AtSRA1 and AtSCAR2: Regulators of Arp2/3 Complex-Mediated Cell Morphogenesis in Arabidopsis thaliana

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Abstract

The actin cytoskeleton plays an important role in cell morphogenesis of plant cells. Plants use different arrays of actin filaments for various cellular functions. The response of dynamic actin arrays to developmental and environmental cues is vital to the growth of the plant. Conversely, static actin filaments support cell architecture and anchor the endomembrane system in the cell. Organelle transport occurs on actin filament 'tracks' which enables cargo to be targeted to specific sites in the cell.

The ARP2/3 complex is a nucleator of actin filament polymerisation and its 7-subunit composition is conserved in both plants and animals. In Arabidopsis, the distorted mutants comprise of 7 members, 4 of which have mutations in subunits of the ARP2/3 complex. Members of this complementation group are affected in the architecture of the actin cytoskeleton leading to aberrant morphogenesis in various tissues. Arabidopsis leaf hair cells referred to as trichomes, are a model system used to investigate the role of actin in cell morphogenesis.

In this study the distorted mutant klunker (klk) was characterised on a genetic, molecular and phenotypic level. The gene affected by the klk mutation was found to be AtSRA1, which is homologous to SRA1, a known regulator of the ARP2/3 complex in Drosophila. The phenotypes observed in the klk mutant were similar to those of ARP2/3 complex mutants with reduced severity. In comparison to the wild type, epidermal pavement cells of klk showed reduced lobing and distorted trichomes. In addition, the hypocotyl growth was reduced and adhesion between epidermal cells was disturbed in dark-grown conditions. In animals, SRA1 and its human homolog PIR121 are known to function as part of a regulatory complex in which the SCAR/WAVE protein activates the ARP2/3 complex.

An Arabidospsis homolog of WAVE1 was identified, and named AtSCAR2. The characterisation of the atscar2 mutant revealed a weak distorted phenotype in the tissues analysed. To learn more about the localisation of the AtSCAR2

isoform, an antibody was raised in mice. Western blotting showed that the antibody was specific for AtSCAR2. Double mutant analysis within the other putative AtSCAR genes revealed redundancy between AtSCAR2 and AtSCAR4. This was apparent as the trichome phenotype of this double mutant was observed to be as severe as that of the strong distorted mutant dis1.

The results presented in this study support the idea that the ARP2/3 complex machinery found in animals is conserved in Arabidopsis with respect to the SCAR/WAVE regulatory pathway.

Publications

The Role of Arabidopsis SCAR genes in ARP2/3-dependent Cell Morphogenesis. Urhig, F. J., Mutondo, M., Zimmermann, I., Deeks, M.J., Machesky, L. M., Thomas, P., Uhrig, S., Rambke, C., Hussey, P.J. and Hülskamp, M.
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The Arabidopsis KLUNKER gene controls cell shape changes and encodes the AtSRA1 homolog.
Saedler R ,Zimmermann I ,Mutondo M ,Hulskamp M .
Shared first authorship
(2004) Plant Molecular Biology, Volume 56, Number 5: 775-782(8)

The Arabidopsis *GNARLED* gene encodes the NAP125 homolog and controls several actin-based cell shape changes.
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Abbreviations and Gene Names

°C	Degrees Celcius	GFP	Green Fluorescent Protein
µ	Micro	GST	Glutathione S-transferase
35s	35s promotor from Cauliflower Mosaic Virus	HSPC300	Hematopoietic stem/ Progenitor Cell Clone 300
ABI2	Abelson Interacting Protein2	kb	kilo base pairs
ABP1	Actin Binding Protein 1	kD	kilo Dalton
ADF	Actin Depolymerising Factor	KLK	KLUNKER
ARP2	Actin-Related Protein 2	Ler	Landsberg erecta
ARP2/3	Actin-Related Protein 2/3 Complex	PIR121	P53-inducible protein-121
ARP3	Actin Related Protein 3	PLP	poly-L-proline-containing
ARPC2	Actin Related Protein Subunit 2	RAC1	ras-related C3 botulinum toxin substrate 1
ARPC3	Actin Related Protein Subunit 3	RNA	Ribonucleic Acid
ATBRICK	BRICK of Arabidopsis	ROP	rho of plants
ATNAP	NCK-associated protein 1 of Arabidopsis	rpm	revolutions per minute
ATPIR	p53-inducible protein-121 of Arabidopsis	RT-PCR	Reverse Transcriptase PCR
ATSCAR	Actin Related Protein	SCAR	Suppressor of cyclic AMP Receptor
ATSRA1	Specifically Rac1-associated protein 1 of Arabidopsis	SD	Standard Deviation
bp	base pair	SH3	Src Homology 3 Domain
CaMV	Cauliflower Mosaic Virus	SHD	SCAR Homology Domain
cDNA	Complimentary DNA	SRA1	Specifically Rac1-associated Protein 1
CLSM	Confocal Laser Scanning Microscopy	UTR	Untranslated region
Col	Columbia	VASP	Vasodilator-stimulated Phosphoprotein
CYFIP	Cytoplasmic FMR1-interacting Protein 2	VCA	Verprolin Homology, Cofilin Homology, Acidic Region
DNA	Deoxyribonucleic Acid	WASP	Wiskott-Aldrich Syndrome Protein
Ds RED	Discosoma species. Red Fluorescent Protein	WAVE	Wiskott-Aldrich Syndrome Protein Family Verprolin-homologous Protein
ER	Endoplasmic Reticulum	WHD2/WH2	WAVE Homology Domain 2
et. al.	et alterni(Lat.) and others	WIP	WASP- interacting Protein
Fig.	Figure	WT	Wild-type
GEF	Guanine Nucleotide Exchange Factor	WW	Two conserved tryptophans domain
GEX-3	gut on Exterior-3	YFP	Yellow Fluorescent Protein

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1 Introduction

1.1 The *Arabidopsis* Trichome: A Tool to Study Cell Morphogenesis

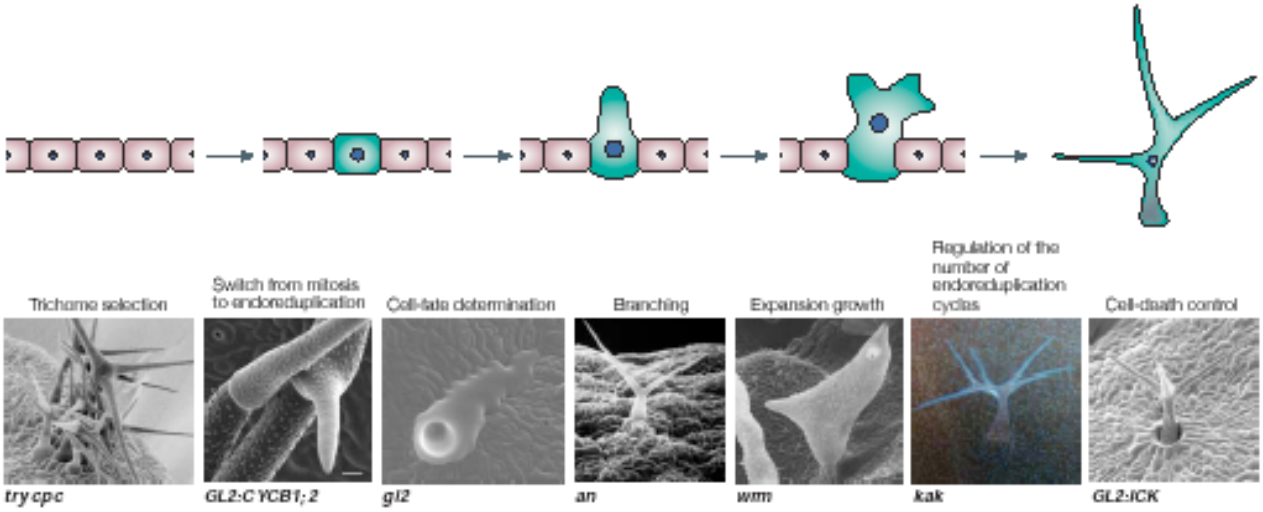
The genetic analysis of trichomes has led to the identification of a large number of genes controlling cell morphogenesis (Marks, 1997; Hulskamp *et al*., 1998) Thus, *Arabidopsis* trichomes can be used as a model system to study a range of developmental processes contributing to cell morphology. This can be done by analysing mutants of the various stages of trichome development (Figure 1). *Arabidopsis* trichomes are polyploid cells and develop three to four branches that are arranged in a predictable manner. After the trichome fate decision is made, the cells are committed and stop dividing. The incipient trichome cell proceeds through four endoreduplication cycles to facillitate cell enlargement. This allows trichomes to reach a final height of upto 0.5 mm. Two successive branching events give the *Arabidopsis* trichome its familiar 3-pronged structure. The primary branching event occurs in a direction parallel to the proximal-distal leaf axis. The primary branch then undergoes a secondary branching event in a plane perpendicular to the first branching plane. Cell elongation in trichomes is found not only at the tip, but also along the whole cell surface (Szymanski *et al*., 1999; Schwab *et al*., 2003). As the whole trichome expands, an increase in vacuolisation also occurs (Hulskamp *et al*., 1994)

1.2 Mutants of Trichome Expansion Growth

A class of eight mutants, the distorted mutants, is affected in the spatial control of cell expansion (Hulskamp et al., 1994). In distorted mutant trichomes, cell expansion is randomised. Cells may elongate at odd angles, may twist and in many cases, individual branches grow enormously while others stop growing. Except for the weakest mutant of this distorted mutant class, all mutants exhibit severe distortions in the organisation of the actin cytoskeleton (Mathur et al.,

2003a,b; Szymanski et al., 1999; Le et al., 2003; Li et al., 2003; Schwab et al., 2003; El-din El-Assal et al., 2004a,b; Saedler et al., 2004a,b). The members of the DISTORTED complimentation group are grl, klk, crk, wrm, dis1, dis2, and spi.

A



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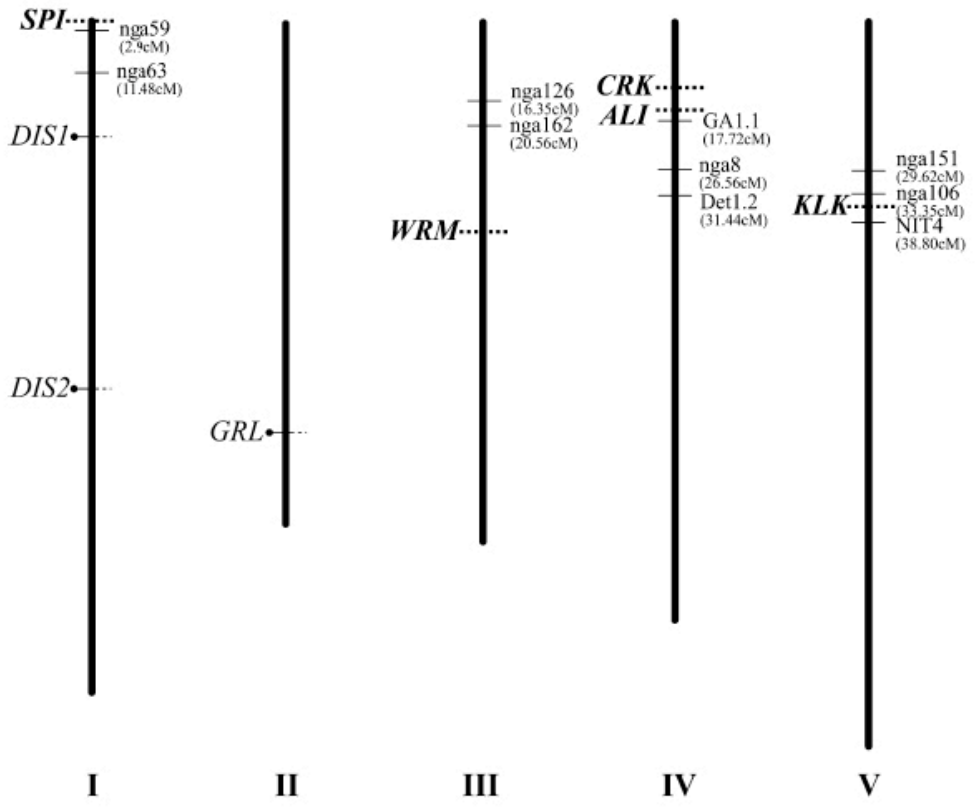

Figure 1 Trichome mutants and the *distorted* group

(A) Range of mutants used to study developmental processes using trichome as a model system (Hulskamp, M. 2004)

(B) Chromosomal locations of distorted mutants (Schwab et al., 2003)

1.2.1 'DISTORTED' Gene Products

It is thought that the *DISTORTED* genes encode proteins involved in the regulation of the actin cytoskeleton because drugs interfering with actin function also result in a distorted trichome phenotype (Mathur *et al*., 1999 ; Szymanski *et al*., 1999) The finding that four *DISTORTED* genes encode subunits of the putative *Arabidpsis* ARP2/3 complex supports this idea. The *WURM (WRM)* gene and the *DISTORTED1 (DIS1)* gene encode the ARP2 and ARP3 subunits, respectively (Le *et al*., 2003; Li *et al*., 2003; Mathur *et al*., 2003a) The CROOKED (CRK) gene encodes a protein with sequence similarity to ARPC5 and DISTORTED2 (DIS2) encodes the ARPC2 subunit. (Figure 2)

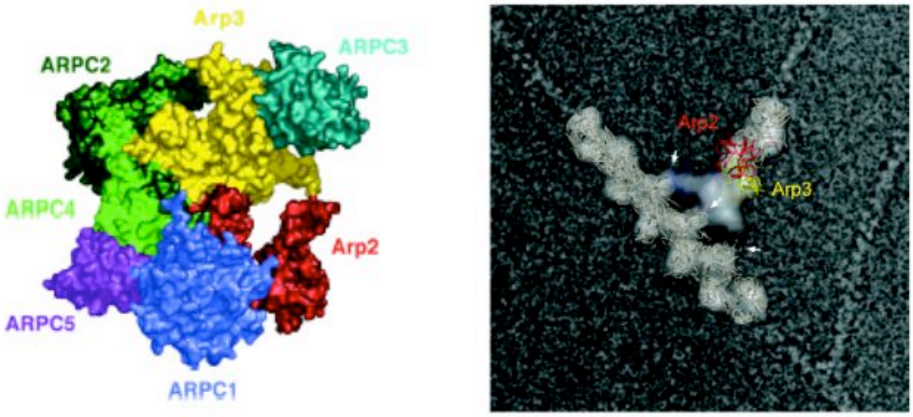

Figure 2 The ARP2/3 complex crystal structure and position on actin filaments

Subunit composition of the ARP2/3 complex and its relative position on actin filaments (Svitkina and Borisy, 1999)

1.3 The Role of the ARP 2/3 Complex in Animals

Purified actin monomers have little ability to produce new actin filaments, as the formation of nucleating dimers and trimers is thermodynamically much more unfavourable than the elongation of pre-existing actin filaments (Mullins et al., 1998). It is therefore necessary for the cell to overcome this rate-limiting step by using other factors. The ARP2/3 complex is one such factor (Higgs, H.N., and Pollard, T.D. 2001). The ARP2/3 complex enables the rapid assembly of actin filaments required for many basic cellular processes such as endocytosis and pathogen invasion (Pollard, T.D and Borisy, G.G. 2003); the networks of filaments created by the action of the ARP2/3 complex are capable of creating a protrusion force at the membrane as seen in amoeboid motility, or a propelling force needed for organelle mobility.

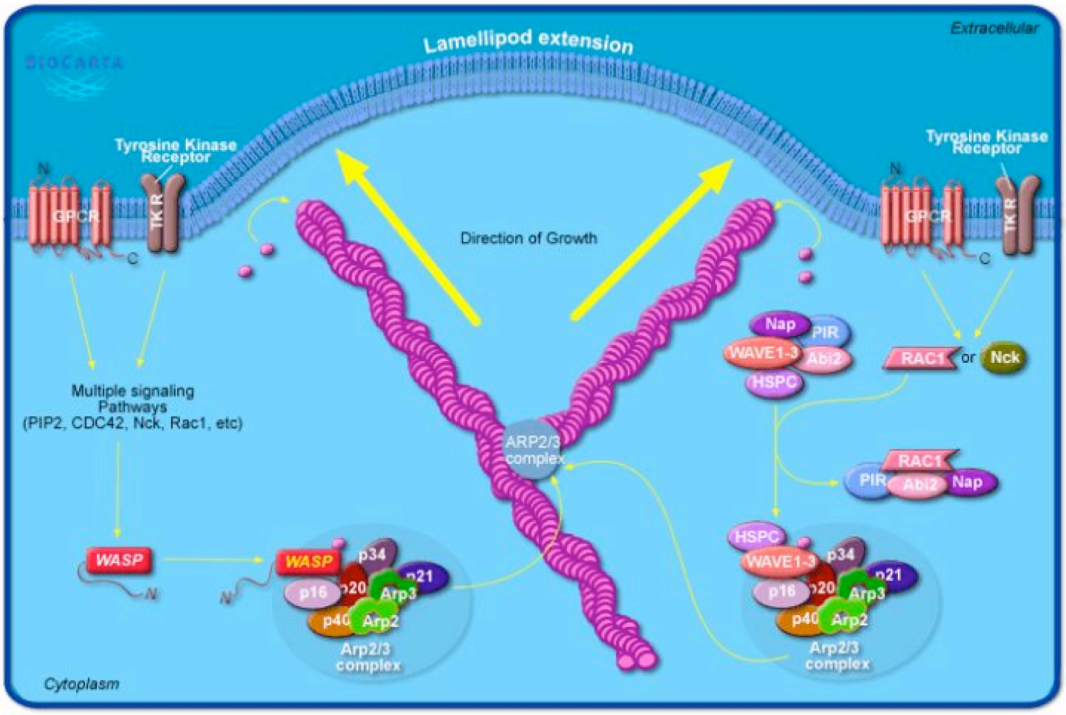

Figure 3 Wasp and WAVE pathways in animals.

A comparison between the WASP family-mediated and WAVE family-mediated activation of the ARP2/3 complex in animals leading to membrane ruffling (www.biocarta.com/)

In animals, the ARP2/3 complex is well studied and is known to consist of seven proteins referred to as ARP2, ARP3 and ARPC1-5 (Machesky and Gould, 1999). Its basic composition is conserved among organisms from yeast to man, which suggests that it arose early in the evolution of eukaryotes (Welch and Mullins, 2002).

Two of the ARP2/3 complex subunits are actin-related proteins (ARP2 and ARP3). Homology models based on the crystal structure of actin reveal similarity (Kelleher et al. 1995), which hints at the function of two ARPs in this, complex (Pollard and Beltzner, 2002). A hypothesis, where a possible role of ARP2 and ARP3 in the complex could be to form a dimer, which is able to bind the slow-growing “pointed” end of an actin filament and thereby allow nucleation of a new filament that grows in the fast-growing “barbed” direction, has been confirmed (Mullins et al 1998; Blanchoin et al., 2000); new daughter filaments were shown to grow at a 70° angle from the sides of mother filaments. The ARP2/3 complex itself was localised to the branch points using electron microscopy of actin filament arrays in lamellipoedia (Svitkina and Borisy, 1999) and in yeast cells, the complex is found in actin patches (Morrell et al., 1999) (Figure 2).

ARPC2 and ARPC4 form a fundamental dimer in the ARP2/3 complex, which is thought to be a scaffold, upon which the rest of the complex members associate (Gournier et al. 2001); of the seven subunits only ARPC2 and ARPC4 are critical for the formation of an intact ARP2/3 complex. Deletion of either one results in smaller subcomplexes or individual subunits. Chemical cross-linking experiments indicate that ARPC2 contacts the mother filament (Mullins et al., 1997; Mullins et al., 1999); branching is inhibited by an antibody against ARPC2. Taken together, this suggests that ARPC2 could be part of the mechanism that anchors the ARP2/3 complex to the mother filament, thereby initiating the nucleation of a new daughter filament by the ARP2/3 complex.

ARPC1 is a WD40 seven-bladed beta propeller protein in which the top faces of all seven the blades show extensive interaction with ARPC4 in the crystal

structure; the remaining four subunits (ARPC2-5) are proteins with novel folds (Robinson et al., 2001).

1.3.1 Properties of the ARP2/3 Complex

In short, the purified ARP2/3 complex has two important properties. Firstly, the complex has been shown to catalyse the de *novo* nucleation of new actin filaments from the sides of existing actin filaments. The complex by itself is thought to be inactive although its highly purified form has been reported to show weak basal nucleating activity. In general, additional factors known as nucleation promoting factors (NFPs) are required to activate the complex (Beltzner and Pollard, 2004), which are themselves responsive to upstream signals.
Secondly, the purified complex is able to cross-link newly formed filaments into actin arrays with a characteristic “Y-branching” formation that is due to the ARP2/3 complex sitting at junctions between mother and daughter filaments. Furthermore, this activity of the ARP2/3 complex can be enhanced by F-actin (Machesky et al., 1999) suggesting a dendritic nucleation mechanism. This property of the ARP2/3 complex is key to protrusion growth of lamellipoedia and fillapoedia. Intracellular pathogens on the other hand, such as *Shigella* and *Lysteria* have harnessed the potential of the ARP2/3 complex, enabling them to utilise the cytoskeletal aparatus for ARP2/3-driven motility through the infected cell.

1.3.2 Activation of the ARP2/3 Complex

Several factors have been identified as activators of the ARP2/3 complex. In animals and yeast, the ARP2/3 complex is thought to be activated locally in growth regions where it triggers actin branching (Welch et al., 1997; Winter et al., 1997). Several proteins have been identified as activators of the ARP2/3 complex. These are ActA from Listeria, the WASP (Wiskott-Aldrich syndrome

protein) protein family, the SCAR (Suppressor of cyclic AMP receptor) protein family, cortactin and yeast ABP1 (Actin binding protein 1). SCAR is also known as WAVE (Wiskott-Aldrich syndrome protein family verprolin-homologous protein) in H. *sapiens* and other mammals. The WASP and SCAR/WAVE proteins contain domains that enable them to respond to a variety of upstream signals by binding to multiple signalling proteins (Higgs and Pollard, 1999).

1.3.3 WASP and Scar/WAVE Proteins

Although the WASP protein family is structurally different from the SCAR/WAVE protein family, the two share important similarities: both WASP and SCAR/WAVE proteins have a functionally conserved 70 to 100-residue region at the C-terminal known as the WA region. This may contain one or two WASP homology (WH) 2 domains, a central (C) linking region, and an acidic (A) region. Collectively, this forms the VCA domain of the protein. The WH2 region can bind actin monomers, while the A region bind the ARP2/3 complex; both WASP and WAVE/SCAR proteins have a region of 110-175 C-terminal residues capable of binding SH3 domain proteins (Higgs and Pollard, 1999).

Mammalian WASP proteins differ from SCAR proteins in three ways. Firstly, WASP proteins contain an EVH1 domain at the N-termanal whereas the corresponding regions of SCAR proteins contain a SCAR homology (SH) domain that is highly similar among the SCAR family proteins. Secondly, the WASP proteins contain a GTPase-binding domain, which SCAR proteins are without; this region of SCAR proteins is notably variable. Lastly, the C region sequences of WASP proteins are similar among the group; the same is true of SCAR proteins. However, the C region of WASP and WAVE/SCAR proteins differs from each other significantly (Higgs and Pollard, 1999).

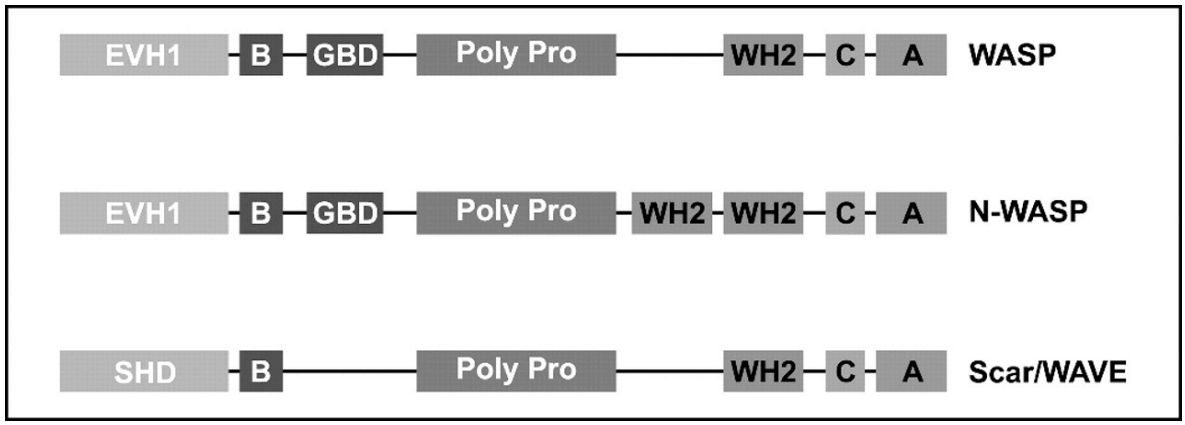

Figure 4 Domain organisations of WASP and SCAR/WAVE proteins

EVH1 , Ena/Vasp homology 1 domain; *B*, basic region; *GBD* , GTPase-binding domain; *Poly Pro* , proline-rich region; *SHD* , Scar homology domain; *WH2 or V* , verprolin-like or WASP homology 2 domain; *C*, central or connecting domain; *A*, acidic domain (Kelly et al., 2006).

The WASP group of proteins is represented in mammals by WASP and N-WASP. Yeasts have Las17 as their only WASP protein. The *Drosophila* homolog is known as *Dm*SCAR.

Every eukaryote so far reported to have the ARP2/3 complex also has one or more WASP or WAVE/SCAR proteins. The members of this family have domains enabling interaction between, the ARP2/3 complex, actin monomers and regulatory inputs. The WASP proteins and WAVE/SCAR proteins have highly conserved actin-binding and ARP2/3-binding domains, whereas greater variability exists in the signaling molecule-binding domains.

1.3.4 Regulation of the ARP2/3 Complex by WASP/SCAR proteins

Many regulatory proteins are known that interact with the ARP2/3 complex and control its site-specific activity. One pathway is controlled by the small GTPase Rac. When activated by the binding of GTP, Rac activates WAVE and HSPC300 (hematopoietic stem/ Progenitor cell clone 300). WAVE1 and

HSPC300 are inhibited by binding to a trimeric complex consisting of NAP125 (NCK-associated protein 1); Abi2 (Abl-interacting protein2) and PIR121 (p53-inducible protein-121, also known as SRA1, CYFIP, GEX2). PIR121 is an isoform of SRA1 (Eden et al., 2002; Blagg and Insall, 2004).

Immunoprecipitations of endogenous and recombinant proteins have created an interaction map of SCAR complex components. The complex forms a chain of interactions that connect the Rac-binding domain of PIR121 to the Arp2/3 complex activation domain of SCAR through NAP125, and ABI. HSPC300 is less tightly associated with the complex, but seems to bind both ABI and SCAR.

In the original model of Rac-mediated activation of the complex, the complete SCAR complex suppresses the ability of SCAR to activate the Arp2/3 complex. Upon binding of Rac·GTP, HSPC300 and SCAR dissociate from the other complex members and SCAR is free to activate the Arp2/3 complex. In this model, some members of the SCAR complex (PIR121, NAP125, and ABI) function as ‘repressors’ of SCAR.

Another model, known as the Activation Model, is generated by experiments showing that the complex does not disassociate upon Rac activation and that the Rac-free SCAR complex is just as effective at activating the Arp2/3 complex in vitro as the Rac-bound complex. The binding of activated Rac to PIR121 induces the complex to localize where the SCAR activation of the Arp2/3 complex can lead to the formation of actin-rich structures. All members of the SCAR-complex cooperate as ‘activators’ to localize SCAR correctly and stabilize SCAR from degradation.

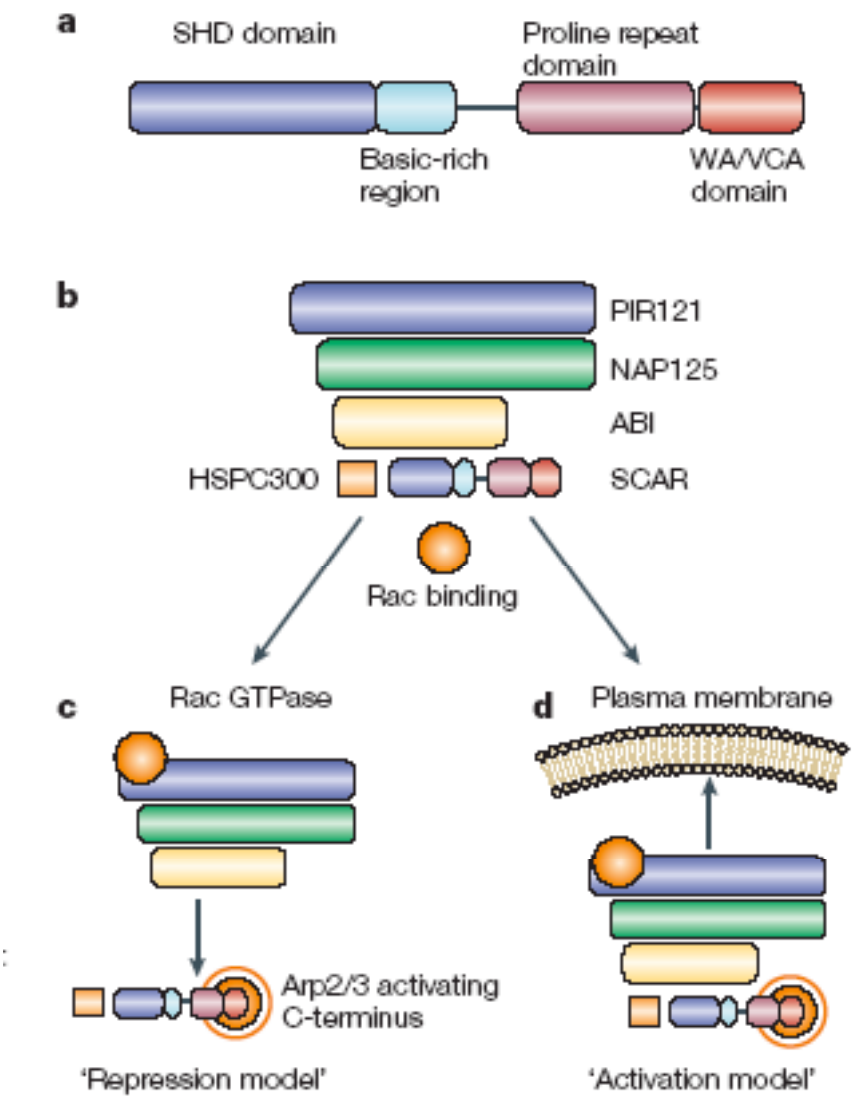

Figure 5 SCAR/WAVE repression and activation models

(A) Typical domain structure of SCAR proteins

(B) Composition of WAVE/SCAR complex thought to regulate the activity of the ARP2/3 complex

(C) and (D) Activation and repression model of WAVE/SCAR-mediated ARP2/3 complex (Deeks and Hussey., 2006) activation

1.4 The ARP2/3 Complex in Higher Plants

In plants, the ARP2/3 complex has not been observed directly by way of high-resolution techniques, as is the case in animals. Literature documenting the biochemical isolation of a functional ARP2/3 complex from plants is also not available. However, strategies involving reverse and forward genetics have enabled the identification and characterisation of various ARP2/3 complex functions in plants. Heterologous complimentation of yeast mutant alleles of ARPC2 and ARP3 by their *Arabidopsis* orthologs further supports the idea of a plant ARP2/3 complex with equivalent function to that existing in animals (Le *et al*., 2003; El-Assal *et al*., 2004). As mentioned earlier, all seven components exist in plant genomes.

Plants survive without components of the Arp2/3 complex, but mutant alleles cause an obvious phenotype that fall into the ‘‘*DISTORTED*’’ group of *Arabidopsis* mutants. In animals, such mutant alleles are often lethal or cause extreme phenotypes. Loss of ARP2/3 complex activity results in altered F-actin levels in a cell. This observation suggests that the loss of F-actin is more severe in animal and yeast cells when the activity of the Arp2/3 complex is disrupted. Experimental evidence shows that RNAi treatment of regulators of the Arp2/3-complex in D. *melanogaster* leads to a 90% drop in F-actin content. Furthermore, mutation in the D. *discoideum* SCAR protein reduces F-actin content by over 50% and Arp2/3-complex mutants in yeast lose their highly visible arrays of actin patches. Therefore, the Arp2/3 complex might only contribute to a small fraction of the total F-actin within a higher-plant cell. Definitive identification of the specific F-actin population that is lost in Arp2/3-complex mutants is required to understand the function of the Arp2/3 complex in plants.

1.5 Aim of Study

At the beginning of this work, four of the eight *distorted* mutants had been shown to harbour mutations in subunits of the ARP2/3 complex. Little beyond this was known about plant-specific activators and regulators of ARP2/3 complex function and the extent to which the ARP2/3 complex machinery had been conserved in plants. Therefore, the questions this study aimed to address were:

1. Which gene is affected by the *klk* mutation and how does this mutation affect the development of the plant?
2. How is *KLK* involved in the putative *Arabidopsis* ARP2/3 complex pathway?
3. Which other factors work in concert with *KLK* to affect actin dynamics via the ARP2/3 complex?
4. Are WASP or SCAR/WAVE proteins conserved in *Arabidopsis*?

2 Results

2.1 Mapping the *klk* locus

2.1.1 Classical Mapping of *klk*

The preliminary mapping of klk resulted in a region of chromosome V being identified as the location of the mutation leading to the klk phenotype (Schwab et al., 2003). This is the region between markers NIT4 and NGA106. A mapping population was established in order to fine-map the klk locus by crossing klk plants (L.erecta background) with wild type plants (Columbia background). It would then be possible to use ecotypic differences to detect recombination events between a marker and the mutant gene. Plants are then scored at the genetic level and SSLPs (simple sequence length polymorphisms) would be detected by PCR. This method was, however, abandonned in the F2 generation as the candidate approach was more successful.

2.1.2 Balistic Rescue

In parallel, another method of fine mapping the klk locus was pursued. Bacterial Artificial Chromosomes (BACs) spanning this region were identified and co-bombarded with GFP:Talin into young leaves. The aim of this experiment was to narrow down the genomic region of the klk locus by rescuing the klk mutant phenotype. GFP:Talin was used to mark transformed cells. Isolation of BAC DNA from E.coli resulted in low yields that were contaminated by large amounts of chromosomal DNA. Furthermore transient transformation efficiency by bombardment of epidermal cells with GFP:Talin was too low to expect to hit a nascent trichome cell at the right stage.

2.1.3 Candidate Approach

The BACs were screened for putative candidate genes homologous to other genes already known to be involved in the ARP2/3 complex pathway. No gene corresponding to the missing components of the ARP2/3 complex were found in the interval between the markers NIT4 and NGA106. Therefore a search for homologs of genes encoding known regulators of the ARP2/3 complex identified At5g18410 that was similar to SRA1 from Drosophila, as the most likely candidate.

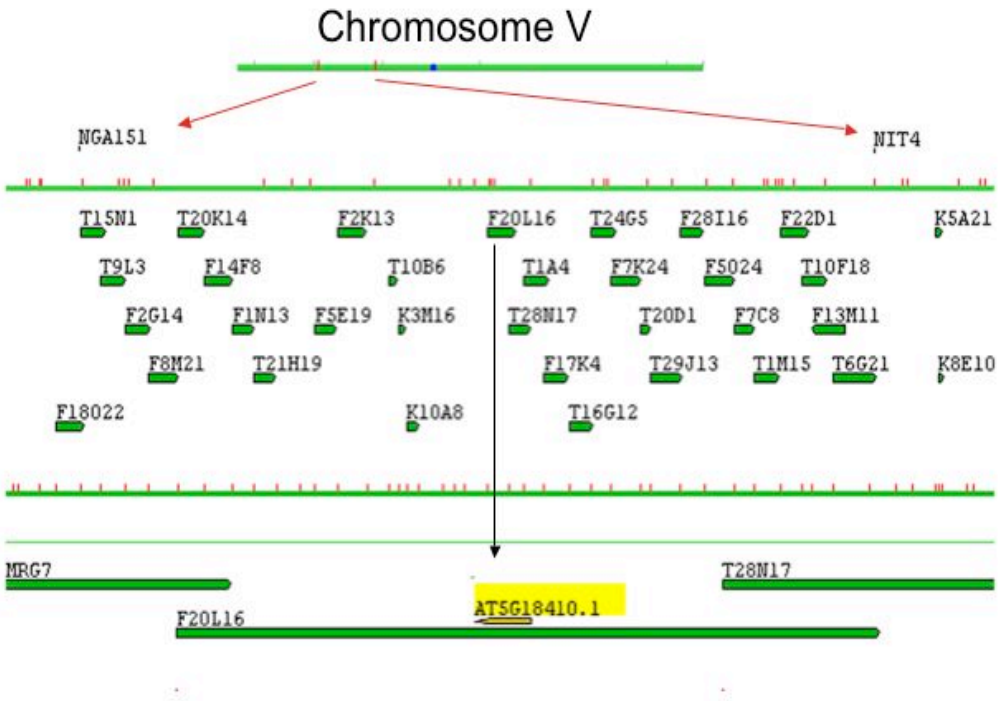

Figure 6 Locus of At5g18410

BACs used in both balistic rescue and candiate approach to map the klk locus. The region on chromosome V between the two markers NGA151 and NIT4 is shown. At5g18410, a putative homolog of PIR121 is highlighted in yellow and is located on BAC F20L16.

2.2 Sequence Analysis of EMS and T-DNA induced *klk* alleles

Five EMS-induced klk alleles were investigated: klk-207, klk-U56, klk-U121, klk-73 and klk-EM1. Sequencing of klk alleles revealed allele-specific mutations indicating that KLK encodes the AtSRA1 homolog. This revealed non-sense codons at amino acid positions 123, 240, 486, 530 and 1121 (see Table 1).
The GABI-Kat T-DNA insertion line 313F03 (Rosso et al., 2003), was shown to be allelic to klk-EM1 by a complementation test. The T-DNA carried an insertion at the nucleotide position 1790 (counting on the genomic DNA from the initiating ATG, Table 1)

Allele	Position of mutation	Mutation	Resulting Codon
klk-207,	370	C-T	stop at 123
klk-U56,	645	G-A	stop at 214
klk-U121,	1461	G-A	stop at 486
klk-73,	1590	G-A	stop at 530
klk-EM1,	3363	G-A	stop at 1121
313F03	1709	-	-

Table 1 Mutations in EMS-induced *klk* alleles

Sequencing of atsra1 gene in five klk alleles revealed stop codons in the positions shown above.

2.3 Expression analysis of wild type and *klk* alleles

The expression of the AtSRA1 gene in different organs was determined by RT-PCR. Expression was observed in all organs analyzed including the roots, the leaves, the seedlings and the flowers (Fig 8B).

The analysis of the RNA levels by RT–PCR (30 cycles) revealed no detectable expression in the GABI-Kat insertion allele and as expected normal expression levels in one EMS-induced allele carrying a point mutation predicted to result in a stop codon (Figure 8C).

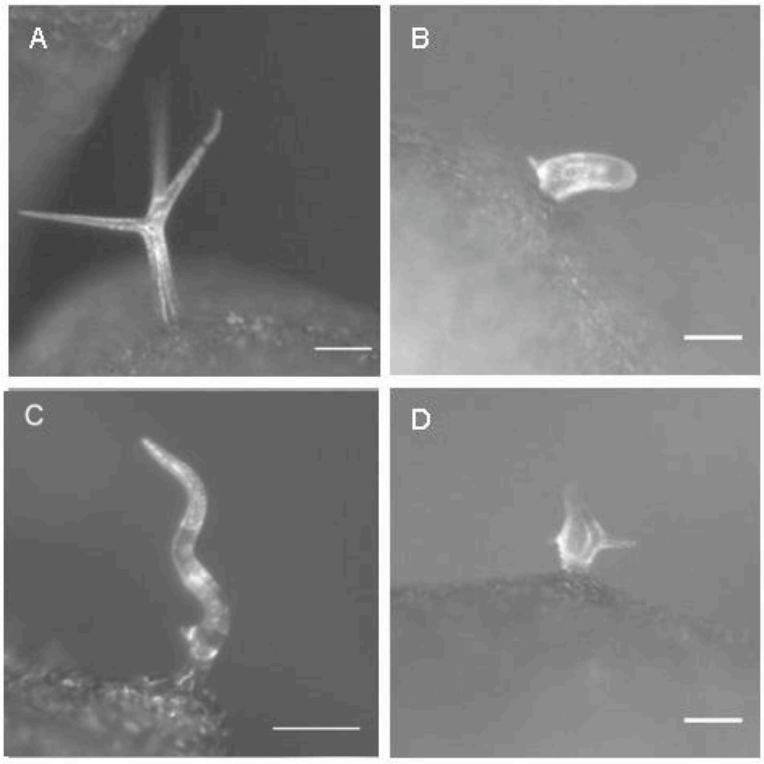

Figure 7 *klunker* Complementation Test

(A) wild type trichome

(B) klk-EM1 trichome

(C) GABI-kat line trichome

(D) Trichome from the resulting double mutant scale bar represents 0.2 mm

2.3.1 Molecular structure of AtSRA1

The exon-intron structure of AtSRA1 differed slightly from the predicted structure (MIPS, Fig. 8A) and contains 30 exons. The open reading frame of the AtSRA1 cDNA encodes a predicted polypeptide of 1282 amino acids

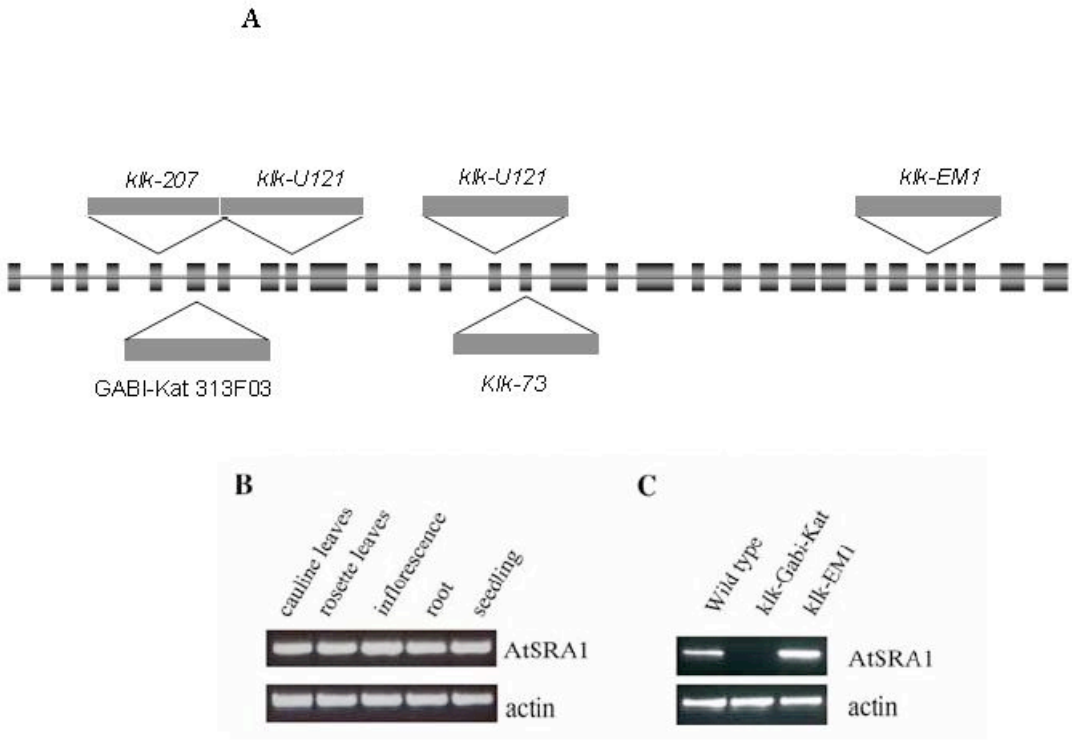

Figure 8 Molecular characterisations of AtSRA1

A) Schematic presentation of the exon-intron structure of the KLK gene. Exons are shown as boxes and introns as lines. The positions of mutations in klk alleles are indicated above.
B) Expression of KLK in different tissues as revealed by RT-PCR.

2.3.2 Protein homology of AtSRA1

The protein homology of AtSRA1 between diverse species was tested using the CLUSTAL-W (Thompson et al.,1994) algorythm; human (CYFIP2/PIR121), Drosophila (SRA1), C.elegans (Gex–2) and Arabidopsis (AtSRA1) were used to generate an alignment. The first 470 amino acids of PIR121 have been previously shown to be a RAC binding domain.

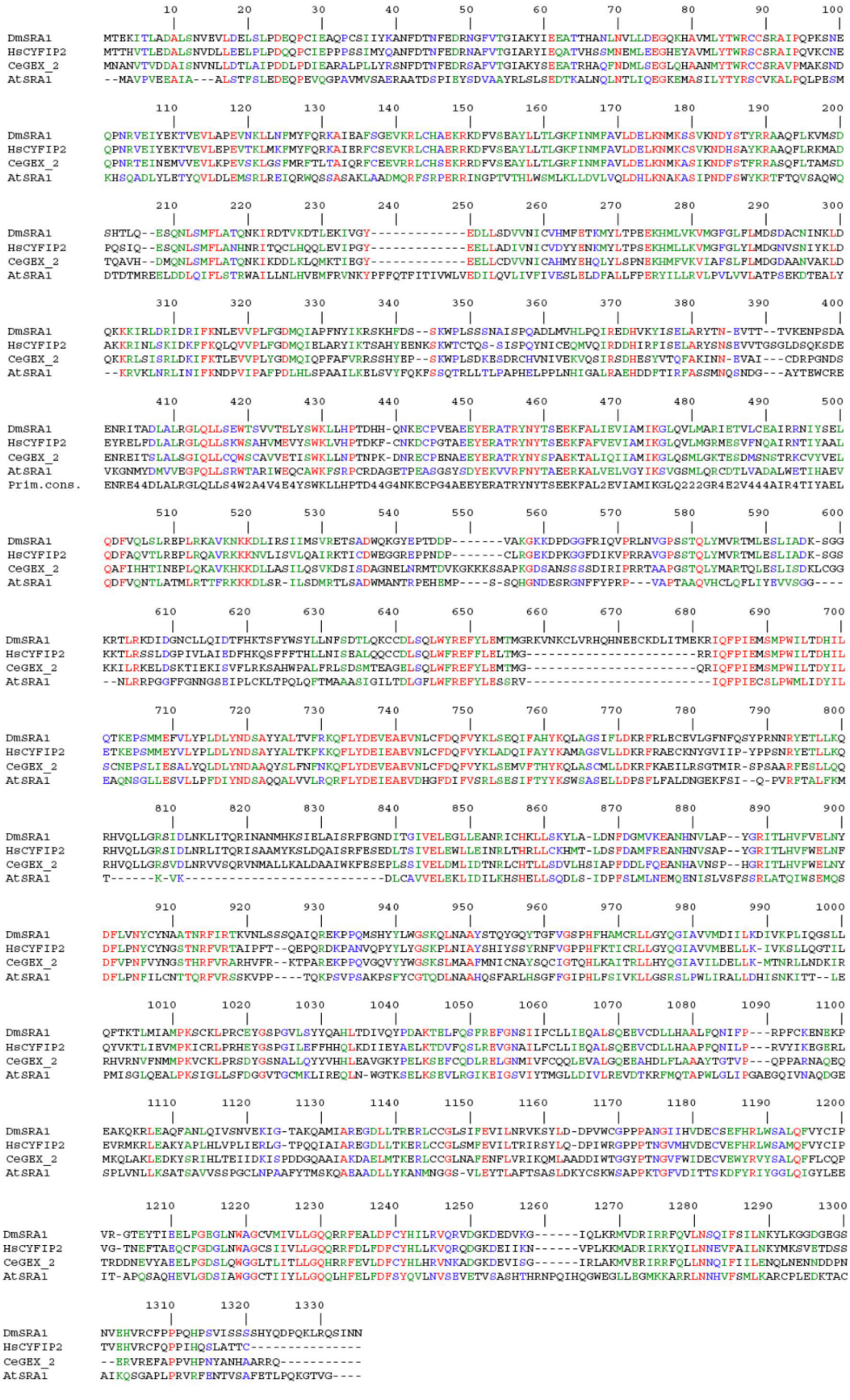
DmSRA1
HsCYFIP2
CeGEX_2
AtSRA1
Prim.cons.

Figure 9 Protein alignment of AtSRA1 with homologs from animals.

Here, homologs from human (CFIP2/PIR121), **Drosophila** (SRA1), **C.elegans** (Gex –2) and Arabidopsis (AtSRA1) are shown. Identity 15.51%, strongly similar 22.25%,, weakly similar 10.11%

2.4 Phenotypic characterisation of the *klk* mutant

The finding that At5g18410 encodes a putative SRA1 homolog in Arabidopsis suggests that KLK acts in a common pathway with the genes encoding components of the ARP2/3 complex. This is also apparent from the comparison of the klk phenotype with the phenotype of known arp2/3 mutants in Arabidopsis.

2.4.1 Epidermal cell phenotypes of *klk*

The trichome phenotype of klk mutants is as strong as in arp2/3 mutants (Figure 3A–C). All trichomes on klk mutant leaves show a distorted phenotype of varying strength. There was no obvious difference in the phenotypic strength between the different klk alleles.

One characteristic phenotype of the arp2/3 mutants is the altered morphology of epidermal pavement cells. While wild-type pavement cells form many lobes, these are reduced in arp2/3 mutants which develop fewer local expansions. This was also observed in klk mutants (Figure 3D–F).

2.4.2 Phenotypic analysis of the *klk* hypocotyl

Phenotypic alterations were also found in the hypocotyl when grown in the dark. Under these conditions hypocotyl cells normally undergo rapid cell elongation. As shown in Table 2 the length of the hypocotyl is drastically reduced in comparison

to the respective wild type. The phenotype of the three klk alleles induced in the Ler background is stronger than that of the Gabi–Kat allele that is induced in the Col background.

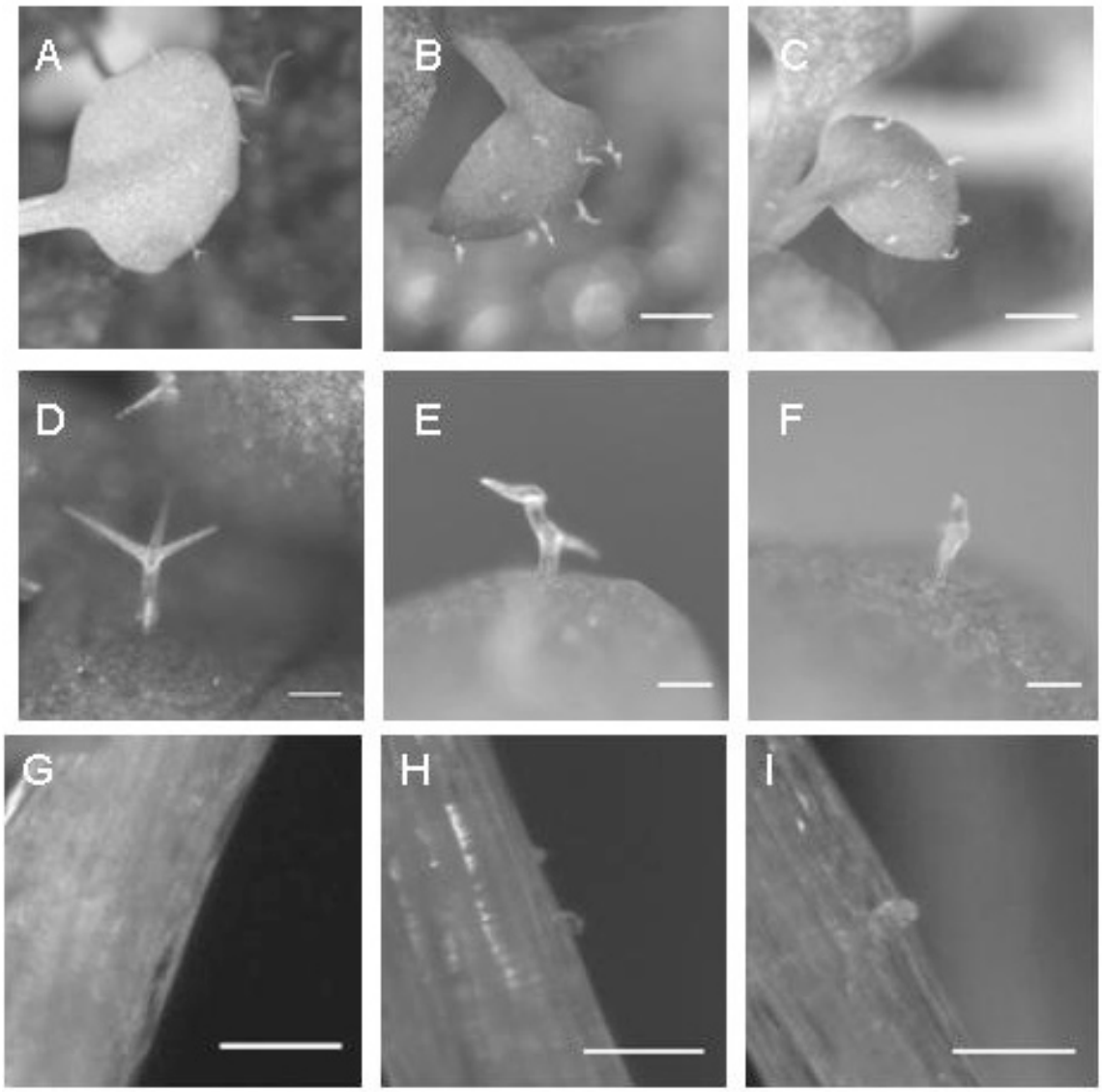

Figure 10 Phenotypic characterisation of *klk* mutants

(A) wild-type leaf trichome. (B) klk-U56 mutant leaf trichomes. (C) klk-73 mutant leaf trichomes. (D) wild type trichome (E) klk-U56 trichome. (F) klk-73 trichome (G) wild type hypocotyls (H) klk-U56 hypocotyl (F) klk-73 hypocotyl.
Note distorted shape of trichomes of both mutants and cell-cell adhesion defect.

2.5 Identification of Putative WAVE1 Homologs in *Arabidopsis*

Five putative WAVE homologs are present in Arabidopsis (Brembu et al., 2004; Frank et al., 2004). They all share a putative WASP homology 2 (WH2) domain. Four of them have an additional verprolin homology/cofilin homology/acidic (VCA) domain.
In this study a functional analysis was made on *AtSCAR2* (SCAR2) gene. *AtSCAR2* encodes a WAVE1 homolog.

2.6 Molecular Characterisation Of AtSCAR2

2.6.1 Analysis of four *AtSCAR2* T-DNA insertion alleles

In order to assess the function of the Arabidopsis Scar/Wave homologs, four insertion mutants in the AtSCAR2 gene were analyzed. For each mutant a homozygous line was isolated and the exact insertion site was determined. The SALK line 57481 has an insertion in the second exon. The SALK line 039449 shows an insertion in exon three 676 bp downstream of the ATG, the SALK line 036419 an insertion in exon six after 2116 bp and the line SALK line 124023 an insertion in exon six after 3503 bp (Figure 11A).

2.6.2 Expression analysis of the *AtSCAR2* alleles

RT-PCRs were carried out by amplifying a fragment located 3' of all the insertions to determine the expression levels in the four mutants. A reduced expression was detected in the SALK line 57481. No expression of AtSCAR2

was found in the other three lines indicating that the latter represent real null-mutants (Figure 11 B).

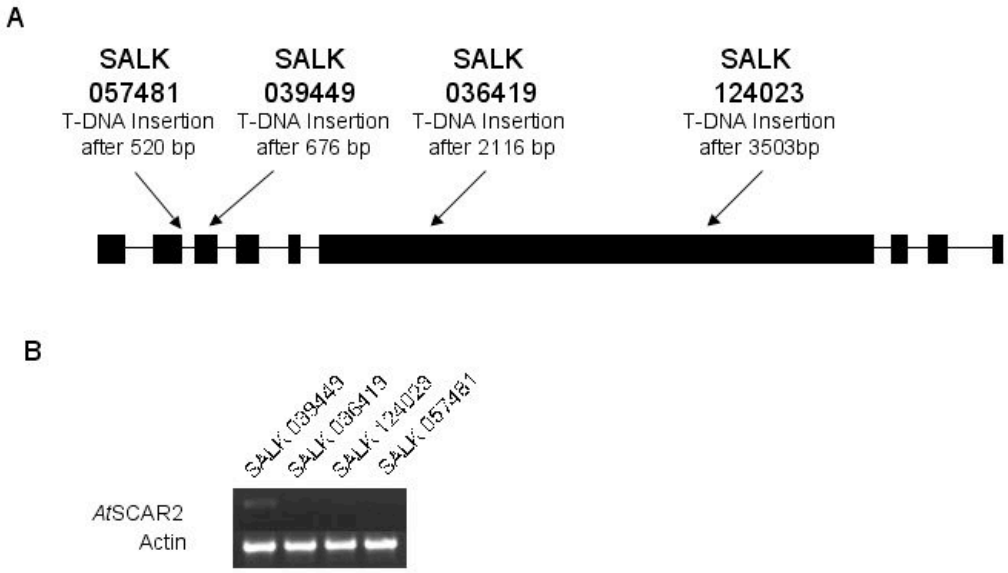

Figure 11 Molecular characterisation of AtSCAR2

A) Gene structure as confirmed by sequencing and the location of mutations in AtSCAR2. Solid boxes represent exons and black lines represent introns. The location of the T-DNA insertions is indicated by arrows. B) RT-PCR analysis of the expression of *AtSCAR2* in the SALK insertion mutants. mRNA was extracted from soil grown mutant plants.

2.7 Phenotypic analysis of *AtSCAR2* mutants

2.7.1 Trichome phenotype of *AtSCAR2*

The most obvious phenotype common to all four atscar2 alleles is the trichome phenotype. Trichomes exhibit a weak distorted phenotype reminiscent to that

seen in the arp2/3 mutants. Trichome branches are often twisted and different in length (Figure 12A-F). An additional phenotype was found in atscar2. In contrast to the arp2/3 mutants, the relative arrangement of the branches was also effected. In order to quantify this, the distance between the first and the second branch point was measured. In wild type plants, the second branch is found on average 17 μm above the first branching point. In the three lines 039449, 036419 and 124023 the branch point was found 34, 33 and 31 μm above the first branch point indicating that the relative positions of branch point is affected in atscar2 mutants.

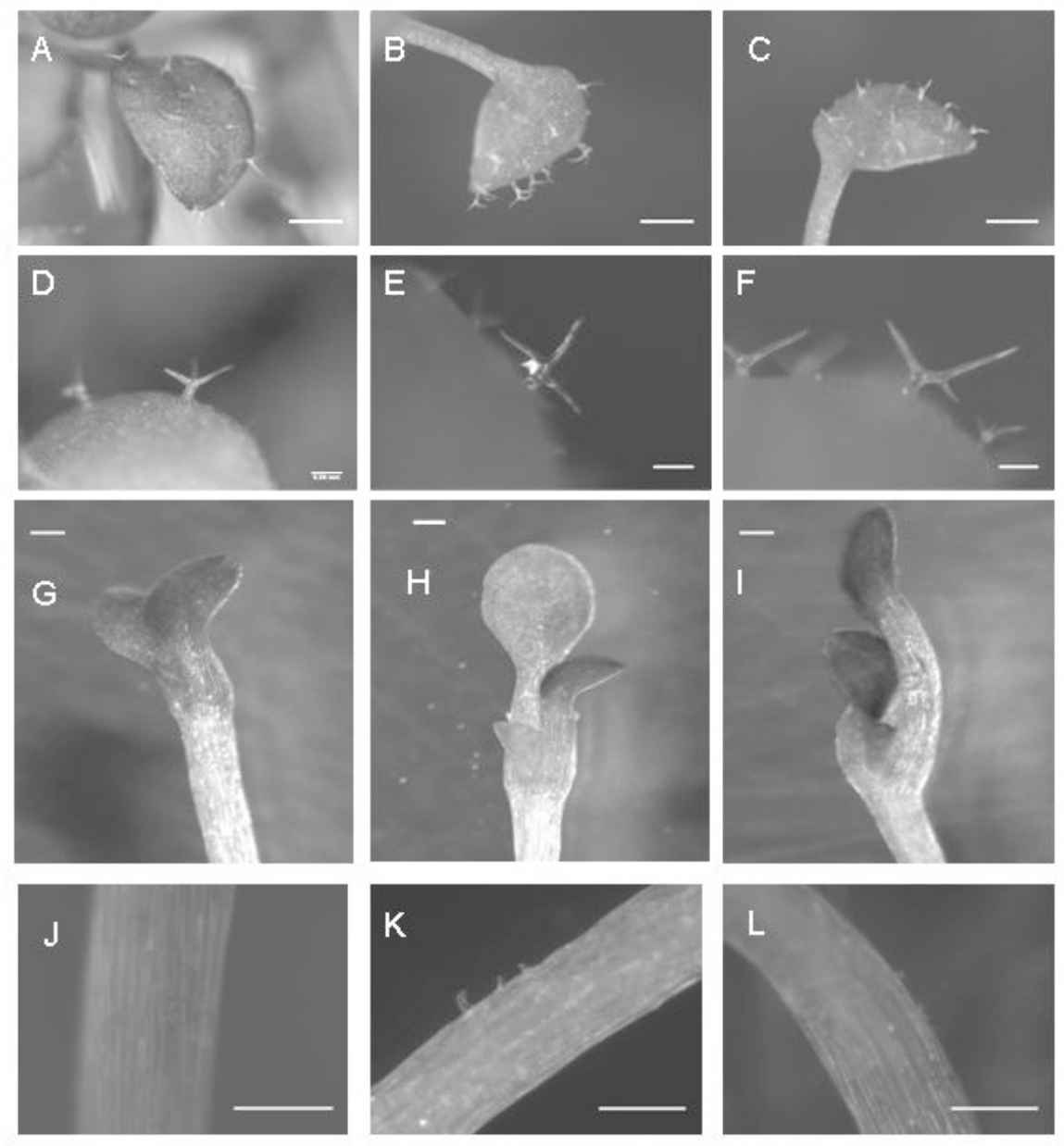

Figure 12 Phenotypic characterisation of *AtSCAR2*

Phenotypic characterisation of wild type and atscar2 mutant plants.

A) wild type leaf. B, C) leaves of SALK 624023 and SALK 039449 respectively

D) wild type trichome. E, F) trichomes of SALK 624023 and SALK 039449 respectively

G) petiole of wild type hypocotyl, H, I) hypocotyl of SALK 624023 and SALK 039449 respectively

J, K, L) dark-grown hypocotyls of wild type, SALK 624023 and SALK 039449 respectively.

Note elongated petiole of atscar2 mutants. Cells tear away from the hypocotyl during challenging growth which leads to the curling phenotype observed here. The branching phenotype of atscar2 mutants is most evident in picture F. The scale bars represent 1 mm.

2.7.2 Epidermal cell phenotype of *atscar2* mutants

In arp2/3 mutants also the hypocotyl cells show morphogenesis defects when grown in the dark. Cytoskeletal mutants are challenged by dark growth because it involves increased cell elongation. This is a suitable system to test for cell-cell adhesion inadequacies. Therefore atscar2 mutants were grown in the dark and tested to see if they had a hypocotyl phenotype. As shown in Figure 12 J-L the atscar2 lines exhibit severe cell form defects such that cells tear out of the epidermal tissue context and bend away from the surface. Root hair growth was normal in the atscar2 mutants.

The cell growth defects described above have in common that cell growth is either reduced or misdirected. It was therefore surprising to find that the petioles of the cotyledons are drastically elongated in atscar2 mutants (Figure 12 J-L). The analysis of the cellular phenotype revealed that the elongation of the petiole is due to increased cell elongation of the petiole cells. In wild type plants, the epidermal petiole cells are on average 0,80 mm long. The average length in homozygous SALK 039449 and SALK 124023 lines is 1,65 mm and 2,2 mm respectively.

2.8 F-actin organisation in *AtSCAR2* mutants

The finding that the phenotype of several cell types in atscar2 mutants is similar to that of arp2/3 mutants. Sequence similarity to WAVE proteins in animals suggests that also the atscar2 mutant have an aberrant F-actin organisation. A comparison was made between the F-actin organisation of pavement cells in wild type plants and atscar2 mutant plants. Therefore a 35S:GFP-Talin construct (Kost et al., 1998) was transiently introduced to individual cells by particle bombardment. In wild type F-actin a small number of thick bundles were observed. Most of the F-actin was seen as finer cables. In atscar2 mutants no fine cables were found. All the F-actin seen was visible as thick bundles.

Although the overexpression of the GFP-Talin protein may generally bias the observations towards more bundled actin, the difference between wild type and mutants as shown in (Fig. 13) was consistently observed. It is therefore possible to conclude that the atscar2 mutants have a disturbed F-actin organisation.

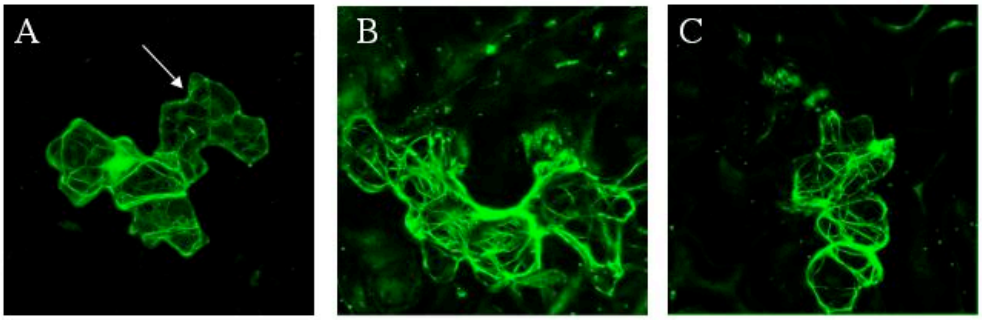

Figure 13 Actin phenotype of *AtSCAR2*

A-C) Individual pavement cells transformed with the 35S:GFP-mTalin construct by particle bombardment.A) Wild-type. Note, the relatively fine actin (arrow)B) SALK_039449. As compared to wild type, little fine actin is observed. C) SALK_ 624023. As compared to wild type, little fine actin is observed.

2.9 Functionally conserved domains of AtSCAR2

It is known from other systems that WAVE1 and WAVE2 interact with the HSPC300 (Eden et al., 2002; Gautreau et al., 2004). In addition, it was shown that AtSCAR3 and AtSCAR1 in Arabidopsis interact with the plant homolog of HSPC300, the BRICK1 gene (Frank et al., 2004). The phenotypic range of atscar2 mutants and the finding that also the actin cytoskeleton is aberrant suggests that also AtSCAR2 functions in this pathway. In order to test this, the interaction between AtSCAR2 and AtBRICK1 was analysed using the Yeast Two Hybrid System. A clear interaction between the two proteins was found (Table 2). In order to determine whether the putative SHD domain of the AtSCAR2 protein was mediating this interaction, only the SHD domain of AtSCAR2 was used for the interaction assays and it was possible to show interaction of this domain with AtBRICK1 (Table 2).

	Bait	Prey	-L-W	-L-W-H
1	pACT2	pAS	-	-
2	pAS-SNF1	pACT-SNF4	++++	++++
3	AtBRICK1	pACT2	++++	+
4	AtBRICK1	AtSCAR2	++++	+++
5	AtBRICK1	AtSCAR2-SHD	++++	++
6	AtSCAR2-WH2	pACT2	++++	-
7	AtSCAR2-SHD	AtBRICK1	++++	-

Table 2 Yeast two hybrid analysis between AtSCAR2 and AtBRICK

Key: ++++ same growth as the control

\- no growth

2.10 Subcellular localization of AtSCAR2

2.10.1 Particle Bombardment

If AtSCAR2 acts as a regional activator of the Arp2/3 complex one might expect that AtSCAR2 is localized in regions of the cell that actively grow such as the root hair tip, the lateral region of trichomes or the lobes of the pavement cells. A 35S:YFP-AtSCAR2 fusion construct was created and transiently expressed in pavement cells. YFP fluorescence predominantly at the cortex of the cell was observed. On occasion, stronger labeling was observed at the tip of the lobes. This was much pronounced in AtSCAR2 mutants presumably because the competition with the endogenous wild type protein results in a reduced local concentration of the YFP-AtSCAR2 protein.

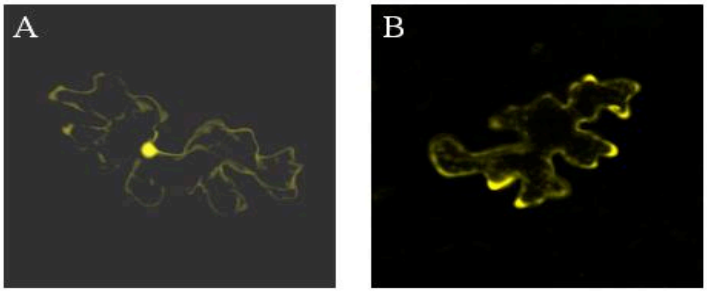

Figure 14 Intracellular localisation of YFP:AtSCAR2

A) Wild type epidermal cell expressing YFP as control

B) Wild-type epidermal cell expressing the YFP-AtSCAR2 fusion protein. The fusion protein seems to be localized in the cytoplasm, and slightly enriched at the lobes. However, this result was not easily reproducible.

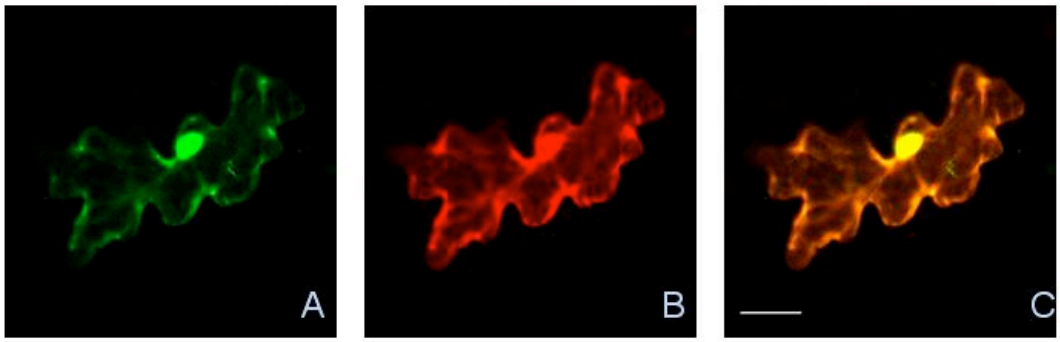

Figure 15 Localisation of GFP:AtSCAR2 with DsRed

Localisation of GFP:SCAR2 cobombarded with DsRED. In wild type cells, SCAR2 protein fused to GFP (A), localises to the cytoplasm which is labeled with DsRed (B). (C) is a merged image of (A) and (B). Scale bar, 20 μm.

2.10.2 Localisation of AtSCAR2 by Infusion in *N. benthamiana*

Another method often used to localise proteins in vivo is by infusion of tobacco leaves with agrobacteria that has been transformed with the construct of choice. This method is more successful for localisation of unstable proteins because the fluorescence is visible for a much longer time than by particle bombardment. An added advantage ist hat a greater region is transformed transiently. This method was used to infuse tobacco plants with GFP:AtSCAR2. As a control, GFP was infused alone. The reulsting pattern of fluorescence was different from the control with respect to the intensity of the signal and the appearance of the tissue. The control tissue showed a clear labeling of the cytoplasm and nucleus. The tissue transformed with GFP:AtSCAR2 however, appeared to have a much denser cytoplasm in some of the lobes of the epidermal cells.

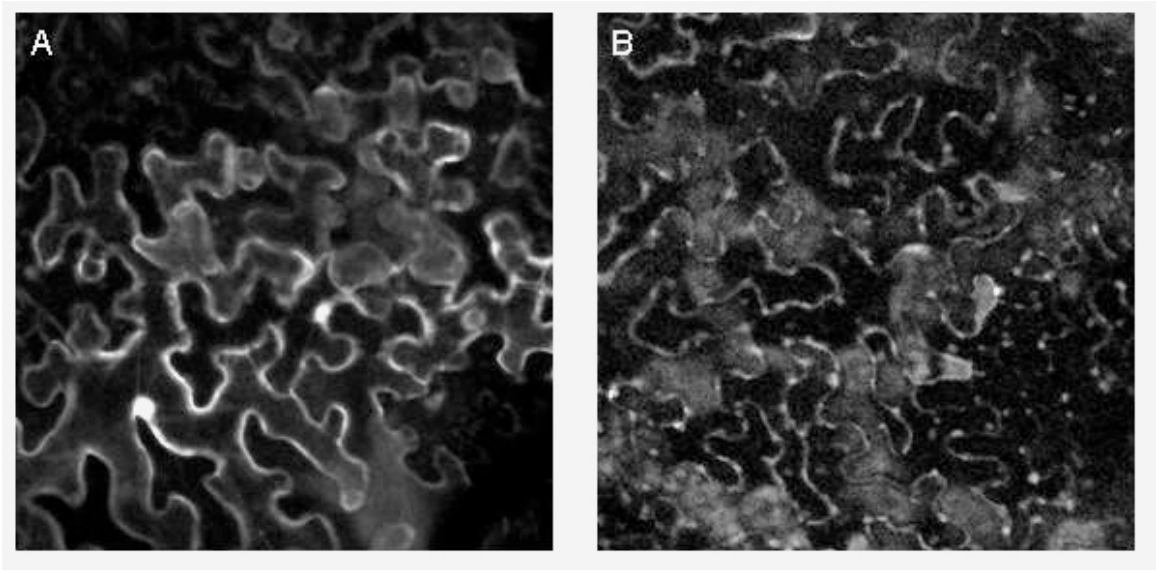

Figure 16: Localisation of AtSCAR2 by infusion

(A) N. benthamiana epidermal cells after infusion with GFP

(B) N. benthamiana epidermal cells infused with GFP:AtSCAR2

Note the dense areas in the lobes in (B) which are absent from (A). The dots in (B) correspond to chloroplasts.

2.11 Raising of an antibody against AtSCAR2

In order to obtain a clear localisation of AtSCAR2, an antibody was raised in mice. This was tested for specificity in order to establish whether it was specific for the whole AtSCAR protein family or AtSCAR2 in particular. The AtSCAR2 antibody was found to be specific for AtSCAR2 exclusively.

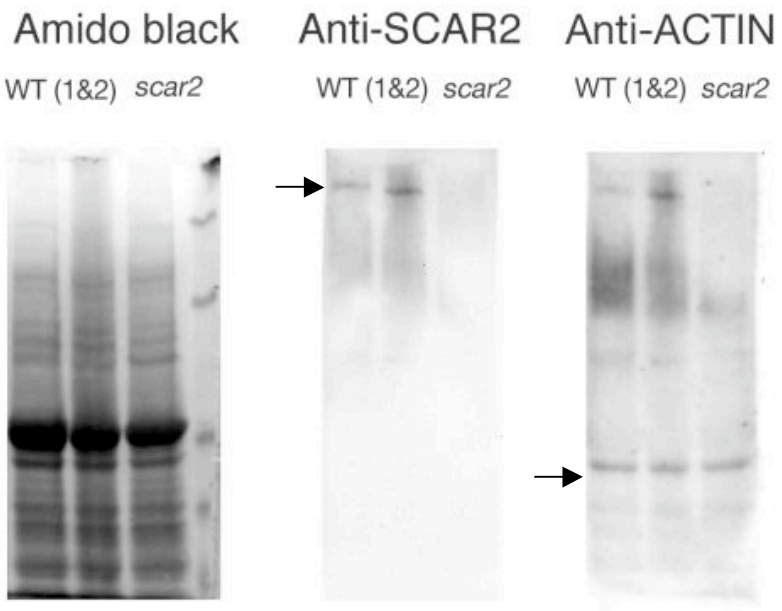

Figure 17 Specificity of AtSCAR2 antibody

Lane 1 contains total protein extract from 8-day-old wild type plants. Lane 2 contains total protein extract from mature wild type plants. Lane 3 contains total protein extract from AtSCAR2 mutant plants.

2.12 Double mutant analysis of the AtSCAR2 family

In order to investigate the role of the other putative members of the SCAR family present in A. thaliana, a double mutant screen was carried out in which atscar1-5 were crossed with each other obtaining all possible combinations. The crossing between atscar2 and atscar3 had to be repeated and thus not results were obtained for this cross. atscar1 and atscar2 displayed linkage which made detection of the double mutant knockout difficult. A strong distorted phenotype was observed in trichomes of plants obtained form the cross between atscar2 and atscar4. PCR was used to confirm that the plants carried both T-DNA insertions homozygously. Detailed characterisation of atscar2/atscar4 mutant plants remains to be done.

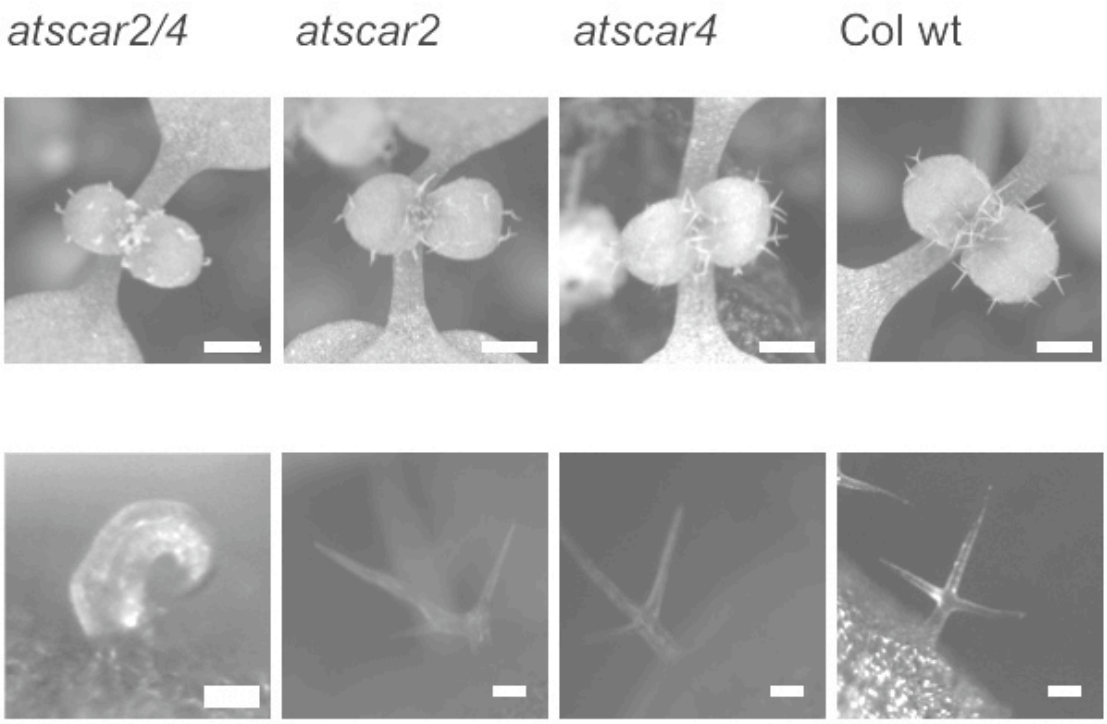

Figure 18 Trichome phenotype of AtSCAR2/AtSCAR4 double mutant

(A) and (E) atscar2 atscar4 double mutant leaves with trichomes that have a strong distorted phenotype.
(B) and (F)atscar2 single mutant leaves and trichome.
(C) and (G) atscar4 single mutant leaves and trichome.
(D) and (H) wild type leaves and trichome

The severity of the distorted phenotype of atscar2/atscar4 double mutants suggests that ARP2/3 complex function is disrupted to the same extent as in severe mutants of the ARP2/3 complex.

3 Discussion

The main goal of this study was to address the general question of how the organisation of actin contributes to the process of cell morphogenesis in plants, and how this is regulated. A combination of reverse and forward genetics was used in an attempt to achieve this goal. The ARP2/3 complex is a central component involved in the actin cytoskeletal organisation in animals and plants. In this study, plant homologs of proteins with a role in the regulation of the ARP2/3 complex have been identified. Functional analysis of the KLK gene and the ATSCAR2 has given some insights into the role of the ARP2/3 complex in plants. These two genes are ubiquitously expressed and necessary for normal cytoskeletal organisation, expansion growth and tissue development in the leaf and cotyledon. The data presented here supports a role for ATSRA1 and ATSCAR2 in the regulation of the ARP2/3 complex in plants.

The mechanisms that control site-specific de novo polymerization of new actin filaments are best studied in animals. Plant systems offer a different perspective on actin-related cell morphogenesis phenomena due to the presence of a cell wall. Interesting questions arise concerning the mechanisms plants have evolved employing a subset of proteins similar to those found in animals. The initiation of new actin filaments is an important cellular process that is mainly carried out by the ARP2/3 complex in animals (Machesky and Gould, 1999). Although the ARP2/3 complex has a pivotal role in actin filament initiation, novel nucleators are still being discovered (Schuldt, A. 2005). Besides cell shape, many types of cell motility are also dependent on the assembly of actin filaments, which can drive membrane protrusions and vescicle movement (Weaver et al., 2003).

3.1 The Existence of a WAVE-Related Regulation Pathway in Plants

The five subunits of the inactive WAVE1 protein complex have been identified in several metazoan organisms (Eden et al., 2002; Bogdan and Klämbt, 2003), as well as the mycetozoan Dictyostelium (Blagg et al., 2003). Plant homologs of NAP125 and HSPC300 have also been reported (Saedler et al., 2004 and Le et al., 2006). In this study, two genes have been identified in the Arabidopsis genome that encodes proteins similar to the mammalian PIR121/CYFIP and WAVE1 proteins.

Multigene families are a common theme when investigating the proteins involved in actin dynamics and cytoskeletal organisation in both plants and animal systems (Ben-Yaacov *et al*., 2001; Zallen *et al*., 2002).. Interestingly, AtSRA1, AtNAP1 and all but one of the ARP2/3 complex subunits in Arabidopsis are single copy genes. A similar situation exists with respect to these genes in all eukaryotic genomes investigated with few exceptions. This suggests that evolutionary pressure exists that inhibits the production of multiple copies of these genes in eukaryotes as a whole. An explanation for this observation could be that higher protein doses have a negative effect on fitness (Brembu *et al*., 2004).

3.2 AtSRA1 is involved in the Regulation of the Actin Cytoskeleton

The evidence presented in this study suggests a role for AtSRA1 in the regulation of the actin cytoskeleton. Firstly, at phenotypic level, the atsra1 mutation affects a similar range of cell types to those affected by the other members of the DISTORTED class of genes. Cells types affected include epidermal pavement cells, trichome and hypocotyl cells.

Secondly, the distorted mutants that have been characterised thus far have all been shown to be involved in ARP2/3-mediated actin cytoskeleton dynamics.

Members of the distorted class of mutants that have been mapped to genes encoding the ARP2/3 complex include wrm, dis1, crk, and dis2. These genes encode ARP2, ARP3, ARPC5, and ARPC2, respectively (Le et al., 2003; Li et al., 2003;Mathur et al., 2003a, 2003b; El-Assal et al., 2004). It is also important to note that klk does not complement the gabi-KAT insertion mutant in AtSRA1. This shows that klk and atsra1 carry mutations in the same gene.

Lastly, the actin cytoskeleton in atsra1 trichomes shows an aberrant organisation, forming thick bundles that are orientated unlike the wild type and increased vacuolisation. It is therefore probable that AtSRA1 operates in the same pathway as the ARP2/3 complex.

3.3 Inactivation of PIR121 in Other Organisms

Inactivation of PIR121, Nap, and ARP2/3 complex subunit homologs also produce similar cellular defects in other organisms. RNA interference depletion of the Nap homolog Kette, the PIR121 homolog Sra-1, and the p20 subunit of the ARP2/3 complex in Drosophila S2 cells all resulted in stellate cell morphology (Kunda et al., 2003; Rogers et al., 2003). During embryogenesis in C. elegans, the PIR121 mutant gex-2 and the Nap mutant gex-3 display defects in ventral enclosure similar to those observed for lines with RNA interference depletion of ARP2/3 complex subunits (Soto et al., 2002; Sawa et al., 2003). All these experiments show that the KLK regulates the ARP2/3 complex.

3.4 AtSCAR2 Defines a Putitive Arabidopsis WAVE Family of Proteins

WAVE and WASP are well-studied regulators of ARP2/3 complex activity in animals (Higgs and Pollard, 2001). WASP proteins are self-inhibiting and the

binding of Cdc42 releases the auto-inhibition resulting in the activation of the ARP2/3 complex. WAVE and HSPC300 interact with the trimeric PIR121/NAP125/Abi2 complex and binding of Rac1 regulates the activity of the complex though it is controversial whether the association with PIR121/NAP125/Abi2 leads to activation or repression. Current evidence shows that WAVE1 and HSPC300 are inhibited by binding to the PIR121/NAP125/Abi2 complex and binding of Rac releases this complex leading to WAVE1-mediated activation of the ARP2/3 complex (Eden et al., 2002). WAVE2, on the other hand, seems to be activated by Abi1, Nap1 and PIR121 and the spatial activation of the ARP2/3 complex is achieved by a re-localization upon activation by Rac (Miki et al., 2000; Innocenti et al., 2004).

In Arabidopsis, 5 genes have been identified as putitive WAVE homologs by bioinformatics. Homozygous T-DNA insertion mutants revealed AtSCAR2 as the only mutant with an obvious trichome phenotype.

3.5 AtSCAR2 Functions in a WAVE-Mediated ARP2/3 Pathway in Arabidopsis

Genetic evidence suggests that AtSCAR2 has a role in a pathway that regulates the ARP2/3 complex in Arabidopsis. A first line of evidence is the observation that the phenotypes of AtSCAR2 plants are similar to those of the *distorted* class of mutants. These mutants have trichome distortions that occur at specific stages of development. A detailed mutant analysis of the AtSCAR2 homolog revealed weak phenotypes in only some of those cell types normally affected in arp2/3 mutants. No root hair phenotype was observed and the hypocotyl cells only occasionally showed a loss of cell-cell adhesion. The pavement cell lobing was only weakly affected and trichomes showed a mild distorted phenotype. In contrast to all other ARP2/3 complex mutants so far described, the relative position of branches was altered.

The second line of evidence is the fact that the actin cytoskeleton of *AtSCAR2* mutants was found to be aberrant. This phenotype was reminiscent of *distorted* mutants *klk* and *grl*, although relatively weaker. This observation places AtSCAR2 in a pathway involving the regulation of actin cytoskeleton dynamics. The similarity described here could be due to a positive regulatory role on the ARP2/3 complex shared by *atscar2, klk and grl* during the development of pavement cells.

A model in which WAVE is stabilised by the WAVE complex proteins is supported by various reports using animal systems (Blagg et al., 2003; Kunda et al., 2003; Rogers et al., 2003). In this model, signaling to the ARP2/3 complex occurs via an interaction between RAC1 and PIR121. Localisation of the fully assembled complex is also RAC1 dependent. This allows the WAVE complex to be channelled to areas within the cell that have high ARP2/3 complex activity needed localised actin-based growth (Innocenti et al., 2004).

3.6 AtSCAR2 is a WAVE1 Homolog in *Arabidopsis*

The WAVE regulatory complex is part of a mechanism for actin nucleation that is necessary in morphogenesis events in eukaryote development. Data presented in this study point to AtSCAR2 as good candidate for the SCAR/WAVE protein in the context of a WAVE regulatory complex. Analysis of the predicted domain organisation of AtSCAR2 supports view.

Homology between mammalian WAVE1 and putative plant SCAR proteins is most obvious at the N and C termini. Mammalian WAVE1 has been shown to have an SHD domain close to the N terminus. This domain defines the WAVE/SCAR family of proteins. A homologous sequence is present at the N terminus of AtSCAR2. This region is followed by a basic region in both WAVE1 and AtSCAR2. The C terminus of WAVE1 has a VCA domain that is necessary for binding actin and the ARP2/3 complex. A highly similar region exists at the C

terminus of AtSCAR2.

The SHD domain of WAVE1 (and other WAVE family proteins) is known to bind HSPC300 in mammalian systems, as a necessary part of its role in the WAVE regulatory complex. It is therefore expected that *Arabidopsis* could have an HSPC300-like protein. In addition to this, the SHD domain of putitive WAVE proteins should be able to bind to such an HSPC300-like protein.

A novel protein has been reported to be a homolog of mammalian HSPC300 (Smith, 2005). It is a single copy gene like many of the main players in the regulation of actin cytoskeleton dynamics. Mutations in this gene lead to a severe phenotype both in Arabidopsis and in maize, where its name was coined due to the brick-like appearance of epidermal pavement cells as a result of abolished cell-lobing.

In this study, the Yeast Two Hybrid System was used to verify the connection between AtBRICK and AtSCAR2. These two proteins were shown to interact when tested in their entirety. Several AtSCAR2 fragments were cloned and tested to establish which part of the protein mediated binding with AtBRICK1. Only the fragment encoding the putative SHD domain was able to maintain binding capacity with AtBRICK1. Therefore this indicates that AtSCAR2 has a conserved SHD domain that is likely to be functional in *Arabidopsis*.

In order to fit the role of a WAVE1 homolog, AtSCAR2 is expected to also have a VCA domain that can interact with the ARP2/3 complex via ARPC3. It has recently been shown that AtSCAR2 does in fact binds to APRC3 in the yeast two hybrid system (Basu *et al*., 2005).

Taken together, these data present a compelling argument for a homologous relationship between AtSCAR2 and WAVE1.

3.7 Localising the activity of the WAVE Regulatory Complex in Plants

Data from this study concerning the localisation of WAVE complex members support a cytoplasmic localisation. Both particle bombardment and stable

transformation of an AtSCAR2-GFP fusion construct in Arabidopsis failed to give a focussed localisation comparable to that observed in animal systems. This could be because AtSCAR2 protein levels are regulated by fast degradation. The observation that AtBRICK1 stabilises AtSCAR2 in vitro (Smith, 2005) suggest that co-bombardment of AtSCAR2 and AtBRICK1 should yield a more focussed localisation. Such experiments were carried yielding the same cytoplasmic. It will be interesting to locate AtSCAR2 protein within the cell using immunolocalisation. The AtSCAR2 antibody raised in this study is expected to be enabling such an experiment due to its specificity for AtSCAR2. This is important because of the similarity within the ATSCAR family.

3.8 Potential Functions of AtSCAR2

AtSCAR2 belongs to an Arabidopsis protein family with five members, four of which contain an Arp2/3 activating VCA domain. Animals and yeast have several varieties of Arp2/3 activators (such as cortactin, WASP and SCAR/WAVE) that often represent families with multiple isoforms. Each family of activators has been attributed broad functional roles (Kaksonen et al., 2003) and in some cases individual isoforms within a family can be attributed a distinct function. To date, no family of Arp2/3 activators other than SCAR have been identified in plants by bioinformatics or experimentally. This suggests that the majority of Arp2/3 regulation may be divided between the four VCA-containing SCAR isoforms.

This is supported by the fact that mutants of NAP1 and PIR121 virtually phenocopy mutants of Arp2/3 components in most tissues. Mutant alleles of AtSCAR2 also produce similar phenotypes in most tissues affected by Arp2/3 dysfunction; however the atscar2 phenotypes are far less severe. Genetic redundancy between isoforms is likely as the expression patterns of the Arabidopsis SCAR homologues overlap extensively.

The phenotypic analysis in this study indicates that among the five SCAR genes the SCAR2 gene encodes the major isoform required for cell morphogenesis with

SCAR4 acting in the same pathway in a redundant manner. The trichome phenotype of AtSCAR2/AtSCAR4 double mutants was similar to that of the most severe distorted mutants. This supports the idea that one of the other SCAR proteins, namely AtSCAR4, is taking over part of the actin-related functions of AtSCAR2 in atscar2 mutants. This then could give rise to the weak phenotype observed in atscar2 mutants. All other scar and scar-like mutants and the double mutants tested in this study provide no hint towards their role in cell morphogenesis though a further level of redundancy may still be possible.

3.9 WAVE-independent ARP2/3 Complex Activation in Plants

The severe phenotypes of the distorted mutants dis1 and dis2 (having mutations in arp3 and arpc2 respectively) are correlated with a lack of ARP2/3 function. The slightly weaker phenotypes of the WAVE complex members klk (atsra1) and grl (atnap1) have been speculated to show that a pathway exists that is independent of the WAVE complex (Basu et al, 2004). This, however, seems unlikely in light of the severe phenotype of the atscar2/atscar4 double mutant, which most probably represents a substantial reduction in WAVE complex activity. Detailed characterisation of the atscar2/atscar4 double mutant phenotype is necessary to establish whether it is indistinguishable from a mutant devoid of ARP2/3 complex function. If this turns out to be the case, then the apparent lack of a WASP-mediated ARP2/3 activation pathway in Arabidopsis would represent an interesting evolutionary diversification between plants and animals.

Proteins involved in the nucleation of actin filaments work in concert with actin modulating proteins that function to balance the pools of the different actin species in accordance with the needs of the cell. The two most-studied actin-modulating proteins are Actin Depolymerising Factor (ADF) that is also known as cofilin, and profilin. Both act synergistically to affect actin dynamics (Didry, 1998). In animals, WAVE, WASP, VASP, WIP and formin proteins form a class of poly-

L-proline-containing (PLP) profilin-binding proteins. Together, these PLP proteins act in the next tier of signal transduction to affect cytoskeletal dynamics. The PLP domain enables PLP proteins to interact with SH3- and WW– domain proteins; multiple inputs into the PLP system confer dynamic responsiveness to the stimuli by the cytoskeleton.

In plants, however, only formins seem to have a conserved PLP domain. This would suggest that formins alone could be responsible for signal transduction affecting profilin in plants, a task performed by 5 protein families in animals. Formins form a large protein family hence this could be plausible. Single mutant analysis of formins has not revealed any observable phenotype, which hints at possible redundancies with the family.

It is interesting to note that the Arabidopsis SCAR proteins, surprisingly, do not have an obvious PLP domain at the sequence level. It would therefore be interesting to test whether members of the AtSCAR family have retained the ability to bind profilin. If they are able to, then mapping this binding domain would enable the detection of perhaps other sequentially diversified PLP proteins (such as a putative WASP protein) that continue to be elusive.

If AtSCARs cannot bind profilin, then it will be interesting to investigate how AtSCARs can be responsive to balance of F-actin and G-actin in the cell; a necessary part of the function of animal PLP domain-containing proteins. AtSCAR sequences are generally longer than their mammalian counterparts and contain extended regions with no homology. These regions could have binding sites for other input proteins unique to plants.

In view of the above observations, it seems that the ARP2/3 complex is not the major actin nucleator in plants. In addition, the actin organisation of ARP2/3 mutants is compromised but similar mutants in animals are not even viable. It is therefore more likely that formins play the most substantial part in fulfilling the cells needs with respect to actin nucleation.

4 Material and Methods

4.1 Materials

4.1.1 Chemicals and Antibiotics

All chemicals and antibiotics used in this study were acquired from Roth (Karlsruhe) and Sigma (Deisenhofen).

4.1.2 Restriction Enzymes

Restriction enzymes were supplied by MBI-Fermentas (St.Leon-Rot) and New England Biolabs (Frankfurt/Main). All other materials for molecular biology were acquired from Amersham, Fermentas, Roche (Mannheim), Sigma-Aldrich(Munich), Clonetech and Invitrogen (Karlsruhe).

4.1.3 Vectors

This study was carried out using the following vectors:

Cloning of PCR Products:
pGEM-T easy (Promega); pBluescript; TOPO (Invitrogen)

Vectors supplied by Invitrogen for Creation of Entry clones (by recombination):
pDONR201; pDONR207

Creation of Destination clones (by recombination):
pEN, pMDC42 (for fluorescent protein fusions); pDEST17, pDEST42, pGAT4 (for bacterial protein expression); pCACT2, pCD2 (for yeast-2-hybrid analysis)

4.1.4 Bacterial Strains

E. coli strain DB3 was used for propagation of Gateway vectors (i.e. those used for cloning by recombination). E. coli strain DH5α was used for propagation of standard constructs. E. coli strain Rosetta and E. coli strain BL21 were used for protein expression in bacteria. The A. tumefaciens strain GV3101 was used for the transformation of plants.

4.1.5 Yeast Strains

The yeast strain AH109 (Halladay and Craig, 1996) was used for yeast cotransformation.

4.1.6 Plant lines

The A. thaliana ecotype L. erecta (Ler) was the background of the mutant allele klunker. All SALK T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center (UK) and were in the A. thaliana ecotype Columbia (Col). The gabi-KAT T-DNA insertion line 31300 was also in the Columbia background (Rosso et al. 2003)

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4.2 Methods:

4.2.1 Plant material and growth conditions

The klk alleles klk-EM1, klk-207, klk-73, klk-U121and klk-U56 are EMS *Arabidopsis* mutants that were induced in the Landsberg erecta background (Hulskamp *et al*., 1994). The gabi-KAT T-DNA insertion line313F03 (Rosso *et al*., 2003) was in a Columbia wild type background. Plants used for this study were grown on soil or *in-vitro* on MS-medium containing 3% sucrose,1% phytoagar and grown at 23°C.

4.2.2 Analysis of T-DNA insertion lines

To confirm the T-DNA insertions in the *AtSCAR2* gene, PCRs on genomic DNA with the following primers were carried out:
SALK_039449: LP_39449 (5´-TATGGCTGGTCTCGTTGGCAT-3), RP_39449 (5´-CACATTCAAAACACCATGAGCA -3);

SALK_036419: LP_36419 (5´- CCACCATGGAGTCGGAAACAG -3), RP_36419 (5-GGCAACGACCATCACGTTCTT -3);

SALK_57481: LP_57481 (5´-TGGAATCCGTTGTTGTAAAGCG -3), RP_57481 (3´-GGTGTCCCTCCATTCCTCCAC 3);

SALK_36419: LP_36419 (5´- CCACCATGGAGTCGGAAACAG -3), RP_36419 (G5´- GCAACGACCATCACGTTCTT –3´);

SALK_124023: LP_124023 (5´- CGGGAGTAGAGAAATTGGCGG -3), RP_124023 (5´- GCTCTGATGGTGGAGACCCAA -3)

and the T-DNA specific primer LBb. The PCR products were sequenced and the exact positions of the inserts determined.

4.2.3 Cloning AtSRA1 Gene:

The KLK gene was identified by allele-specific mutations in the Arabidopsis AtSRA1 homolog (At5g18410) in several klk alleles. The genomic DNA sequence of the Arabidopsis SRA1 homolog was obtained from the MIPS Arabidopsis database (http.//mips.gsf.de/cgi-bin/proj/thal/). The cDNA sequence covering the region between the ATG and the stop codon was amplified using the following primer combination:
klkfw:5'-ATGGCGGTTCCTGTAGAGGAAG –3';
klkrev: 5'-TCAGCCAACGGTGCCTTTCTGAGG-3'.
The pGEM-Teasy® vector (Promega) was used for sub-cloning the cDNA fragment that were generated. Homology searches were performed using BLAST programs. Amino acid alignments were carried out using the ClustalW multiple sequence alignment based programs.

4.2.4 Cloning the ATSCAR2 Gene

cDNA encoding the AtSCAR2 protein was amplified by PCR using the primers WAVE4_fw (5´-attB1-ccATGCCGTTGACGAGGTACCAATCTCG-3) and WAVE4_rev (5´-attB2_tTCAAGAATCACTCCAACTATCTG-3). The resulting PCR product was recombined by Gateway BP reaction into pDONR201.

4.2.5 Cloning the AtBRICK1 Gene

The cDNA of AtBRICK was amplified with the primers:
BRICK1 for 5'- attB1-GCATGGCGAAAGCTGGAGGGATCAC-3' and

BRICK1 rev 5'attB1- GTCACGTCGCAAACAGAGAAG-3'. The resulting PCR product was recombined by Gateway BP reaction into pDONR201.

4.2.6 Preparation of Expression Clones

All constructs necessary for expression of the target gene in bacteria or in planta were created using recombination between 'entry' clones and 'destination' vectors via Gateway® Technology (Invitrogen)

4.2.7 RNA Isolation

100 mg of plant tissue was snap-frozen in an eppendorf tube and ground to a fine powder using a homogenizer. 600 μl of TRIzol reagent was added and the tissue was allowed to thaw in the liquid. After homogenization, a further 400 μl of TRIzol reagent was added. The samples were incubated at room temperature for 5 minutes. 0.2 μl of chloroform per 1 ml of TRIzol reagent was added and the samples were vortexed for 10 seconds. After allowing the samples to rest for 3 minutes at room temperature, centrifugation was done at no more than 12000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube. The RNA was precipitated by adding 250 μl of 3M NaAc and 250 μl of isopropanol. The samples were allowed to stand for 10 minutes at room temperature and then centrifuged again at 4°C for 10 minutes at no more than 12000g. The supernatant was removed and the pellet washed once with in 1 ml of 75% ethanol (briefly vortexing). The RNA was pelleted by centrifuging at no more than 7500g for 5 minutes at 4°C. The ethanol solution was removed from the RNA pellet and the pellet was not allowed to dry completely as this impeded the dissolution of the pellet in water. The pellet was then dissolved in 20 μl of RNAse-free water and digested with DNAse to remove any DNA left in the samples. All solutions were made with RNAse-free water.

4.2.8 Preparation of Complimentary DNA

All reverse transcription experiments were done with the 'RevertAid™ H Minus First Strand cDNA Synthesis Kit' supplied by Fermentas. The manufacturer's instructions were followed accordingly. RT PCR was carried out for 33 cycles.

4.2.9 Analysis of Gene Expression

Total RNA was isolated from the following tissues of wild type A.thaliana plants: seedlings, roots, rosette leafs, cauline leafs, stem and inflorescence. RNA was treated with DnaseI (MBI). First strand cDNA was then synthesized with the RevertAid H-Minus First Strand cDNA Synthesis Kit (MBI fermentas).
Thirty-three cycles of PCR were performed with the indicated primers:
ATSRA1 primers: KLK__fw: 5'-GCGGTTCATGCAAACAGCTCC-3' and
KLK_rev: 5'-TCAGCCAACGGTGCCTTT-3'.
AtSCAR2 primers: Wave4–fw 5'-ACCAGCCACCATGGAGTCGGAAAC-3' and
Wave4-rev: 5'-CCTTTCTTCCATCATTGCCTCCCT-3'
Different dilutions of cDNA preparations were amplified with actin specific primers to yield equal amounts of cDNA for each tissue. The normalized dilutions were then used for amplification with ATSRA1 or AtSCAR2 -specific primers. Actin was used as a control:

4.2.10 Preparation of Genomic DNA

100 mg of plant tissue was homogenized in 400 µl of 2X CTAB buffer. The samples were incubated at 65°C for 30-60 minutes. 1 volume of phenol/chloroform/isoamyl alcohol (48:48:4) was added and the samples were vortexed throroughly. The samples were centrifuged at maximum speed for 2 minutes. The aqueous layer was transferred to a fresh tube and the extraction

was repeated using chloroform/isoamyl alcohol (94:4). A 0.7 volume of isopropanol was added to the samples and they were mixed by inversion. They were incubated at room temperature for 10 minutes. The DNA was pelleted by maximum-speed centrifugation at room temperature. The supernatant was discarded and the DNA pellet washed with cold 70% ethanol. The pellet was secured by a brief centrifugation step at maximum speed. The supernatant was discarded and the DNA pellet was dried and dissolved in 50 µl of Tris buffer, pH8.

4.2.11 Preparation of Plasmid DNA

Plasmid DNA from A. tumefaciens was isolated using the Qiaquick plasmid miniprep kit supplied by Qiagen; the Plasmid Miniprep Kit I supplied by PEQLAB (Erlangen) was used for isolation of plasmid DNA from E. coli. The manufacturer's protocol was followed in both cases.

4.2.12 DNA manipulation

DNA manipulation and cloning were carried out according to Sambrook and Russel (2001). All polymerase-chain reaction (PCR)-amplified fragments were sequenced prior to further investigation. Sequencing was carried out on an ABI 310 Prism automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing reactions were performed using Big-Dye kit 1.1 or Big-Dye kit 3.1 (Perkin Elmer Applied Biosystems, Foster City, CA).

4.2.13 Plant Growth Conditions

Seeds were sown on moist freshly prepared Arabidopsis culture soil and then allowed to stratify at 4°C in the dark for three days, while covered to retain moisture. Plants were grown at 22°C under constant light for 16 hours followed by 8 hours of darkness. The lid was removed when the seeds had germinated.
Rapid hypocotyl growth was induced by growing the seedlings for 8 days under low-light conditions. Plates bearing the seeds were wrapped with aluminum foil and a 2 mm aperture created on the top surface of the foil made it possible for limited light to enter.

4.2.14 Crossings

Suitable flowers were identified on the female parent. All other flowers and siliques were removed from the stem. Sepals, anthers, and petals were removed from the flowers (to be crossed) using jewellers' forceps. The carpels were left intact. The plants were allowed to recover for a day. A flower from the male parent was then pressed open at the base using forceps and its pollen was dusted onto the stigma of the female parent. Pollen was considered suitable for crossing if it was bright yellow and sticky. The stigma was considered ready for crossing if its papillae appeared erect forming a crown on the surface. The adhesion of the pollen on the stigma was checked after crossing. After a successful crossing, the siliques were seen to elongate after three days.

4.2.15 Preparation of competent *Agrobacterium* cells

200 ml of YEB media was inoculated with 1 ml form an overnight culture of the chosen strain of Agrobacterium. This was cultured at 28°C until an $O.D_{600}$ of 0.5-0.8 was reached. The cells were pelleted by centrifugation at 5000 rpm for 10

minutes at room temperature. The pellet was washed with streile TE buffer. The cells were resuspended in 1/10 of the original volume of YEB media. The cells were aliquoted and snap-frozen in liquid nitrogen and stored at –80°C.

4.2.16 Transformation of *Agrobacterium*

1-5 µg of DNA from a standard miniprep was added to a thawed Agrobacterium aliquot and snap-frozen in liquid nitrogen for 5 minutes. The cells were then incubated at 37°C for 2 minutes. 1 ml of YEB media was then added and the cells were incubated at 28°C for 2 hours on a bench top shaker at 800rpm. The transformed cells (10-20 µl) were plated on the appropriate antibiotics and incubated for 2 days at 28°C. Individual colonies were checked for the construct of interest by PCR.

4.2.17 Stable Transformation of *Arabidopsis*

Plants were transformed according to the “floral dip” method (Clough and Bent 1998). To increase plants fitness they were grown at 18°C until the first flowers appeared on inflorescence. Four days prior to plant transformation a 5 ml pre-culture in YEB medium of the transgenic A. tumefaciens strain was incubated for two days at 29°C. 1ml of this pre-culture was used to inoculate the final 200 ml culture, which was incubated for another two days at 29°C. The cells were pelleted at 5800 rpm for 12 minutes. The pellet was resuspended in 5% (w/v) sucrose solution containing 0.05% (v/v) Silwett L-77. Inflorescence were dipped for approximately 20 seconds and afterwards covered with a lid. The lid was removed after two days and after that plants were grown under standard growth conditions.

4.2.18 Transient transformation of *N. benthamiana*

Nicotinia benthamiana was used with Agrobacterium tumefaciens strain GV3101 (with pMP90 Ti plasmid). Plants were 5-6 weeks old andgrown at 21°C, 14 hrs light, 10 hrs dark. Fresh infiltration medium was made on the day of infiltration.

Infiltration medium:

MES and sodium solutions were stored in the fridge.

Stock solutions

500 mM MES (0.976 g in 10 ml).

20 mM $Na_3PO4.12H_2O$ (trisodium orthophosphate) (0.076 g in 10 ml.

200 mM Acetosyringone (3',5'-dimethoxy-4' hydroxy acetophenone) (0.0392 g in 1 ml made with DMSO.

Infiltration medium (for 50 ml)

250 mg D-glucose

5 ml MES

5 ml $Na_3PO_4.12H_2O$

25 μl acetosyringone if using 200 mM, 5 μl if using 1M.

Cell preparation

5 ml aliquots from an overnight *Agrobacterium* culture was spun down and resuspended in 1 ml of infiltration medium at room temperature. This was spun down again and the supernatant was removed. The cells were resuspended in 1 ml of infiltration medium. The cells were spun for 5 minutes at 4000 rpm and the supernatant was removed. 1 ml of infiltration medium was added at room temperature. And this was repeated again.

Leaf infiltration

Cells were taken up into a 1 ml syringe with no needle. The syringe was placed firmly but gently on the underside of a young leaf. The liquid was gently pushed into the leaf tissue. It was then possible to see the extent of infiltration clearly as the liquid entered the mesophyll air spaces. This procedure was performed in a

large pastic bag to stop infiltration solution from squirting on to the surrounding area. The infiltrated leaves were marked with a thread and the plants were incubated for 2-4 days. Leaf squares were then cut out and analysed by microscopy.

4.2.19 Seed Sterilisation

Seeds were sterilised by rinsing first with water and soaking in 70% ethanol for 10 minutes. The seeds were then rinsed thoroughly with water before plating on apropriately supplimented MS media.

4.2.20 Protein Isolation and antibody production

All protocols for expression of His-tagged constructs were carried out according to „Molecular Cloning: A Laboratory Manual (third edition) by J. Sambrook. Injection of mice and collection of antisera was carried out at Durham University

4.2.21 Yeast Techniques

The yeast strain AH109 (Halladay and Craig, 1996), was maintained in standard yeast full media or selective drop-out media (Clontech) using standard conditions.
Transformation of plasmid or library DNA into yeast was done according to the LiAc transformation method (Gietz et al., 1995). Co-transformed cells were plated onto synthetic dropout medium lacking leucine and tryptophan and onto synthetic dropout medium lacking leucine, tryptophan and histidine supplemented with 3 mM 3-aminotriazole (3-AT) (Sigma-Aldrich, Munich, Germany) to investigate interaction of the hybrid proteins. Recombinant hybrid proteins were tested for self-activation and non-specific protein-binding properties.

4.2.22 Particle Bombardment

Particle bombardment was carried out using a particle accelerator driven by helium (PDS-1000/ He; BIO-RAD, Hercules, CA, USA). Microcarriers of 1.0 μm diameter were prepared for DNA coating by washing several times with 70% ethanol and resuspending in a 50% v/v glycerol solution. The microcarriers were then coated with DNA of expression constructs by vortexing with 2.5M $CaCl_2$ and 0.1M spermidine. The coated microcarriers were washed again with 70% ethanol and resuspended in absolute ethanol for application onto macrocarriers. Particle delivery was carried out at a pressure of 27 inches of Mercury. Helium at a pressure of 900 psi was used for microcarrier acceleration. The bombarded cells were then placed at 22°C in an illuminated growth chamber and analyzed after 16 hours.

4.2.23 Microscopy and image processing

Epidermal surfaces were analyzed with agarose impressions (Mathur and Koncz, 1997). Light microscopy was done with a LEICA DMRE microscope equipped with a high-resolution KYF70-3CCD JVC camera and a frame grabbing DISKUS software (DISKUS, Königswinter). A Spectrophotometric Confocal Laser Scanning Microscope (Leica TCS-SP2 AOBS) was used to visualize fluorescence from fusion proteins under investigation. Images were processed using the Adobe Photoshop 6.0 and ImageJ software.

4.2.24 Bioinformatic Tools

PCR-Primers and constructs were designed using the Vector-NTI-suite 9 software (InforMax, Paisley PA4 9RF United Kingdom). DNA and protein sequence homology searches were performed with the BLAST search algorithm (Altschul et al. 1990) from NCBI. Amino-acid sequences were aligned with

ClustalW to obtain multiple sequence alignments. Prediction programs were used such as PROSITE, NCBI conserved Domain search and PSORT (ExPASy Proteomics Server).

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6 Zusammenfassung

Das Aktin Zytoskelett ist wichtig für die Zellmorphogenese in Pflanzen. Verschiedene Funktionen erfordern unterschiedliche Arten von Aktin Filamenten. Die Antwort dynamischer Aktin Filamente auf entwicklungsbiologische und umweltbedingte Reize ist unverzichtbar für das Wachstum der Pflanze. Im Gegensatz dazu vermitteln weniger dynamische Aktinfilamente die Stabilisierung der Zellarchitektur und verankern das Endomembransystem. Der Transport von Organellen ist ebenfalls abhängig von Aktinfilamenten und vermittelt den gerichteten Transport zu spezifischen Bereichen der Zelle.

Der ARP2/3 Komplex dient als Polymerisationsstart von Aktinfilamenten. Sowohl in Pflanzen als auch in Tieren besteht dieser Komplex aus sieben Untereinheiten. Die Arabidopsis distorted Gruppe umfasst sieben Mutanten, wovon vier Mutationen in Untereinheiten des ARP2/3 Komplexes tragen. Mitglieder dieser Komplementationsgruppe sind in der Architektur des Aktin Zytoskelett betroffen was zu Morphogenesedefekten in verschiedenen Geweben führt. Blatthaare auch besichend als Trichome von Arabidopsis dienen in dieser Studie als Modellsystem für die Rolle von Aktin in der Zellmorphogenese.

In dieser Arbeit wurde die Distortet Mutante klunker (klk) auf genetischer, molekularer und zellbiologischer Ebene untersucht. Das Gen welches in klk betroffen ist ist AtSRA1, ein Homolog von SRA1 welches als Regulator des ARP2/3 Komplexes in Drosophila bekannt ist. Der Phenotyp von klk, obwohl schwächer, ähnelt dem von ARP2/3 Mutanten. Verglichen mit dem Wildtyp haben klk Pflanzen verzerrte Trichome und Blattepidermiszellen welche deutlich weniger verzahnte Umrisse haben. Zusätzlich ist bei Dunkelwachstum die Hypokotyllnge und der Gewebezusammenhalt der Epidermis reduziert. Von SRA1 aus Tieren und dem humanen Homolog PIR121 ist bekannt, dass sie als Teil des regulatorischen SCAR/WAVE Komplexes den ARP2/3 Komplex aktivieren.

Im Weiteren wurde ein Homolog von WAVE1 identifiziert und AtSCAR2 genannt. Die Charakterisierung der atscar2 Mutante zeigte einen schwachen distorted Phenotyp in den analysierten Geweben. Um mehr Informationen über die Lokalisierung von AtSCAR2 in pflanzlichen Zellen herauszufinden, wurde ein Antikörper aus Mäusen isoliert. Western Analyse zeigte, dass dieser Antikörper spezifische für die AtSCAR2 Isoform ist. Die Analyse von Doppelmutanten mit weiteren vermuteten AtSCAR Genen ergab Redundanz zwischen AtSCAR2 und AtSCAR4. Dies konnte durch einen Trichomphenotyp belegt werden, welcher ähnlich ausgeprägt ist wie der in der starken distorted Mutante dis1.

Die Ergebnisse dieser Studie unterstützen die Hypothese dass die ARP2/3 Maschinerie im Hinblick auf den regulatorischen SCAR/WAVE Komplex zwischen Pflanzen und Tieren konserviert ist.

7 Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die von mir vorgelegte Dissertation ist von Prof. Dr. Martin Hülskamp betreut worden.

Moola Mutondo

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