the actin cytoskeleton (Ball et al., 2002). Both WASP and N-WASP EVH1 domains interact with WASP interacting protein (WIP), which also binds actin (Ramesh et al., 1997; Volkman et al., 2002). The corresponding region in WAVE proteins constitutes a unique domain called the Scar homology domain (SHD). Although no proteins have been reported to interact with SHD, recent studies indicate that a leucine zipper-like motif in the SHD is important in localisation of WAVE at the tips of filopodia in the growth cone of neuronal cells (Nozumi et al., 2003). WASP proteins also contain a CRIB domain, positioned next to the proline-rich region, that binds Cdc42 with high affinity (Abdul-Manan et al., 1999; Rudolph et al., 1998).

Regulation of WASP activity by Cdc42 and PIP2

Under resting conditions, WASP and N-WASP are maintained in an inactive state through autoinhibition; the VCA region is masked by binding of the CRIB domain to the amphipatic helix of the C region (Miki et al., 1998; Panchal et al., 2003). Activated Cdc42 will bind to the CRIB, directly competing with the VCA region. A small region of basic residues immediately N-terminal to the CRIB domain binds phosphatidylinositol-4,5-diphosphate (PIP2). Current models suggest a mechanism of cooperative activation of WASP and N-WASP by Cdc42 and PIP2, as shown in Figure 4. Binding of Cdc42 to the CRIB displaces the C region, whereas binding of PIP2 to the basic region will displace the A region, thereby releasing the VCA and activating the Arp2/3 complex (Blanchoin et al., 2000; Prehoda et al., 2000; Rohatgi et al., 2000).

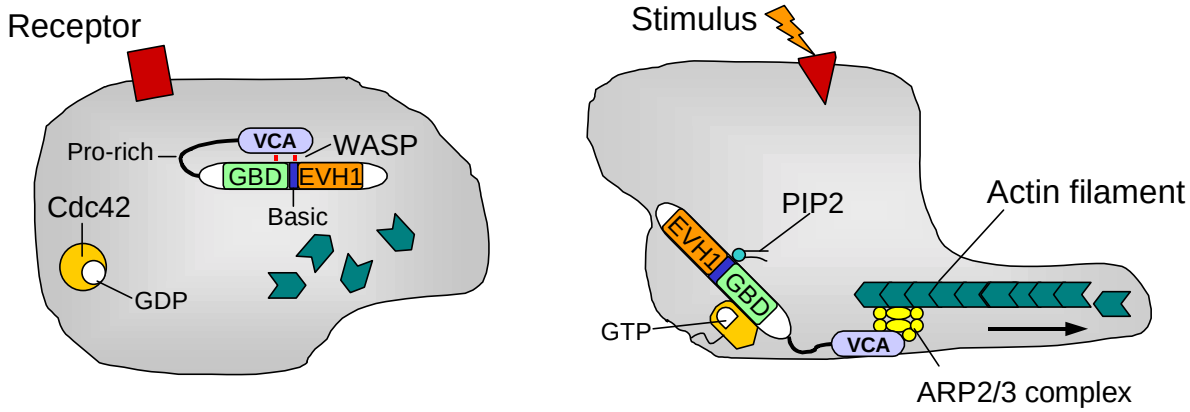

Figure 4. Regulation of WASP activity. In the inactive state of WASP, the central (C) region of the VCA domain interacts with the GBD domain, and the acidic region interacts with the basic region adjacent to the GBD. Cooperative binding of active Cdc42 to the GBD and of PIP2 to the basic region exposes the VCA domain, resulting in Arp2/3 complex activation. Abbreviations: EVH1, Ena/VASP homology 1; GBD, GTPase-binding domain; PIP2, phosphatidylinositol-3,4-diphosphate. Inspired by Takenawa and Miki (2001), and Ridley and Cory (2002).

Regulation of WAVE activity by a regulatory protein complex, Rac and Nck

The WAVE proteins lack a CRIB domain (Fig. 3), suggesting that they are neither binding any Rho GTPases directly nor being regulated by autoinhibition. In accordance with these presumptions, WAVE is constitutively active *in vitro* (Machesky et al., 1999). The WAVE proteins contain a basic region, similar to WASP and N-WASP. Although PIP2 has not been shown to bind this region in WAVEs, it is required for Arp2/3-mediated actin polymerisation from WAVE2 (Suetsugu et al., 2001). Eden and co-workers (2002) purified a WAVE1-containing protein complex by column chromatography and immunoprecipitation, and identified the other proteins in the complex as PIR121, Nap125, HSPC300 and Abi-2 (Abelson interactor). This protein complex is unable to stimulate actin polymerisation, indicating that WAVE1 is inactive. Interestingly, when active Rac or the SH3 domain-containing adapter protein Nck was added to the inactive WAVE1 complex, PIR121, Nap125 and Abi-2 dissociated from HSPC300-WAVE1, which regained the ability to activate the Arp2/3 complex (Fig. 5). Thus, WAVE1 appears to be kept inactive through interaction with a regulatory protein complex, and its activation by Rac is indirect rather than direct,

through the binding and dissociation of the regulatory complex by Rac. The role of HSPC300 in WAVE1-mediated activation of the Arp2/3 complex is still uncertain. In a similar experiment, Soderling and colleagues (2002) identified a novel Rac GTPase activating protein, WAVE-associated RacGAP protein (WRP), which binds to WAVE1 through its SH3 domain. WRP is able to inhibit Rac *in vivo*, suggesting that WRP functions as a signal terminator of Rac.

Recently, the molecular architecture of the mammalian WAVE complex was resolved (Gautreau et al., 2004; Innocenti et al., 2004). Interactions between Sra-1, Nap1, Abi-1, WAVE2 and HSPC300 were studied using *in vitro* cotranslation of the different subunits followed by specific immunoprecipitations (Gautreau et al., 2004) or tandem mass spectrometry (Innocenti et al., 2004). Abi and Nap appear to constitute the core of the WAVE complex. Sra-1 is a peripheral subunit interacting with Nap, whereas WAVE interacts with Abi through its' SHD domain, as well as with HSPC300. The proposed architecture of the WAVE complex is shown in fig. 5.

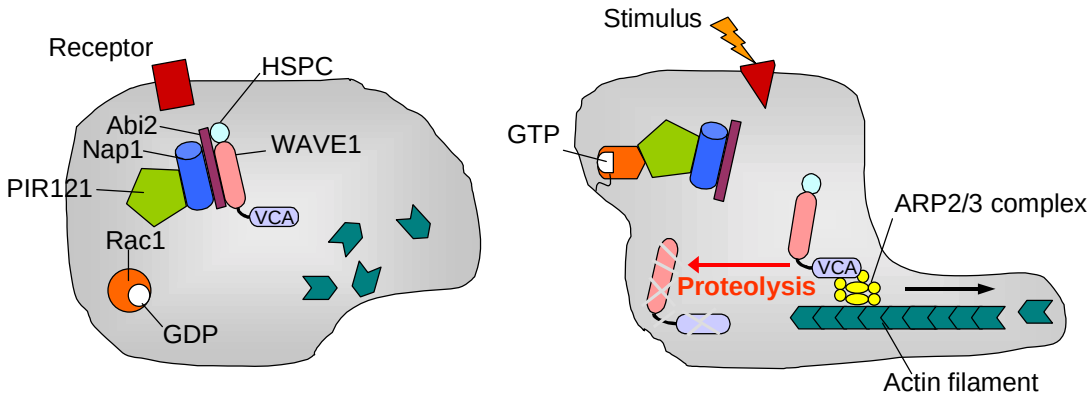

Figure 5. Modulation of WAVE activity by a regulatory protein complex and Rac. Inactive WAVE1 is part of a regulatory complex consisting of Abi2, Nap1, PIR121 and HSPC300. Active Rac1 binds PIR121, leading to dissociation of the PIR121-Nap1-Abi2 subcomplex from WAVE1-HSPC300. The uncomplexed WAVE1 is able to activate the ARP2/3 complex, leading to actin polymerisation. The indicated interactions between the different WAVE complex subunits are based on results by Gautreau et al. (2004). *Dictyostelium* mutants for PIR121 contain highly reduced amounts of WAVE, indicating that free active WAVE is rapidly degraded through a proteolytic mechanism (Blagg et al., 2003). Abi2, Abelson interactor 2; Nap1, Nck-associated protein 1; PIR121, 121F-specific p53 inducible RNA; WAVE1, WASP family verprolin-homologous protein 1. Modified from Ridley and Cory (2002).

Subunits of the WAVE regulatory complex

Prior to the publication by Eden et al. (2002), experiments performed in several organisms already indicated a role for the different subunits of the WAVE1 regulatory complex in organisation of actin cytoskeleton. Unfortunately, no common nomenclature for the subunits exists, neither within nor between species. For clarity, I will use the protein names employed by Eden et al. (2002) in combination with the names used in the specific experiment. Table 2 summarises the nomenclature of the WAVE regulatory complex subunits.

Table 2. WAVE regulatory complex nomenclature in *H. sapiens*, *Drosophila*, and *C. elegans*

Standardised names in this thesis	*H. sapiens*	*Drosophila*	*C. elegans*
WAVE	WAVE, SCAR	WAVE, SCAR	SCAR, GEX-1
PIR121	PIR121, Sra-1, CYFIP, SHYC	CYFIP, Sra1	GEX-3
Nap	Nap, Nap125, HEM	Kette	GEX-2
HSPC300	HSPC300	HSPC300	HSPC300
Abi	Abi	Abi	Abi

Nap/HEM-like proteins were first identified in human tissue (Hromas et al., 1991), and later in mouse, *Drosophila* and *C. elegans* (Baumgartner et al., 1995). Vertebrates appear to have two copies of Nap (Nck-associated protein)/Hem proteins. Nap1/HEM-2 was also cloned in a screen for proteins binding to the SH3 domain of human Nck (Kitamura et al., 1996). A similar screen for proteins binding to human Rac1 also identified Nap1 (Kitamura et al., 1997). However, *in vitro* experiments indicated that binding of Nap1 to Rac1 is indirect rather than direct. Another screen for Rac1-associated proteins identified a protein closely related to PIR121, called p140Sra-1 (140 kDa Specifically Rac1-associated protein), as well as HEM-2/Nap1 (Kobayashi et al., 1998). The association between Sra-1 and Rac1 is localised to the N-terminal 400 residues. Sra-1 also appears to interact with actin (Kobayashi et al., 1998) as well as profilin II (Witke et al., 1998).

Transcriptome analysis of cells treated with the DNA-damaging agent doxorubicin shows that PIR121 (121F-specific p53 inducible RNA) expression is highly induced by the treatment, suggesting that PIR121 might be a p53-induced gene involved in apoptosis (Saller et al., 1999). However, the mechanisms behind the induction of

PIR121 and the possible effects of higher PIR121 expression in apoptosis are unknown. Yet another role for PIR121 was discovered with the finding of interaction between the two PIR121 proteins (called CYFIP1 and CYFIP2) with the fragile X mental retardation protein (FMR-1), an RNA-binding protein associated with polysomes as part of a messenger ribonucleoprotein (mRNP) complex (Schenck et al., 2001). PIR121/Sra-1 is conserved throughout the metazoa, however, only vertebrates seem to have two genes encoding PIR121-like proteins. Moving the focus from human cells to *Drosophila*, Schenk and colleagues (2003) found that loss of PIR121 function leads to defects in axon growth, branching and pathfinding in the neural system, similar to the phenotypes of FMR1 and Rac1 mutants in *Drosophila*. Genetic interaction studies of PIR121, FMR1 and Rac1 indicates that PIR121 acts as a Rac1 effector that antagonises FMR1 function (Schenck et al., 2003). By analogy to the WAVE1 regulatory complex in mammals, PIR121 in *Drosophila* may bind and inhibit FMR1 activity. Active Rac1 will bind PIR121, and release active FMR1.

In parallel with the work on PIR121/CYFIP, the lethal *Drosophila* mutant *kette*, which also has defects in axonal patterning of the central nerve system, was identified. The Kette gene encodes the *Drosophila* homologue of Nap/HEM. Expression of constitutively active and dominant negative Rac1 mutants in neuronal cells of wild type embryos phenocopies the *kette* mutant. However, expression of constitutively active Rac1 in *kette* mutants partially rescues the mutant phenotype, suggesting an antagonistic relationship between Nap/Kette and Rac1 (Hummel et al., 2000). Bogdan and Klämbt (2003) recently studied the relationship between Nap/Kette and the WAVE and WASP proteins. The Nap/Kette protein antagonises WAVE function, but surprisingly, it appears to activate WASP-dependent actin polymerisation. The SH3-containing protein Abi, which is also present in the WAVE1 regulatory complex (Eden et al., 2002), interacts both with Nap/Kette and WASP, suggesting that it operates as a linker between Nap/Kette and WASP.

Mutant analyses in *C. elegans* also suggest a critical role for Nap and PIR121 in embryogenesis. The GEX-2 and GEX-3 (gut on exterior) genes, the mutation of which leads to defects in cell migration and organisation during embryogenesis, were identified as homologues of PIR121 and Nap, respectively (Soto et al., 2002). A third *gex* mutant, *gex-1*, is mutated in the *C. elegans* WAVE homologue (Soto, M.C., Brownwell, D., Priess, J., Mello, C.C., "The *C. elegans* WAVE homolog GEX-1 acts

with the Arp2/3 complex to regulate cell movements", poster at the 14th biennial international *C. elegans* Conference, 2003).

All of the subunits constituting the WAVE1 regulatory complex have been found in the mycetozoan *Dictyostelium*. Surprisingly, *Discoideum* cells defect in PIR121 function contain very small amounts of intact WAVE/Scar protein, suggesting that active WAVE/Scar is rapidly degraded through an unknown mechanism (Blagg et al., 2003). This result is confirmed by two groups using RNA interference to study cell morphology in *Drosophila* S2 cells (Kunda et al., 2003; Rogers et al., 2003). Both groups investigated the effects of RNAi depletion of proteins involved in actin regulation. RNAi down-regulation of two Arp2/3 complex subunits (ARPC2 and ARPC4), WAVE/Scar, Nap/Kette, PIR121 and Abi all resulted in a similar phenotype with stellate cell shape, consistent with the theory that these proteins participate in a common pathway. Furthermore, WAVE/Scar protein is absent in cells expressing RNAi of Nap/Kette, PIR121 and Abi, similar to the observations by Blagg et al. (2003).

A recent publication presents an alternative model for the role of the WAVE regulatory complex in controlling WAVE activity (Steffen et al., 2004). Murine Sra-1 co-immunoprecipitated with Nap1, Abi-1 and WAVE2, as well as with constitutively activated Rac1. Nap1 and Sra-1 were shown to be translocated to the tip of lamellipodia upon microinjection of constitutively activated Rac1 into cultured cells. Furthermore, RNAi ablation of Nap and Sra-1 resulted in highly reduced formation of lamellipodia; neither overexpression of WAVE2 nor microinjection of constitutively activated Rac1 rescued this phenotype. These results support a model in which Nap1 and Sra-1 stay associated with WAVE2 upon activation by Rac1 and are essential components of a protein complex necessary for formation of lamellipodia (Steffen et al., 2004). WAVE1 and WAVE2 may be differentially regulated; the same could be the case for PIR121 and Sra-1. These differences might explain the discrepancies between the results by Eden et al. (2002) and Steffen et al. (2004). WAVE1 is continuously distributed along the leading edge, whereas WAVE2 and WAVE3 are localized at the initiation sites of microspikes on the leading edge and at the tips of elongating filopodia (Nozumi et al., 2003), illustrating the differences between the WAVE family members. Fig. 6 shows a model illustrating the findings by Steffen et al. (2004).

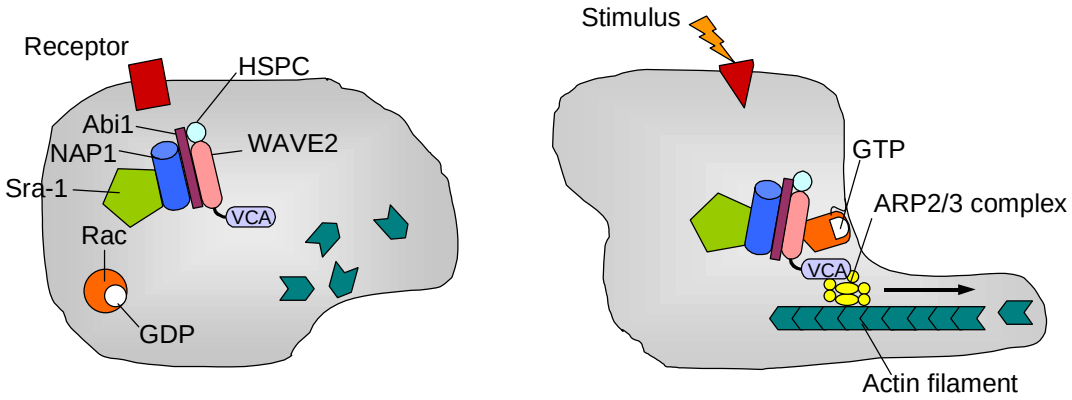

Figure 6. Alternative model for regulation of WAVE activity, based on the results by Steffen et al. (2004). In this model, the WAVE regulatory complex stays intact upon activation by Rac1, and is essential for activation of the ARP2/3 complex by WAVE. Abi1, Abelson interactor 1; Nap1, Nck-associated protein 1; Sra-1, specifically Rac1-associated protein; WAVE2, WASP family verprolin-homologous protein 2.

The function of HSPC300, the small protein associated with WAVE1, has not been studied in metazoa. In maize, three *brick* mutants have epidermal leaf cells without lobes, similar to what is observed for the Arp2/3 complex mutants in *Arabidopsis*. Local enrichments of cortical actin are found in wild type cells at developing lobes. In the *brk* mutants, this diffuse actin is absent (Frank and Smith, 2002; Frank et al., 2003). The *BRK1* gene encodes a HSPC300 homologue, indicating that this protein is important for proper activation of the Arp2/3 complex, at least in plants.

To summarise, WAVE/Scar proteins appear to exist as part of a regulatory protein complex in unstimulated cells. The other subunits of the regulatory complex are Nap/Kette/GEX-3, PIR121/Sra-1/CYFIP/GEX-2, Abi and HSPC300/BRK1. The regulatory complex functions not solely to keep WAVE inactive; it also protects WAVE from rapid degradation and might also be important for correct intracellular localisation of WAVE. Upon stimulation, activated Rac or Nck bind to PIR121, thereby dissociating the regulatory complex. Active WAVE stays associated with HSPC300 and activates the Arp2/3 complex, which leads to increased actin polymerisation and branching of actin filaments, producing the driving force for cell locomotion or morphogenesis. In order to avoid excessive actin filament nucleation of the constitutively active, uncomplexed WAVE, a yet unknown proteolytic system degrades WAVE, halting further activation of Arp2/3 unless more WAVE protein is

produced and released from its regulatory complex. In an alternative model, the WAVE regulatory complex is important for activation of WAVE and stays associated with active WAVE.

AIMS OF THE STUDY

The major aim of this study was to increase the knowledge about the structure and function of RAC-like GTPases in plants, using *Arabidopsis thaliana* as a model system. In order to do these studies, we wanted to clone the entire RAC/ROP gene family in *Arabidopsis* and perform functional anlyses on selected genes. We also wanted to investigate possible effectors of RAC/ROP GTPases.

RESULTS AND DISCUSSION

Cloning of a family of RAC/ROP GTPases in Arabidopsis (paper I)

In an effort to identify Ras-like proteins in plants, degenerate PCR-primers previously used in amplification of Ras GTPases in a number of metazoan organisms were employed for low stringency RT-PCR, with *Arabidopsis* cDNA as template. Sequencing of cloned PCR products identified six RAC/ROP-like GTPases (*AtRAC4*, *7*, *8*, *10* and *11*, and *ATR2a*, which was later identified as AtRAC3)(Paper I, Fig. 1). Interestingly, no genes with similarity to Ras-like genes were found. A mixed probe of *AtRAC4* and *AtRAC10* were used to screen an *Arabidopsis* cDNA library, and resulted in the cloning of full-length cDNAs encoding five RAC GTPases, named *Arac1* (*AtRAC1*) to *Arac5* (*AtRAC5*) (Paper I, Fig. 3), as well as a partial cDNA (*Arac6*/*AtRAC6*). Thus, including cDNA clones and sequenced PCR products, ten RAC/ROP-like GTPases were identified. This was the first major investigation of the RAC/ROP gene family in *Arabidopsis*. Analysis of the amino acid sequence encoded by the full length cDNA clones suggested that the plant RAC/ROP proteins have a secondary structure similar to Ras proteins (Paper I, Fig. 4). The amino acid sequence of the Arac proteins and other plant Rac proteins was compared with metazoan Rho family GTPases. The plant Rac proteins were found to constitute a subfamily more related to Rac and Cdc42 than to Rho GTPases (Paper I, Fig. 5). Analyses of *Arac1* to *Arac5* expression in tissues at selected developmental stages were performed. Due to the generally low expression levels of *Arac* genes, RT-PCR was used. *Arac1*, *Arac3*, *Arac4*, and *Arac5* were expressed in all tissues examined (Paper I, Fig. 6, Fig. 8A and C). In contrast, *Arac2* appeared to be preferentially expressed in roots and stems (Paper I, Fig. 7 and Fig. 8B). RT-PCR studies by Li et al. (1998) on *AtRAC1*, *AtRAC3*, *AtRAC4* and *AtRAC5* confirmed these results. Promoter studies of *AtRAC3* (Lemichez et al., 2001) and *AtRAC4* (Li et al., 2001) also indicate that these two genes are ubiquitously expressed.

Genomic analysis of the RAC/ROP GTPases in Arabidopsis (paper II)

The complete *Rac* gene family in *Arabidopsis thaliana*, consisting of eleven members (*AtRAC1* to *AtRAC11*), was presented (Paper II, Fig. 1). Based on amino acid similarity and genomic structure, the *AtRAC* gene family can be divided into two groups; group I consists of *AtRAC1* to *AtRAC6*, *AtRAC9* and *AtRAC11*; group II is constituted by *AtRAC7*, *AtRAC8* and *AtRAC10*. Group II *AtRAC* genes have an extra exon at the 3' end, which is probably the result of the insertion of an intron in the last exon of an ancestral *RAC* gene (Paper II, Fig. 2A). While the group I AtRAC proteins all have a C-terminal consensus motif for geranylgeranylation, the group II AtRACs appear to lack this motif. Instead, they contain a cystein-containing motif, suggesting that they may be subject to a different C-terminal modification (Paper II, Fig. 2B). Indeed, work by Lavy et al. (Lavy et al., 2002) show that the group II AtRACs likely are palmitoylated. Clones of nine *AtRAC* genes were isolated from a genomic library made from the Landsberg erecta ecotype, and the genomic structure was characterised (Paper II, Fig. 4). Comparison of the genomic sequence of *AtRAC* genes from the Landsberg and Columbia ecotypes (the latter was sequenced by the *Arabidopsis* genome sequencing project (The Arabidopsis genome initiative, 2000) revealed great variation in the number of polymorphisms and indels; *AtRAC5* contains no polymorphisms, whereas *AtRAC2* has about one single nucleotide polymorphism (SNP) per 50 bp (Paper II, Fig. 3, Table 1). The polymorphism frequency may be correlated to expression levels, through transcription-coupled DNA repair (Hanawalt, 1989). AtRAC2 has low expression levels compared with the other *AtRAC* genes, and may therefore be more prone to accumulation of polymorphisms due to reduced transcription-coupled DNA repair.

The evolution of RAC/ROP GTPases in plants (paper II)

In order to study the evolution of the plant *RAC* gene family, the genes flanking the different *AtRAC* genes were compared (Paper II, Fig. 7). Several of the *AtRAC* genes were created as part of large duplications, as neighbouring genes have (at least partly) conserved positions. These duplications appear to have occurred at different

points of time during evolution. Gene pairs created by duplication include *AtRAC1* and *AtRAC6*, *AtRAC4* and *AtRAC5*, and *AtRAC8* and *AtRAC10*.

A protein alignment was made of the known plant RACs together with metazoan and mycetozoan Rac and Cdc42 (Paper II, Fig. 5). Phylogenetic trees based on this alignment confirm that the plant RAC proteins emerge as a sister group to the Rac/Cdc42 proteins. Searches for Rho type GTPases in lower eukaryotes such as amoebas and slime molds reveal that whereas Rac-like proteins are found in several of these phyla, Rho and Cdc42 has so far just been identified in fungi/yeast and animalia (Winge, 2002). Thus, the plant RAC proteins have probably evolved from a Rac-like ancestor. The phylogenetic analyses on protein (Paper II, Fig. 5) and DNA sequences (Paper II, Fig. 6) confirmed that the plant RAC GTPases can be divided into two main groups. A further division into smaller subgroups is also possible. The group II of RAC/ROPs can be divided into two subgroups; one containing AtRAC7-like proteins, the other containing homologues of AtRAC8 and AtRAC10. Monocot species are represented in both of the subgroups, indicating that they originated before the split between dicotyledons and monocotyledons. In group I, AtRAC2 and possibly AtRAC9 constitute one subgroup; *AtRAC2*-like genes have been found in conifers, suggesting that AtRAC2 belongs to an ancient subgroup. The other AtRACs in group I belong to a subgroup that apparently have expanded in dicotyledons; thus, some of the duplication events leading to the generation of new genes within this subgroup probably occurred after the monocot/dicot split. A recent study on monocot RAC/ROP GTPases (Christensen et al., 2003) supports our general conclusions. Due to the sequencing of new *RAC/ROP* homologues by several genome and EST projects, Christensen et al. (2003) were able to group *AtRAC9* more firmly together with the *AtRAC2*-like genes. *AtRAC9* is believed to be more rapidly evolving than other *RAC*s, leading to its divergent sequence. *AtRAC9* has very low expression levels (Winge et al., unpublished); as mentioned for *AtRAC2*, this may result in reduced transcription-coupled DNA repair, leading to a faster accumulation of mutations.

AtRAC2 is specifically expressed in primary xylem (paper III)

The tissue-specific expression of *AtRAC2* indicated by RT-PCR prompted us to investigate further the expression pattern of *AtRAC2*. A 1.3 kb fragment of the genomic region immediately upstream of *AtRAC2* including 22 bp of the *AtRAC2* coding sequence was cloned in front of the reporter genes GUS and GFP5-ER, and transgenic *Arabidopsis* plants carrying these gene constructions were generated by *Agrobacterium*-mediated transformation (Paper III, Fig. 1A). All transgenic lines investigated displayed similar expression patterns, albeit with varying levels of expression. Transgenic *AtRAC2*:*GUS* plants showed *GUS* expression in developing primary xylem elements in root, hypocotyl, leaves and stem (Paper III, Fig. 2). Expression in roots first appeared in the protoxylem (Paper III, Fig. 2A); GUS staining seemed to precede the visual appearance of secondary cell walls (Paper III, Fig. 2B). The same expression pattern was seen when visualising GFP in *AtRAC2:GFP5-ER* plants (Paper III, Fig. 3). GUS staining later appeared in metaxylem cells (Paper III, Fig. 2B, C and D). In hypocotyls and developing leaves (Paper III, Fig. 2E), weak GUS staining was observed. GUS staining in stems of *AtRAC2:GUS* plants was restricted to developing primary xylem cells (Paper III, Fig. 2F and G). Mäule reagent, which visualises lignin, only weakly stained GUS positive cells of stem cross-sections, indicating that *AtRAC2* expression mainly precedes lignification of xylem elements in the stem (Paper III, Fig. 2H).

Further data supporting the results from the promoter studies recently came from the "gene expression map of the *Arabidopsis* root" published by Birnbaum et al. (2003). Using cell sorting of GFP fluorescent protoplasts from different GFP promoter lines with cell type specific root expression, Birnbaum et al. (2003) were able to compare gene expression in different tissues and developmental zones of the *Arabidopsis* root. In the published dataset, *AtRAC2* showed very low expression in most root tissues, but had a pronounced expression peak in the stele of the root elongation and differentiation zone. The stele includes vascular tissue; thus, the expression data for *AtRAC2* published by Birnbaum et al. (2003) confirms the observed root expression pattern for *AtRAC2:GUS*. Other genes in the same dataset showing expression patterns similar to *AtRAC2* included the HD-ZIP transcription factors *AtHB-8* and *REVOLUTA* (*REV*) / *INTERFASCICULAR FIBERLESS* (*IFL*), the cellulose synthase catalytic subunits *AtCesA4* (*IRX5*), *AtCesA7* (*IRX3*) and *AtCesA8* (*IRX1*), and the

xylem-specific cysteine proteases *XCP1* and *XCP2*. The gene products of all these genes have been implicated in differentiation or development of xylem. Both *AtHB-8* (and *REV/IFL*) are expressed in the provascular tissue as well as in developing vascular tissue (Baima et al., 1995; Baima et al., 2001). Mutation of *IFL* blocks the normal differentiation of interfascicular fiber cells, which are layers of long cells located between the vascular bundles of the inflorescence stem (Zhong and Ye, 1999). Loss-of-function mutants of AtHB-8 show no visible phenotypes, but overexpression of *AtHB-8* appears to stimulate vascular tissue differentiation, as the number of xylem cells increases (Baima et al., 2001). AtCesA4, AtCesA7 and AtCesA8 are all implicated in secondary cell wall synthesis. Mutation of each gene results in collapsed xylem vessels as a consequence of reduced thickness of the secondary cell wall, the mutants were therefore called *irregular xylem* (*irx*)(Taylor et al., 1999; Taylor et al., 2000; Taylor et al., 2003). The xylem specific proteases are thought to be involved in the final stage of xylem differentiation, in which the xylem cell undergoes programmed cell death, forming an empty xylem vessel (Funk et al., 2002; Zhao et al., 2000). The co-expression of *AtRAC2* with these genes in roots supports the observed GUS staining pattern in roots of *AtRAC2:GUS* plants.

Functional studies of AtRAC2 (paper III)

In an effort to resolve the function of AtRAC2, we subjected transgenic *AtRAC2:GUS* plants to a number of treatments, including salt stress, osmotic stress, oxidative stress and application of different hormones. None of these treatments led to any observable(?) changes in localisation or intensity of GUS staining, indicating that *AtRAC2* expression is mainly developmentally regulated. Expression of several other *AtRAC* genes also appears to be rather non-responsive to different treatments (Brembu et al., unpublished results). Probably, the more detailed spatial and temporal control of AtRAC activity is achieved on protein level, through interactions with regulatory proteins such as GAPs and GEFs. However, the long lifetime(?) of the GUS protein could possibly mask a reduction in *GUS* expression.

A T-DNA insertion mutant of AtRAC2 (*atrac2-1*) was obtained, and RT-PCR analysis showed that *AtRAC2* expression was absent in the mutant (Paper III, Fig. 1B). No observable phenotypes were observed in the *atrac2-1* mutant, neither in general

morphology nor in organisation of the vascular tissue. Transgenic plants expressing wild type AtRAC2 and constitutively activated AtRAC2 (Gly15Val) under control of the viral CaMV 35S promoter were created (Fig. 1A). Transgenic plants overexpressing wild type AtRAC2 did not show any notable phenotypes. Considering the low endogenous expression levels of AtRAC2, this is somewhat surprising. AtRAC2 protein activity is perhaps buffered by increased RACGAP activity or higher levels of RhoGDI complexing with AtRAC2. In contrast, transgenic plants overexpressing constitutively active (CA) AtRAC2 displayed a different phenotype. Cotyledons and rosette leaves of *CA-atrac2* plants elongated faster than wild type plants, resulting in a more elongated leaf shape of *CA-atrac2* plants (Paper III, Fig. 4A and B). Scanning electron microscopy (SEM) studies revealed that the shape of leaf epidermal cells was changed in *CA-atrac2* plants. The pavement cells of *CA-atrac2* plants have a smooth outline with highly reduced number and length of lobes compared to wild type pavement cells (Paper III, Fig. 4C and D). The phenotype was characterised by calculating the ratio between the area (μm^2) and the perimeter (μm) of pavement cells; cells with long lobes will have a lower ratio than cells with shorter lobes. The ratio of cell area to cell perimeter was significantly higher in *CA-atrac2* expresssing plants compared to wild type and atrac2-1 plants (Paper III, Fig. 4E), in accordance with the previous observations. However, cell size was not affected (Paper III, Fig. 4F).

The subcellular localisation of AtRAC2 was determined by creating transgenic plants carrying GFP fusion constructs in which GFP was fused to the N-terminal of wild type AtRAC2 or *CA-atrac2*, under control of the CaMV 35S promoter (Paper III, Fig.1A). Both GFP-AtRAC2 and GFP-*CA-atrac2* were predominantly localised to the plasma membrane (Paper III, Fig. 5). This localisation has also been observed for the AtRAC GTPases AtRAC3 (Bischoff et al., 2000), AtRAC4 (Jones et al., 2002; Fu et al., 2002), AtRAC6 (Kost et al., 1999), AtRAC7 (Lavy et al., 2002; Ripel et al., unpublished), AtRAC8 (Lavy et al., 2002; Zheng et al., 2002) and AtRAC10 (Lavy et al., 2002; Brembu et al., unpublished). The morphology of pavement cells expressing GFP-*CA-atrac2* was similar to that of pavement cells expressing *CA-atrac2* (alone), with reduced length and number of lobes. AtRAC2 fused to GFP therefore seems to be functionally active.

Plants contain evolutionarily conserved subunits of a WAVE-like regulatory complex (paper IV)

With the sequencing of the *Arabidopsis* genome, it became evident that the Arp2/3 complex was conserved in plants. An *ARP2* homologue had earlier been identified in *Arabidopsis* (Klahre and Chua, 1999). However, no functional studies had been performed on the Arp2/3 complex in plants. Moreover, the mechanisms regulating Arp2/3 complex activity appeared not to be conserved in plants, as no plant homologues of the nucleation promoting factors (NPFs) known in animals had yet been identified. When Eden and colleagues (2002) reported the finding of a WAVE1 regulatory protein complex, we searched sequence databases for plant protein sequences with similarity to subunits of the protein complex. Putative *Arabidopsis* homologues were identified for three of the subunits: PIR121, Nap125 and HSPC300. In maize, an HSPC300 homologue was disrupted in the mutant *brick1* (*brk1*), which displayed epidermal leaf cells without lobes (Frank and Smith, 2002). We therefore called the *Arabidopsis BRICK1* homologue *AtBRK1*. The putative Nap125 and PIR121 homologues were previously uncharacterised in plants, and were designated *AtNAP* and *AtPIR*, respectively. The full length mRNA transcripts of *AtNAP* and *AtPIR* were sequenced, and comparison with the genomic sequence (the *Arabidopsis* Genome Initiative, 2000) showed that AtNAP has 23 exons, whereas AtPIR has 31 exons (Paper IV, Fig. 1B and C). The AtNAP and AtPIR cDNA sequences have some significant differences compared to the putative coding sequence submitted by the *Arabidopsis* Genome Initiative (2000). *AtNAP* contains four additional exons and one additional intron, whereas *AtPIR* has non-canonical splicing acceptor and donor sites in intron 12. Another notable feature of *AtNAP* and *AtPIR* is the presence of a 5' UTR intron in both genes. *AtNAP* and *AtPIR* encode large proteins of 1396 and 1282 amino acids, respectively (Paper IV, Fig. 2). Both AtNAP and AtPIR show moderate similarity to their metazoan homologues; AtNAP contains 15% identical and 32% similar amino acid residues compared to human HEM-2/Nap1, whereas AtPIR contains 28% identical and 49% similar amino acid residues compared to human PIR121 (Paper IV, Table I). In wild type plants, *AtBRK1*, *AtPIR* and *AtNAP* were expressed in all tissues studied (Paper IV, Fig. 1D).

AtNAP and AtPIR T-DNA insertion mutants show defects in polar cell expansion (paper IV)

We obtained putative T-DNA insertion lines from the SALK T-DNA insertion mutant collection (Alonso et al., 2003) and from the FLAG collection at INRA-Versailles (Bechtold et al., 1993) and verified the insertions by sequencing. The lines SALK_014298 (*atnap-1*), SALK_038799 (*atnap-2*) and SALK_009695 (*atnap-3*) contained T-DNA insertions in AtNAP exon 9, exon 20 and exon 22, respectively (Paper IV, Fig. 1B). The line SALK_106757 (*atpir-1*) had a T-DNA insertion 15 bp upstream of the second exon containing the start codon of *AtPIR* (Paper IV, Fig. 1C). The first intron of *AtPIR* contains a putative branch point sequences nine bp upstream of the T-DNA insertion site in *atpir-1*; thus, the splicing efficiency is likely to be dramatically reduced in *atpir-1*. The line EXM115 (*atpir-2*) carried a T-DNA insertion in exon 14 of AtPIR. RT-PCR verified that *AtNAP* expression was absent in *atnap-1* and that *AtPIR* expression was highly reduced in *atpir-1* (Paper IV, Fig. 1E). All the insertion mutants for AtNAP and AtPIR that were studied displayed similar phenotypes. Detailed analyses were done on *atnap-1* and *atpir-1*. The most apparent phenotype was the abnormal morphology of trichomes on leaves and stems (Paper IV, Fig. 3 and 4), comparable to the "*distorted*" class of trichome mutants (Hülskamp et al., 1994). Trichomes of *atpir-1* and *atnap-1* plants had reduced length but increased diameter compared to wild type trichomes. Secondary and tertiary branches were generally highly stunted in *atnap-1* and *atpir-1* trichomes, and the overall shape of the mutant trichomes varied greatly compared to the regular and uniform shape of wild type trichomes. Other epidermal cell types were also affected in the mutants. The lobes of leaf pavements cells were significantly shorter in the *atnap-1* and *atpir-1* mutants (Paper IV, Fig. 5A, B and C). The phenotype was quantified by calculating the ratio between the area (μm^2) and the perimeter (μm) of pavement cells, as in paper III. The ratio was significantly higher in mutant cells compared with wild type cells, confirming the observed phenotype (Paper IV, Fig. 5D).

The chromosomal locations of *AtNAP* and *AtPIR* were compared with the map positions of the characterised "distorted" class of trichome mutants. *AtNAP* colocalised with the *gnarled* (*grl*) mutant, whereas *AtPIR* was positioned close to the map position of another mutant, *klunker* (*klk*). Seeds from the *grl-247* allele were

generously donated by Martin Hülskamp, University of Köln, Germany. Upon crossing, *atnap-1* failed to complement *grl*; none of the heterozygous F1 progeny showed reversion to normal trichome phenotype. The *AtNAP* transcript was sequenced in the allele *grl-247*, and revealed a C-T transition in the second exon, leading to the formation of a stop codon which terminates AtNAP translation after only sixty amino acids in *grl-247* (Paper IV, Fig. 1B). Thus, *AtNAP* is allelic to *GRL*. Although requested for more than a year, *klk* seeds were not provided, and we could therefore not verify that *AtPIR* is allelic to *klk*.

AtNAP and AtPIR are involved in regulation of the actin cytoskeleton (paper IV)

Earlier studies have suggested that organisation of the actin cytoskeleton is affected in the "distorted" mutant class (Mathur et al., 1999; Szymanski et al., 1999). *atnap-1* and *atpir-1* were therefore crossed with transgenic plants expressing the actin-binding domain of mouse talin fused to yellow fluorescent protein (YFP). The talin domain binds actin filaments, making *in vivo* visualisation of the actin cytoskeleton possible (Kost et al., 1998). In wild type trichomes, the actin cytoskeleton was organised in a fine and highly branched, cortical mesh. Actin filaments were mainly longitudinally oriented, extending to the tips of trichome branches (Paper IV, Fig. 6A and D). In contrast, the actin cytoskeleton in *atnap-1* and *atpir-1* trichomes appeared to be less branched, instead forming thick actin cables (Paper IV, Fig. 6B, C and E). Actin filaments were more transversely oriented in mutant trichomes. The density of actin cables were frequently observed to be very high at branchpoints, especially when branch growth was stunted. Thus, disruption of AtNAP and AtPIR apparently leads to defects in the organisation of the actin cytoskeleton. As mentioned in the introduction, mutants of Arp2/3 complex subunits in *Arabidopsis* also display "distorted" phenotypes, including disturbed organisation of the actin cytoskeleton (Mathur et al., 2003a; Mathur et al., 2003b; Le et al., 2003; Li et al., 2003; El-Din El-Assal, 2004). Including unpublished data, seven genes have been identified whose inactivation leads to "*distorted*" phenotypes. Considering the chromosmal localization, each of the seven genes probably corresponds to one of the *DISTORTED* genes (Schwab et al., 2003). As shown in Table 3, the verified and putative *DISTORTED* genes encode AtNAP, AtPIR and five of seven subunits of the

ARP2/3 complex. Furthermore, a double cross between *dis1* (AtARP3 mutant) and *klk* (putative AtPIR mutant) showed an additive phenotype, indicating that AtARP3 and AtPIR act in the same process (Schwab et al., 2003). Taken together, the data strongly suggest that AtPIR and AtNAP are involved in regulation of the actin cytoskeleton through the Arp2/3 complex.

Table 3. Molecular identity of the *distorted* mutants

Mutant	Gene mutated	At locus ID	Reference
wurm	*AtARP2*	At3g27000	Mathur et al., 2003a; Le et al., 2003
distorted1	*AtARP3*	At1g13180	Mathur et al., 2003a; Le et al., 2003
distorted2	*AtARPC2*	At1g30825	El-Din El-Assal et al., 2004
alien	*AtARPC4*?[1]	At4g14510	Li et al., 2003[2]
crooked	*AtARPC5*	At4g01710	Mathur et al., 2003b
gnarled	*AtNAP*	At2g35110	Brembu et al. (Paper IV)
klunker	*AtPIR*?[1]	At5g18410	Brembu et al. (Paper IV)
spirrig	N.A.	-	-

[1]These genes map to the vicinity of the corresponding mutant allele, and T-DNA insertion mutants show *distorted* phenotypes, but the genes have not been sequences in the mutant background
[2]Reported as unpublished results

The phenotypes of *atnap-1*, *atpir-1* and mutants of the ARP2/3 complex are surprisingly mild compared to corresponding mutants in metazoa. A possible explanation could be that the large family of formins in *Arabidopsis* are able to carry out most of the functions usually performed by the ARP2/3 complex. Only a subset of cells undergoing rapid polar extension may be dependent upon proper function of the ARP2/3 complex.

A family of putative WAVE-like proteins in plants - the missing link between RAC/ROP GTPases and the Arp2/3 complex?

Do plant RAC/ROP GTPases regulate branching and nucleation of actin filaments through the Arp2/3 complex? Several authors have stated that plants do not contain proteins with similarity to WASP or WAVE proteins (Vantard and Blanchoin, 2002; Mathur and Hülskamp, 2002; Li et al., 2003; Smith, 2003). However, low stringency searches using conserved C-terminal VCA region resulted in the finding of a small family of four *Arabidopsis* genes (At1g29170, At2g34150, At2g38440 and At5g01730) encoding putative proteins which contained a C-terminal motif with moderate similarity to VCA regions (residues 947-1016 in At1g29170). An alignment of the four VCA-containing *Arabidopsis* proteins with human N-WASP and mouse

WAVE1, WAVE2 and WAVE3 revealed that most of the residues believed to be critical for proper function of the VCA region (Paunola et al., 2002; Panchal et al., 2003) were conserved in the *Arabidopsis* proteins (Fig. 7).

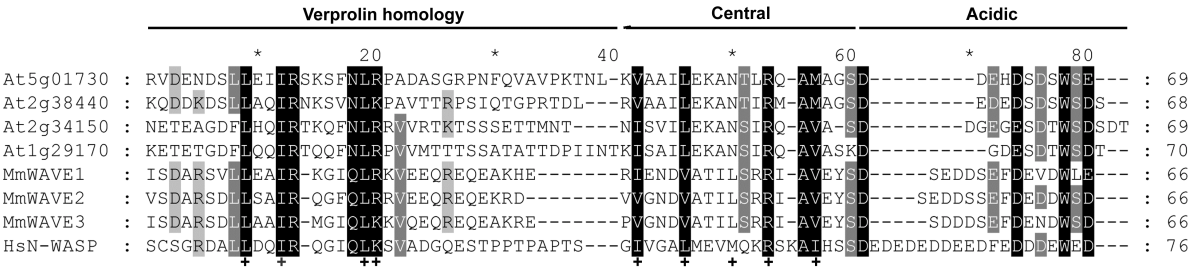

Figure 7. Amino acid alignment of the VCA region of the *Arabidopsis* WAVE-like proteins, mouse WAVE1-3 and human N-WASP. The alignment was produced with the GeneDoc program. Amino acid residues reported to be essential for VCA activity are indicated with crosses below the alignment.

All of the four VCA-containing *Arabidopsis* proteins have an N-terminal region (residues 1-176 in At1g29170) with similarity to Scar homology domains (SHD), as well as a small region (residues 177-188 in At1g29170) of basic residues (Fig. 8). Thus, these proteins are *Arabidopsis* WAVE homologue candidates. A fifth *Arabidopsis* gene, At4g18600, encodes a protein that also contains an N-terminal motif similar to the SHD domain, but lacks a C-terminal VCA-like region. Deeks and Hussey (2003) also noted that At1g29170 and At4g18600 contain SHD domains, but failed to identify the VCA region in the former protein.

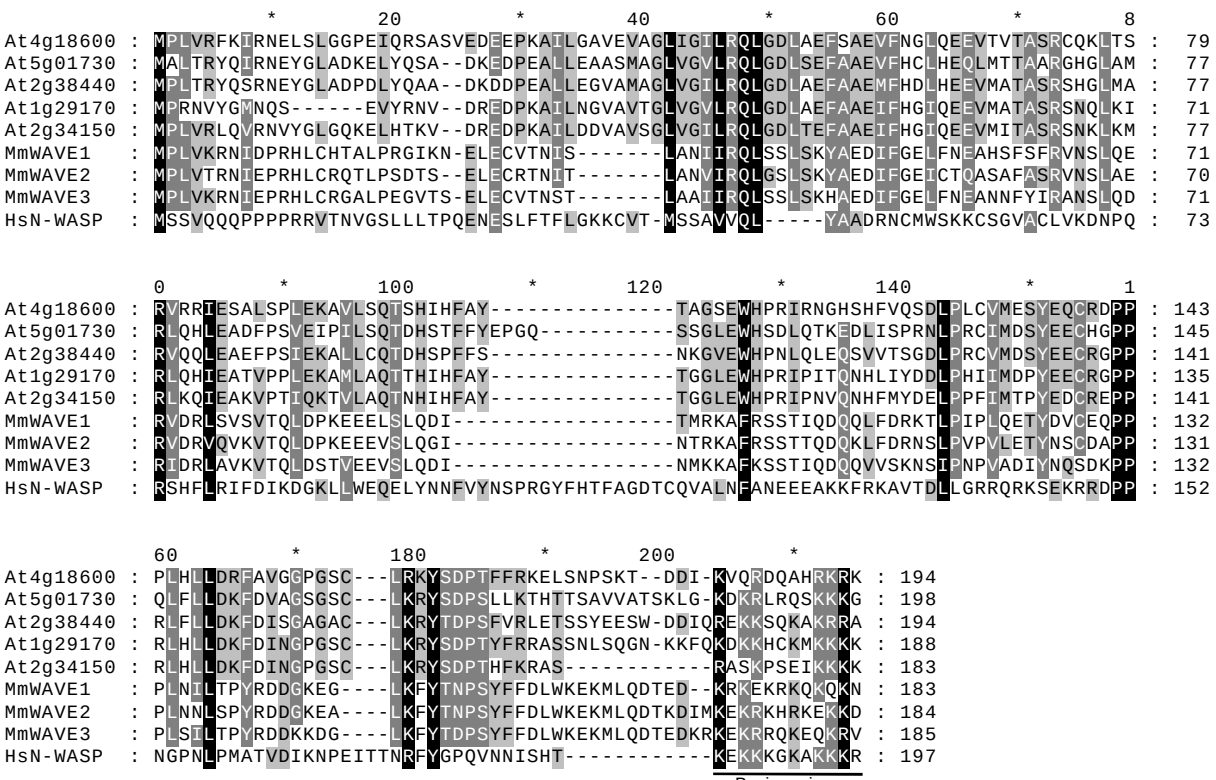

Figure 8. Amino acid alignment of the SHD domain and the adjacent basic region (underlined) of the *Arabidopsis* WAVE-like proteins and mouse WAVE1-3, and the EVH1 domain of human N-WASP. The alignment was produced with the GeneDoc program.

The *Arabidopsis* WAVE-like proteins have a large central region, varying from about 660 (At2g34150) to about 1700 residues (At4g18600), which lack any similarity to other proteins. This region is also divergent within the family. Based on sequence similarity in parts of this region, At1g29170 and At2g34150 constitute one sub-group of the *Arabidopsis* WAVE-like proteins. At2g38440 and At5g01730 comprise another sub-group, although the similarity is less prominent. Due to the large central region, the *Arabidopsis* WAVE-like proteins are substantially larger than any known WAVE/SCAR proteins (Fig. 9). Another significant difference is the lack of a proline-rich region in the *Arabidopsis* WAVE-like proteins; only a short motif of 8 to 15 residues is rich in prolines. The proline-rich region in WAVE/WASP proteins is thought to bind SH3 domains in otherwise diverse proteins. The *Arabidopsis* genome appears to contain only five genes encoding proteins with putative SH3 domains (Lam et al., 2001; Brembu, personal observations). Other proteins may regulate the activity of the WAVE-like proteins through binding to the large central domain. It is

also interesting to note the presence of a basic region in the *Arabidopsis* WAVE-like proteins, localised next to the SHD domain as in metazoan WAVEs (Fig. 8). Although PIP2 has not been reported to bind the basic region of WAVE proteins, it is tempting to speculate that PIP2 might act as a regulator of WAVE activity in plants. Expressed sequence tags (ESTs) for all the putative WAVE homologues were found in sequence databases, indicating that all five genes are expressed.

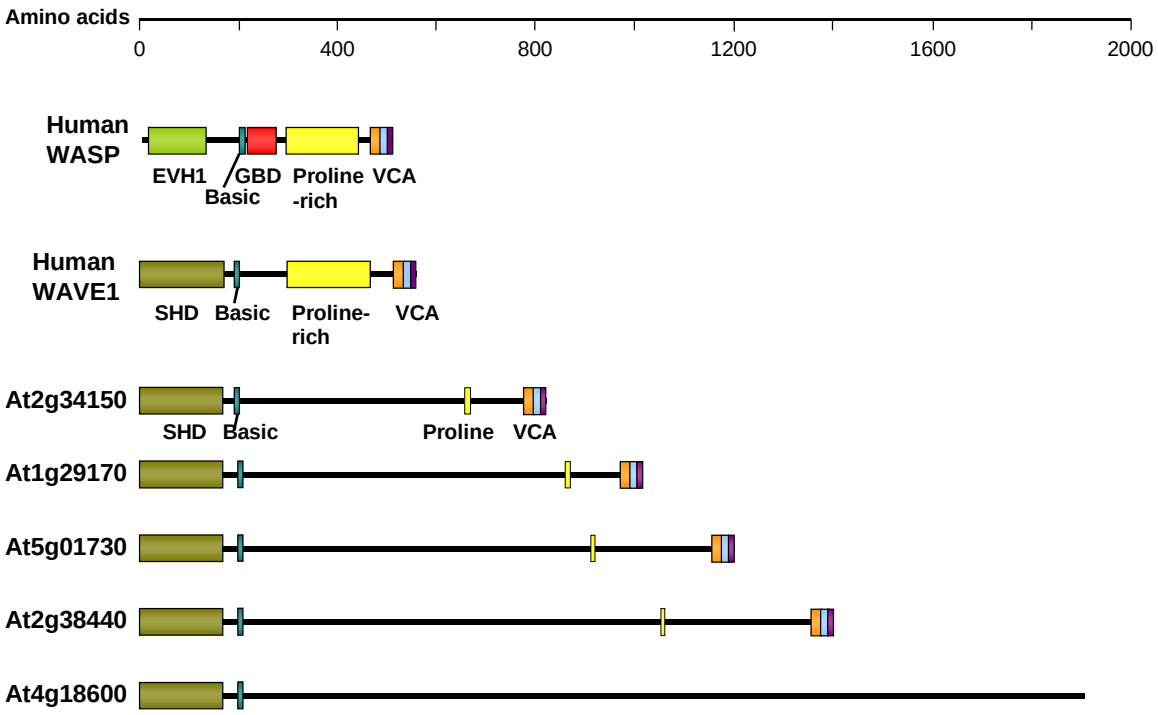

Figure 9. Domain structure of the *Arabidopsis* WAVE-like proteins, Human WASP and human WAVE1. Abbreviations: EVH1, Ena/Vasp homology domain 1; GBD, GTPase-binding domain; SHD, Scar homology domain.

A model for ARP2/3-mediated regulation of actin organisation in plants

A model for regulation of actin filament branching and polymerisation by the ARP2/3 complex in plants is presented in Fig. 10. In general, the mechanism appears to be well conserved between plants and other eukaryotic organisms (for comparison, see Fig. 5). In unstimulated *Arabidopsis* cells, *Arabidopsis* WAVE-like proteins exist in an inactive protein complex together with AtNAP(/GRL), AtPIR(/KLK) and AtBRK1. No genes encoding clear homologues of Abi has been found in plants, but other proteins may take part in the complex. A developmental or external/environmental signal

leads to activation of AtRAC GTPases, through as yet unknown receptors. Although no interactions between AtPIR and AtRACs have been shown, the N-terminal, putatively Rac-interacting domain of AtPIR is relatively well conserved; thus, it is not unlikely that an AtRAC-AtPIR interaction exists. AtRAC binding of AtPIR leads to dissociation of the regulatory complex, releasing now active WAVE associated with AtBRK1. The WAVE-AtBRK1 protein pair binds and activates the ARP2/3 complex, which in turn initiates new actin filaments branching from existing filaments. No interaction between any of the components in this model has been shown in plants, but the mutant studies support the assumption of a functional connection between AtNAP, AtPIR and the ARP2/3 complex. The fact that clear homologues of Abi have not been identified in plants is puzzling, considering the central position of Abi in the core of the WAVE regulatory complex in mammalian cells (Gautreau et al., 2004; Innocenti et al., 2004). The highly divergent central domain of the plant WAVE homologues indicates that a putative plant Abi conunterpart may share little similarity with animal Abi. Homologues of other components interacting with the WAVE1 regulatory protein complex in mammalian cells, such as Nck (Eden et al., 2002) and the GAP WRP (Soderling et al., 2002) have not been found in plants; novel, plant-specific mechanisms for regulation of WAVE activity may well have been developed.

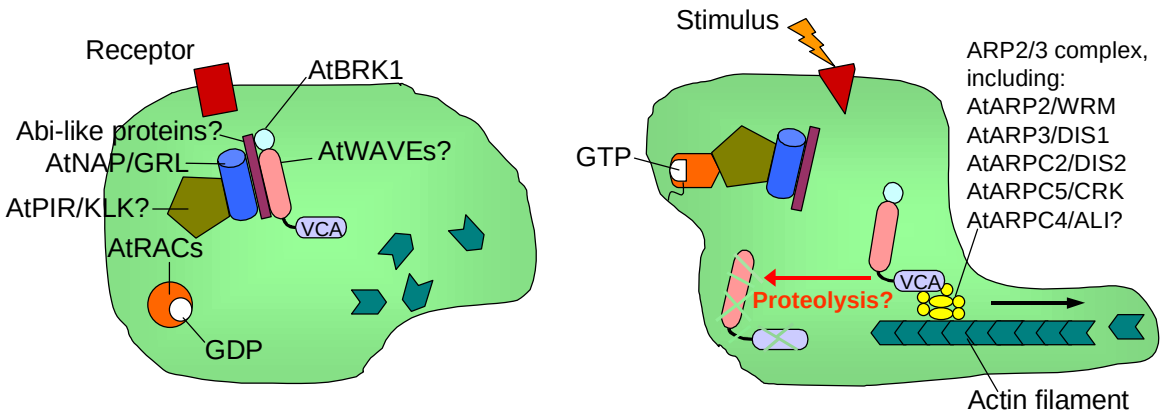

Figure 10. A model for regulation of the Arp2/3 complex through WAVE-like proteins in *Arabidopsis*. With a possible exception of Abi, homologues of all the subunits of the WAVE regulatory complex and the ARP2/3 complex are conserved in *Arabidopsis*. All characterised mutants of the *distorted* class are affected in genes encoding proteins involved in this process.

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