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The role of *Arabidopsis SCAR* genes in ARP2-ARP3-dependent cell morphogenesis

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The actin-nucleating ARP2-ARP3 complex controls cell shape in plants in many different cell types. Its activity is controlled by a multimeric complex containing BRK1 (also known as HSPC300), NAP1, SRA1, ABI and SCAR/WAVE. In this study, we focus on the function of the five putative SCAR homologues in *Arabidopsis* and we provide biochemical evidence that AtSCAR2 can activate the ARP2-ARP3 complex in vitro. Among the single mutants, mutations in only *AtSCAR2* result in a subtle or weak phenotype similar to *ARP2*, *ARP3* and other 'distorted' mutants. Double-mutant analysis revealed a redundancy with *AtSCAR4*. Systematic application of the yeast two-hybrid system and Bimolecular Fluorescence Complementation (BiFC) revealed a complex protein-interaction network between the ARP2-ARP3 complex and its genetically defined regulators. In addition to protein interactions known in other systems, we identified several new interactions, suggesting that SPIKE1 may be an integral component of the SCAR/WAVE complex and that SCAR proteins in plants might act as direct effectors of ROP GTPases.

KEY WORDS: ARP2-ARP3, Actin, SCAR/WAVE, Trichomes, *Arabidopsis*

INTRODUCTION

In plants the morphogenesis of both diffuse as well as tip-growing cells depends to a large extent on the actin cytoskeleton (Mathur and Hulskamp, 2002; Smith, 2003). Recently it was shown that one of the key regulators of actin organisation, the ARP2-ARP3 complex, is required for higher plant development (El-Assal et al., 2004b; Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; Saedler et al., 2004a). In animals and yeast this complex consists of seven subunits, two subunits that are related to actin (ARP2, ARP3) and five additional components (ARPC1-C5) (Machesky and Gould, 1999). The activated ARP2-ARP3 complex binds to existing actin filaments and initiates the formation of new filaments (Amann and Pollard, 2001; Blanchoin et al., 2000). This in turn creates a dynamic branched network of filamentous actin that is thought to drive the formation of local membrane protrusions. Consistent with this, the ARP2-ARP3 complex and its regulators are localised to mammalian lamellipodia (Machesky, 1997) and other actin-driven cell extensions. Recent work has revealed in some detail the role of the ARP2-ARP3 complex in early endocytotic events in yeast, where cortical actin patches generated by ARP2-ARP3 complex activity guide endocytotic internalisation (Kaksonen et al., 2003). The ARP2-ARP3 complex is required for related processes in animals and protists (Insall et al., 2001; Merrifield et al., 2004), and is also utilised in some instances for organelle motility (Chang et al., 2003).

In plants, mutant phenotypes have been described for mutations in *ARP2*, *ARP3*, *ARPC2* and *ARPC5*. Consistent with a function of the ARP2-ARP3 complex in generating new branched actin networks, all four mutants display F-actin organisational phenotypes (Mathur et al., 1999; Schwab et al., 2003; Szymanski et al., 1999). The four mutants belong to a class of eight trichome mutants that has been collectively called the 'distorted' group due to their uncoordinated trichome cell expansion phenotype (Hulskamp et al., 1994). A more detailed phenotypic analysis of the *arp2* mutants revealed defects in additional cell types. Elongating hypocotyl cells tear out of the epidermal cell layer and may bend outwards when rapid cell expansion is triggered by dark growth conditions (El-Assal et al., 2004b; Le et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; Saedler et al., 2004a). Normally, pavement cells form many lobes that tightly interlock to form a continuous leaf epidermal surface. *ARP2-ARP3* complex mutations and mutations of ARP2-ARP3 complex regulators affect the development of leaf epidermal lobes by reducing the ability of lobes to intercalate and adhere (Basu et al., 2004; El-Assal et al., 2004a; Frank et al., 2003; Le et al., 2003; Li et al., 2004; Mathur et al., 2003a; Mathur et al., 2003b; Saedler et al., 2004a; Zimmermann et al., 2004). Tip-growing root-hair cells show reduced or wavy growth in *arp2* and *arpc5* mutants when root hairs are challenged to elongate rapidly (Li et al., 2003; Mathur et al., 2003b).

In the absence of ARP2-ARP3 complex activity, plants grow fairly normally and complete their life cycle, indicating that the ARP2-ARP3 complex has special regulatory functions in some cell types rather than an essential role in all actin-related processes. This raises the question of how the activity of the ARP2-ARP3 complex is regulated. Recent reports indicate that a regulatory pathway similar to that known in animals is also operating in plants. In animals and *Dictyostelium*, the SCAR/WAVE (suppressor of cAMP receptor from *Dictyostelium*/Wiskott-Aldrich syndrome protein-family verprolin-homologous protein) complex regulates ARP2-ARP3 complex activity (Machesky and Insall, 1998; Machesky et al., 1999). The small GTPase RAC1 binds to the pentameric SRA1-NAP1-ABI-SCAR-HSPC300 complex and either triggers the dissociation of the SRA1-NAP1-ABI complex from the SCAR-HSPC300 subcomplex (Eden et al., 2002) or activates the complete complex (Innocenti et al., 2004). The SCAR subunit in turn binds and activates the ARP2-ARP3 complex.

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Two of the eight *Arabidopsis* 'distorted' genes encode SRA1 and NAP1. The corresponding mutants show an aberrant actin phenotype and affect the same cell types as the *arp2-arp3* complex mutants except for the root hairs, indicating that they regulate most aspects of the ARP2-ARP3 complex function (Basu et al., 2004; Brembu et al., 2004; Deeks et al., 2004; El-Assal et al., 2004a; Saedler et al., 2004b; Zimmermann et al., 2004). Recently it was shown that HSPC300 is conserved in plants (as BRICK1, BRK1), and the analysis of the corresponding maize and *Arabidopsis* mutants also revealed a distorted phenotype and an involvement in the regulation of actin (Djakovic et al., 2006; Frank and Smith, 2002; Le et al., 2006). Least is known about the direct activators of the *Arabidopsis* ARP2-ARP3 complex, the AtSCAR proteins. Five putative SCAR homologues are present in *Arabidopsis* (Brembu et al., 2004; Frank et al., 2004). They all share a SCAR homology domain (SHD) and in addition, four of them share a VCA (verprolin homology-central-acidic) domain. Frank and co-workers showed that ZmSCAR1, AtSCAR3 and AtSCAR4 can activate the ARP2-ARP3 complex in vitro and that AtSCAR1 and AtSCAR3 bind to the *Arabidopsis* HSPC300 homologue. Thus, the biochemical evidence indicates that SCAR homologues act in the ARP2-ARP3 activation pathway. Recently two publications reported that mutations in the *AtSCAR2* gene result in a similar range of phenotypes to *arp2-arp3* complex mutants (Basu et al., 2005; Zhang et al., 2005a). Also the regulation of the multimeric SRA1-NAP1-ABI-SCAR-HSPC300 complex might be conserved as *Arabidopsis* PIR121 interacts with ROP2 (Basu et al., 2004). ROP2 is one of 11 small Rac-like GTPases found in plants. ROPs form a unique class and have therefore been termed 'Rho proteins of Plants' (Vernoud et al., 2003; Yang, 2002; Zheng and Yang, 2000).

In this study we analyse the role of the five *Arabidopsis* SCAR homologues in ARP2-ARP3 complex-dependent actin regulation. Only *atscar2* mutants but none of the other four *atscar* mutants show 'distorted'-related phenotypes. Our double-mutant analysis revealed that *AtSCAR4* and *AtSCAR2* act redundantly. The detailed analysis of protein–protein interactions by two-hybrid analysis revealed a complex interaction network including not only interactions already known from other species but also new ones. Supported by Bimolecular Fluorescence Complementation (BiFC) analyses of protein interactions in vivo (Walter et al., 2004), we provide evidence that SPIKE1 interacts with several members of the *Arabidopsis* SCAR and ABI protein families, and that AtSCAR2 interacts directly and specifically with the activated form of ROP7 at the plasma membrane.

MATERIALS AND METHODS

Plant material and mutant characterisation

All mutant lines were obtained from the Nottingham *Arabidopsis* Stock Centre and GABI-Kat. Seeds were sterilised with 100% ethanol and treatment with 4% $NaHCl_2$ solution with 0.01% Triton X100 followed by several washings with sterile water. Plants were grown on MS media with 1% glucose under continuous light at 22°C or in soil under long day conditions at 20°C.

To confirm the T-DNA insertions in the *AtSCAR* genes, PCRs on genomic DNA with the following primers were carried out:

AtSCAR1: GK_447E03: 447E03_fw (5′-CCACTGTTGCTCAGACA-TTGAC-3′), 447E03_rev (5′-GTGCTGCTACATCAGAGCTAC-3′) and the T-DNA specific primer 8409.

AtSCAR2: SALK_039449: LP_39449 (5′-TATGGCTGGTCTCGTT-GGCAT-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 (5′-GGTGTCCCTCCATTCCTCCAC-3′); SALK_36419: LP_36419 (5′-CCACCATGGAGTCGGAAACAG-3′), RP_36419 (5′-GCAACGACC-ATCACGTTCTT-3′); SALK_124023: LP_124023 (5′-CGGGAGTA-GAGAAATTGGCGG-3′), RP_124023 (5′-GCTCTGATGGTGGAGAC-CCAA-3′) and the T-DNA specific primer LBb1.

AtSCAR3: SALK_087926: LP_087926 (5′-GCTGGGAAAAGGAT-TAGGAGAA-3′), RP_087926 (5′-AAATTTTTGTTGCAAGTTAATT-GAT-3′); SALK_630493: LP_630493 (5′-GCAAATGTCTTCCTGGA-GTGA-3′), RP_630493 (5′-TGTCTTGACCCAAGGTGTCTG-3′) and the T-DNA specific primer LBb1.

AtSCAR4: SALK_616410: LP_616410 (5′-CATGTGATGTCCGTG-AATCCT-3′), RP_616410 (5′-CTATGGAATCAATCCCTTGGC-3′); GK_126B09: 126B09_fw (5′-GGCCAGTCTGGTTATCGTGATCG-3′), 126B09_rev (5′-CCAGGATTTGGTCAGCCGCAG-3′) and the T-DNA specific primers LBb1 (SALK) and 8409 (GK).

AtSCAR-like: SALK_555413: LP_555413 (5′-GAGCGCTGACTCT-ATCCGTCT-3′), RP_555413 (5′-CTCTGTTTCATTTCTGCTGCG-3′); SALK_504474: LP_504474 (5′-GTGCTTCACTTTGTTCTTGCG-3′), RP_504474 (5′-GTAGGCTCTGAGTGGCATCCT-3′); GK_411H03: 411H03_fw (5′-CATCAGATTCCCACAGCTCAG-3′), 411H03_rev (5′-GAAGGTGAAGTACATAATGAGCC-3′) and the T-DNA specific primer LBb1 (SALK) and 8409 (GK). The PCR products were sequenced and the exact positions of the inserts determined.

Constructs and recombinant DNA

Full-length cDNAs encoding the proteins investigated in this study were amplified by PCR using primers containing attB1 and attB2 sites for GATEWAY recombination as described by Invitrogen. PCR products were recombined into pDONR201 (Invitrogen), and the resulting clones were further transferred to yeast two-hybrid vectors (pAS-attR and pACT-attR; J.F.U., unpublished), and to BiFC vectors (pBatTL; J.F.U., unpublished) by GATEWAY recombination (Invitrogen). Details of the numerous constructs and primers will be provided on request. The cDNA encoding the N-Terminal 346 amino acids of AtSCAR2, which contains the SCAR homology domain, was amplified with the primers WAVE4_fw (see above) and WAVE4_F1_rev (5′-attB2-tcatCCA CGGACCTCATTGTATGTAG-3′). For protein expression, the C-terminal fragment of AtSCAR2 encoding the last 153 amino acids containing the VCA domain was amplified using the primers 384VCAGATF (5′-attB1 TCCCTGATGCTTCAAATGCA-GAAACTG-3′) and 384VCAGATR (5′-attB2-GATTTCAAGAATCA-CTCCAACTATCTG-3′). A C-terminal fragment encoding the last 236 amino acids of AtSCAR2 containing the VCA domain was amplified for use in yeast two-hybrid assays using the primers WAVE4_rev (5′-attB2-tTCAAGAATCACTCCAACTATCTG-3′) and WAVE_F2_fw (5′-attB1-CCTTATCAGGAGTGCATAGAGG-3′).

The amplified cDNAs were inserted into pDONR201 or pDONR207 (Invitrogen). For localisation and rescue experiments, SCAR2 cDNA (Deeks et al., 2004) was transferred to GFP vector pMDC43 (Curtis and Grossniklaus, 2003) to form a GFP-SCAR2 fusion. Functionality of the GFP-SCAR2 fusion protein was proven by rescue of the *scar2* mutant. Rescued plants were verified by testing homozygosity of the *scar2* mutant alleles and the presence of the rescue construct.

Analysis of gene expression

Total RNA was isolated by using TRI reagent (Molecular Research Centre) from the following tissues of wild-type *Arabidopsis* plants: seedlings, roots, rosette leaves, cauline leaves, stems and inflorescence. RNA was treated with DNaseI (MBI). First strand cDNA was then synthesised with the RevertAid H-Minus First Strand cDNA Synthesis Kit (MBI).

A total of 33 cycles of PCR were performed with the indicated primers. Different dilutions of cDNA preparations were amplified with actin-specific primers to yield equal amounts of cDNA for each tissue. The normalised dilutions were then used for amplification with AtSCAR/WAVE-specific primers. The following primers were used for expression analysis:

wave1-fw (5′-TTCGGAAATAAGTAGTGGCACTCATAG-3′); wave1-rev (5′-CCTCTTGCTTTCTCGGATACCTTCTG-3′); wave2-fw (5′-TGGTCGGGAGATTGTGGGAG-3′); wave2-rev (5′-TCCTTTCTGCGG-AAACCACTAG-3′); wave3-fw (5′-CCACAGGACGCATATGAGGG-3′) wave3-rev (5′-GGCCAATCCGTCTTCCAGACA-3′); wave4-fw (5′-ACCAGCCACCATGGAGTCGGAAAC-3′) wave4-rev (5′-CCTTTCTT-

CCATCATTGCCTCCCT-3′) wave5-fw (5′-CCTATATAGCCCCTCAG-AAGACCTACTTGTGC-3′) wave5-rev (5′-GCATCTTTCAGAGACGG-GCATGAGAT-3′).

cDNA isolated from each of the *atscar* and *atscar-like* mutants was treated in the same manner. The normalisation of cDNA was done by performing PCR with elongation factor 1-specific primers. The equally concentrated dilutions of cDNA were used to carry out the expression analysis of *AtSCAR* and *AtSCAR-LIKE* in each of the mutant alleles. The following primers were used to amplify a fragment 3′ of the T-DNA insertion (42 cycles):

AtSCAR1: fw (5′-CAGAAGCTGGAGACTTCTTGC-3′), rev (5′-GAGAGACTCGAGATCATGTGTCGCTATCGCTCCATG-3′).

AtSCAR2: fw (5′-ACCAGTGAGGCTGACAATTATGTGGACG-3′), rev (5′-AAACTTCCATCCCCACCCCAGCTG-3′).

AtSCAR3: fw (5′-GCATGGTGACTTCTGCTCC-3′), rev (5′-GAGAG-ACTCGAGATTACGTATCACTCCATGTATCGC-3′).

AtSCAR4: fw (5′-GAGCCTCAGGTTGATCACC-3′), rev (5′-GAG-AGAGCGGCCGCACTCACTCGCTCCAGCTATCTG-3′).

AtSCAR-LIKE: fw (5′-CCTATATAGCCCCTCAGAAGACCTACTTG-TGC-3′), rev (5′-GCATCTTTCAGAGACGGGCATGAGAT-3′).

Protein expression

The AtSCAR2 VCA domain was transferred using LR enzyme mix into GST fusion vector pGEX-4T-1 (Amersham) containing the gateway cassette (Invitrogen). This plasmid was transformed into *Escherichia coli* BL21 DE3 pLysS Rosetta 2 expression cells (Novagen). Cells were induced with IPTG at a final concentration of 1 mM at 37°C for 3 hours. GST-tagged protein was recovered using glutathione sepharose 4B (Amersham) and dialysed against G-buffer. After buffer exchange the protein was snap-frozen in liquid nitrogen and stored at –80°C.

Actin polymerisation assays

Polymerisation assays were performed using 4 mM actin in G-buffer (2 mM Tris-HCl pH 8, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.02 % NaN_3) with 10 % of monomers pyrenelated. Bovine ARP2-ARP3 was supplied by Cytoskeleton (Denver, CO, USA), and handled according to the manufacturer's instructions. Polymerisation was induced by the addition of 10× KME (500 mM KCl, 10 mM $MgSO_4$, 10 mM EGTA, 100 mM Imidazole pH 6.5). Pyrene fluorescence was monitored using a PTI spectrofluorimeter equipped with a rotating multi-sample turret. For a general description of this method, see Mullins and Machesky (Mullins and Machesky, 2000).

Particle bombardment

Particle bombardment was carried out using a helium Helios gene gun (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 1 mg of plasmid per 0.5 mg of gold (equivalent to one cartridge) as described by Kemp et al. (Kemp et al., 2001). Helium at a pressure of 150 psi was used for microcarrier acceleration. The bombarded cells were then placed at 22°C in an illuminated growth chamber and analysed after 24 hours.

Cell culture, protoplasting and transfection

Arabidopsis cell suspension culture (Columbia ecotype; grown in MS medium supplemented with 0.5 mg/L NAA and 0.1 mg/L KIN) was maintained as described previously (Mathur and Koncz, 1998a). Protoplast isolation and polyethylene glycol-mediated transfection was performed according to Mathur and Koncz (Mathur and Koncz, 1998b). The transfected cells were incubated at 23°C for 16 hours in the dark before microscopic observation.

Yeast transformation and yeast two-hybrid assays

Yeast strains AH109 (Halladay and Craig, 1996) and Y187 (Harper et al., 1993), were maintained in standard yeast full media or selective dropout media (Clontech) using standard conditions. Transformation of plasmids into yeast was done according to the LiAc transformation method (Gietz et al., 1995). Interaction of hybrid proteins was tested by crossing singly transformed cells or by co-transformation of yeast cells with subsequent plating onto synthetic dropout medium lacking leucine and tryptophan and onto synthetic dropout medium lacking leucine, tryptophan and histidine supplemented with 3-20 mM 3-aminotriazole (3-AT) (Sigma-Aldrich, Munich, Germany). Recombinant hybrid proteins were tested for self-activation and nonspecific protein-binding properties. The adequate 3-AT concentrations suppressing unspecific reporter activation were determined individually for every bait construct.

RESULTS

Molecular characterisation of *Arabidopsis scar/wave* mutants

In order to assess the function of the *Arabidopsis* SCAR/WAVE homologues, we analysed several insertion mutants in the four *AtSCAR* genes and in *AtSCAR-LIKE*. For each line we isolated a homozygous mutant line and determined the exact insertion site. The GABI-KAT line 447E03 (*scar1-1*) has an insertion in the fourth exon of *AtSCAR1*, 2369 bp downstream of the ATG. The SALK line 039449 (*scar2-1*) has an insertion after 498 bp in the second intron of *AtSCAR2*, the SALK line 036419 (*scar2-2*) in the sixth exon after 2116 bp, and the SALK line 124023 (*scar2-3*) in the sixth exon after 3503 bp. The SALK line 630493 (*scar3-1*) has an insertion in the third exon of *AtSCAR3*, 1221 bp downstream of the ATG. In the SALK line 616410 (*scar4-1*) the insertion was found in the third intron of *AtSCAR4* after 694 bp, and in the GABI-KAT line 126B09 (*scar4-2*) in the seventh exon after 2235 bp. The SALK line 555413 (*scar-like1*) has an insertion in the first intron of *AtSCAR-LIKE* 480 bp downstream of the ATG, the SALK line 504474 (*scar-like-2*) has an insertion in exon four after 1473 bp, and the GABI-KAT line 411H03 (*scar-like-3*) in exon seven after 6591 bp (Fig. 1A). RT-PCRs were carried out by amplifying a fragment located 3′ of all the insertions to determine the expression levels in the mutants. We detected no expression in at least one allele of each of the four *AtSCAR* genes and *AtSCAR-LIKE*, indicating that these represent real null-mutants (Fig. 1A).

In a previous study it was shown that *AtSCAR2* is normally only expressed in cotyledons, siliques and rosette leaves but not in flower buds or roots (Frank et al., 2004). In our hands, *AtSCAR2* expression was found in all tissues analysed including the root, the cotyledons, the stem, the rosette leaves, the cauline leaves and the inflorescence (Fig. 1B). In addition the expression profiles of the other *Arabidopsis* SCAR homologues were different to that previously published (Fig. 1B). In particular, we found that all *Arabidopsis* SCAR homologues are expressed in roots. In addition we showed that *AtSCAR1* transcript is specifically absent in seedlings and that *AtSCAR-LIKE* is absent in the stem.

Phenotypic analysis of *atscar* and *atscar-like* mutants

In order to test whether the four *AtSCAR* genes and *AtSCAR-LIKE* gene are involved in ARP2-ARP3 complex-dependent cell morphogenesis, we analysed cell types known to be affected in *arp2-arp3* complex mutants including trichomes, epidermal pavement cells, root hairs and hypocotyls cells.

All three *atscar2* alleles analysed in this study exhibited a weak distorted phenotype reminiscent of *arp2-arp3* complex mutant trichomes. Branches of *atscar2* trichomes are often twisted and variable in length (Fig. 2A-F). The effect on branch formation was studied using the criteria developed in Zhang and co-workers (Zhang et al., 2005b). The branch positioning defect was quantified by measuring the distance between the first and the second branch point of 100 trichomes of ten leaves in each mutant line. Also, the relative arrangement of *atscar2* branches was affected (Fig. 2D,E,F) in a

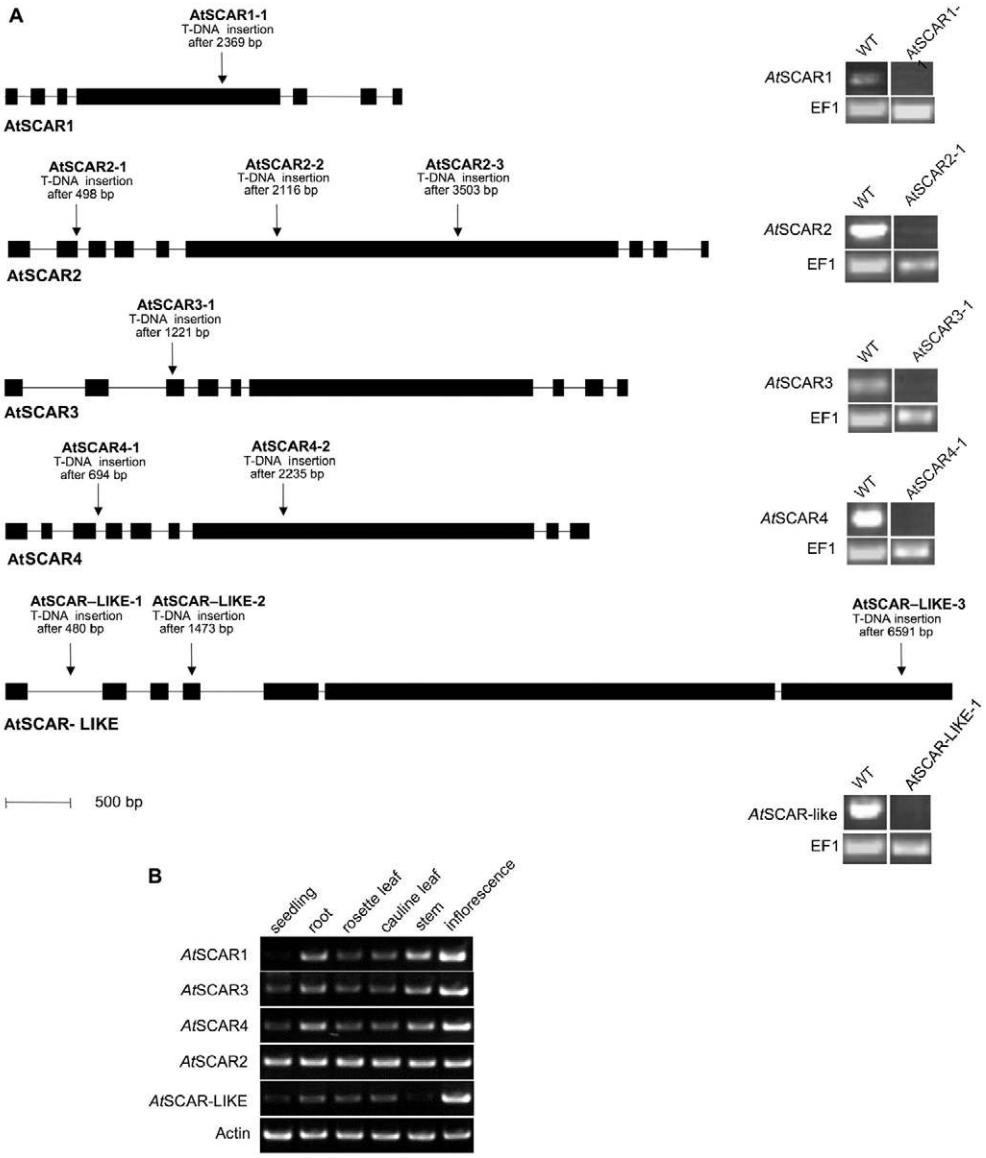

Fig. 1. Molecular characterisation of the *AtSCAR* genes. (**A**) Gene structures of AtSCAR genes as confirmed by sequencing, and the location of mutations in their respective genes. Black boxes represent exons and black lines represent introns. The locations of the T-DNA insertions are indicated by arrows. To the right RT-PCRs using primers 3′ of the insertion site are shown to confirm that no RNA transcript is produced by the relevant alleles. The expression of elongation factor1 was used as a control. (**B**) Expression analysis of *AtSCAR1*, *AtSCAR2*, *AtSCAR3*, *AtSCAR4* and SCAR-LIKE by RT-PCR in various plant organs. The expression of actin was used as a positive control.

manner similar to *arp2-arp3* complex mutants (Basu et al., 2005). In wild-type trichomes, the second branch is found on average 11 μm above the first branching point. In the three alleles of *atscar2* analysed in more detail, SALK-039449 (*scar2-1*), SALK-036419 (*scar2-2*) and SALK-124023 (*scar2-3*), the branch point was found 34, 33 and 31 μm above the first branching point. Using the Student's *t* test we confirmed that the interbranch zone lengths of the three mutants are significantly different from wild type (P=0.0001, t=9.27). This indicates that the relative positions of branch points are affected in *atscar2* mutants. The distance between the two branch points in the mutant lines of the other three *AtSCAR* genes and *AtSCAR-LIKE* was not significantly different from wild type (Fig. 2Q).

Epidermal cell lobe phenotypes associated with *ARP2-ARP3* complex mutations and mutations of ARP2-ARP3 complex regulators suggest that ARP2-ARP3 complex activity affects the development of plant epidermal lobes by influencing either the magnitude of lobe expansion (Brembu et al., 2004; Frank et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; Saedler et al., 2004a; Saedler et al., 2004b) or cell–cell adherence following lobe intercalation (Basu et al., 2004; El-Assal et al., 2004b; Le et al., 2003). Surprisingly, the *atscar2* mutants showed well-developed epidermal lobes. In order to detect a mild lobing phenotype we quantified the lobe formation by calculating the complexity of 100 individual pavement cells on ten mature rosette leaves per mutant line using the formula: complexity= $(\text{perimeter})^2/(4\times p\times \text{area})$. Compared with wild-type plants (complexity=2.4) none of the mutant lines of the four *AtSCAR* genes and *AtSCAR-LIKE* showed a significant difference concerning the pavement cell complexity. In *arp2-arp3* complex mutants the hypocotyl epidermal cells also show morphogenetic defects when

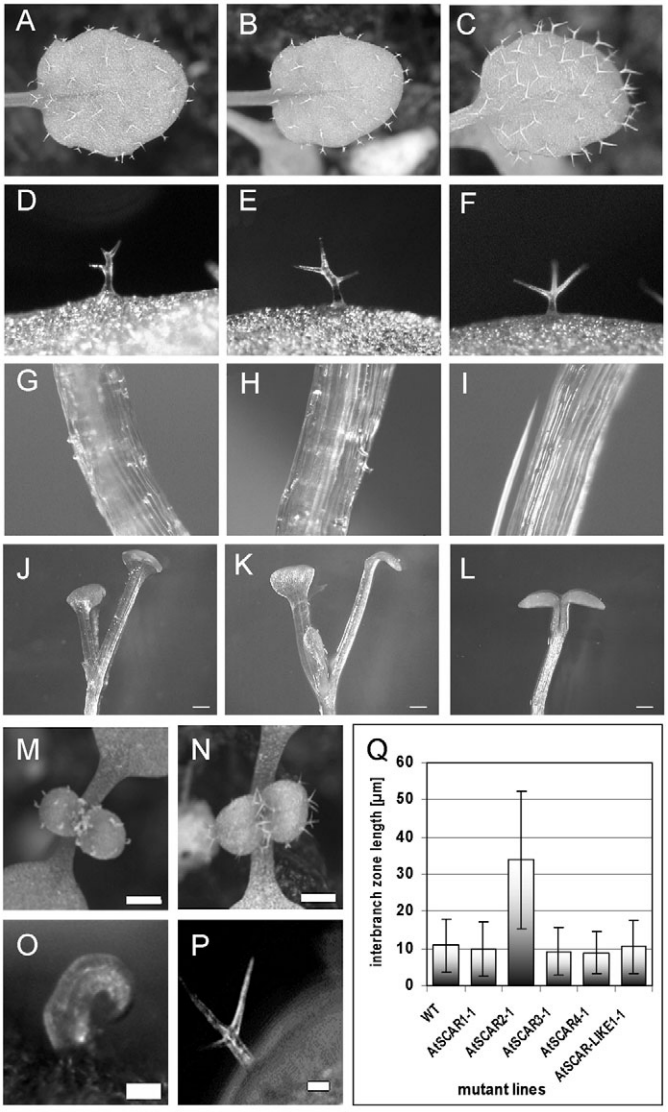

Fig. 2. Phenotypic characterisation of wild-type and *atscar* mutant plants. (**A**) Leaf overview of the mutant SALK-039449. (**B**) Leaf overview of the mutant SALK-124023. (**C**) Wild-type leaf. (**D**,**E**) Trichomes on leaves of the mutant *SALK-039449* and *SALK-124023*, respectively. The mutants have slightly distorted trichomes and the branches are not regularly arranged as can be seen by the larger distance between the two branching points. (**F**) Wild-type trichome. (**G**,**H**) Dark-grown hypocotyl of the mutants *SALK-039449* and *SALK-124023*, respectively. Some cells are disconnected from their neighbouring cells and bend outwards. (**I**) Dark grown wild-type hypocotyl. (**J**,**K**) 8-day-old dark-grown seedlings of the mutant *SALK-039449* and *SALK-124023*, respectively. The seedlings already show developed petioles, cotyledons, and the first pair of true leaves. Note the long petioles. (**L**) 8-day-old dark-grown wild-type seedlings. (**M**) Overview of rosette leaves of the *atscar2; atscar4* double mutant. (**N**) Overview of rosette leaves of the *atscar4* single mutant. (**O**) Trichome of the *atscar2; atscar4* double mutant displaying the distorted phenotype. (**P**) Trichome of the *atscar4* single mutant. (**Q**) Measurements of the distance between the lower and upper branch point of trichomes. Only *atscar2* mutants show differences to wild type. Scale bars: 1 mm in L, M and N; 0.4 mm in O; 0.2 mm in P.

grown in the dark. During etiolation, hypocotyl cell elongation is drastically increased and represents a challenging mode of growth for some cytoskeletal mutants. As shown in Fig. 2G-I, the dark-grown *atscar2* lines occasionally exhibit severe cell expansion and adherence defects such that in some instances cells tear out of the epidermal tissue layer and bend away from the surface. This phenotype was absent in the other *atscar* and *atscar-like* mutants. Root hair growth was normal in all *atscar* and *atscar-like* mutants.

The aspects of the *atscar2* phenotype described above are due to either reduced or misdirected cell growth. It was therefore surprising to find that the petioles of the cotyledons are drastically elongated in *atscar2* mutants (Fig. 2J,K,L). This phenotype was also found in a mutant affecting ARP2-ARP3 complex function, the *distorted1* mutant, and in a regulator of the ARP2-ARP3 complex, the *nap1* mutant called *gnarled* (Brembu et al., 2004; Deeks et al., 2004; El-Assal et al., 2004a; Li et al., 2004; Zimmermann et al., 2004). The analysis of the cellular phenotype revealed that the elongation of the petiole is due to increased cell elongation of petiole cells. In wild-type petioles the epidermal cells are, on average, 0.82 mm long. The average length in homozygous SALK-039449 (*scar2-1*) and SALK-124023 (*scar2-3*) lines is 1.67 mm and 2.1 mm, respectively. This twofold increase in average cell length indicates that the ARP2-ARP3 pathway counteracts cell elongation in these cells. The petioles in the other *atscar* and *atscar-like* mutants were normal.

Double-mutant analysis of the Arabidopsis SCAR genes

One explanation for the finding that *atscar2* mutants show a weak phenotype and that the other *atscar* mutants show no obvious phenotype is a redundancy of gene function between members of the *AtSCAR* family. We tested this possibility by creating various double mutants. A striking enhancement of the *atscar2* phenotype was found when *atscar2* mutants were combined with *atscar4* alleles. Both *atscar2; atscar4* double mutants and mutants homozygous for *atscar2* and heterozygous for *atscar4* showed an equally severe phenotype indistinguishable from strong ‘distorted’ mutants (Fig. 2M-P). This indicates that in an *atscar2* mutant background the reduction of *AtSCAR4* gene dosage leads to a severe phenotype reflecting a redundant action between the two genes. All other double-mutant combinations tested, including *atscar1; atscar3*, *atscar1; atscar5*, *atscar3; atscar4*, *atscar2; atscar-like* showed no obvious actin-related phenotype.

AtSCAR2 VCA activates the ARP2-ARP3 complex

The similarity between aspects of the *atscar2* phenotype to *arp2-arp3* complex mutants would suggest that AtSCAR2 activates the *Arabidopsis* ARP2-ARP3 complex. Rabbit muscle skeletal G-actin (10% pyrenelated) at a concentration of 4 mM was polymerised in the presence of bovine ARP2-ARP3 (500 nM) and either AtSCAR2 GST-VCA (160 nM) or GST only. In animals, the VCA domain brings together actin monomers and the actin-nucleating ARP2-ARP3 complex and consists of three homology domains (V, which binds to an actin monomer; C, cofilin homology domain; A, acidic region that associates with the ARP2-ARP3 complex) (Stradal et al., 2004). Fig. 3 shows that ARP2-ARP3 activity is stimulated by AtSCAR2 GST-VCA, whereas AtSCAR2 GST-VCA alone does not accelerate polymerization. Consequently AtSCAR2 has the potential to act as an activator of the ARP2-ARP3 complex in vivo.

AtSCAR2:GFP is localised to the cytoplasm

Epidermal lobe phenotypes exhibited by mutant subunits of the ARP2-ARP3 complex and by mutant *arp2-arp3* complex regulators suggest that ARP2-ARP3 complex activity affects the development of pavement cell lobes and the subsequent intercalation of lobes between neighbouring epidermal cells. Mutations of the maize

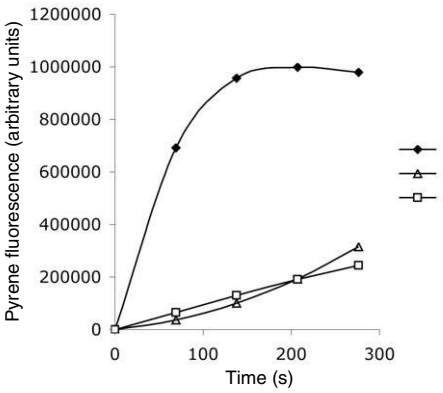

Fig. 3. In vitro activation of bovine ARP2-ARP3. Graph showing pyrene fluorescence (arbitrary units) plotted against time (seconds) of actin polymerisation assays (10% pyrenelated actin) in the presence of 500 nM bovine ARP2-ARP3 with 160 nM GST (triangles), 160 nM AtSCAR2 GST-VCA only (squares), and ARP2-ARP3 with AtSCAR2 GST-VCA (diamonds).

HSPC300 homologue *BRICK1* and *Arabidopsis ARP2* and *ARP3* are associated with the loss or mis-localisation of F-actin structures within epidermal lobes (Frank et al., 2003; Li et al., 2003). The AtSCAR2 protein fused to GFP was created and proven to be functional by demonstrating that it can rescue the *atscar2* mutant phenotype. As we could not detect fluorescence in these lines, we expressed AtSCAR2-GFP in single epidermal pavement cells transiently by particle bombardment. AtSCAR2-GFP localises to the cytoplasm of initiating cell lobes without any preference to particular cell regions (Fig. 4). Occasionally we found apparent concentrations in lobes, but this was always colocalised with co-transformed cytoplasmic RFP.

Protein–protein interactions between components of the SCAR/WAVE and ARP2-ARP3 complexes and putative upstream regulators

Arabidopsis retains five SCAR isoforms, yet mutant alleles of only one isoform produce 'distorted'-like phenotypes. Combining *atscar2* alleles with *atscar4* alleles is sufficient to reproduce the null-phenotype of *arp2-arp3* complex mutants. The requirement for a total of five isoforms could be explained by functional specialisation between the AtSCAR isoforms. We addressed the question of functional specificity among the members of the protein families involved in the ARP2-ARP3 complex regulatory pathway by analysing protein–protein interactions. In *Arabidopsis* NAP1, SRA1 and HSPC300/BRICK1 are single copy genes, whereas SCAR/WAVE is represented by five, ABI by four, and ROP by eleven homologous genes. We used a systematic yeast two-hybrid approach to test all possible combinations of interactions between components of the ARP2-ARP3 complex, the SCAR/WAVE activator complex, representative ROPs and SPIKE1 and whether they can dimerise. We applied BiFC technology to detect and confirm interactions in planta. With this technique we focused on the most relevant and in particular the new unexpected interactions. In total we tested 67 different protein combinations with the BiFC system. In 45 cases we could reconfirm the yeast two-hybrid result in planta (Table 1).

In addition to known or expected interactions, the resulting network of interactions revealed several interesting novel possible functional relationships (Fig. 6, Table 1).

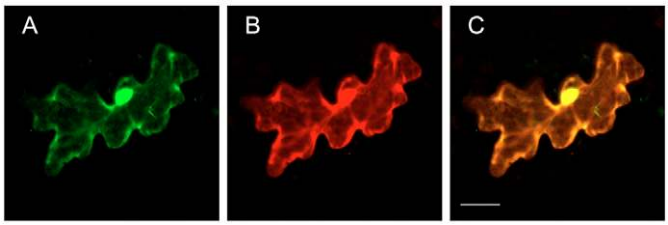

Fig. 4. Localisation of AtSCAR2-GFP. In wild-type cells, SCAR2 protein fused to GFP (**A**), localises to the cytoplasm, which is labelled with DsRed (**B**). (**C**) is a merged image of (A) and (B). Scale bar: 20 μm.

Protein–protein interactions within the SCAR/WAVE complex

The array of yeast two-hybrid analyses revealed partnering selectivity between the members of SCAR/WAVE and ABI proteins (Fig. 6, Table 1). Interaction with all four ABI proteins was found in the case of AtSCAR3 only. AtSCAR2 interacts with ABI1, 2 and 4, AtSCAR1 and AtSCAR-LIKE interact with ABI 1 and 2, whereas no interaction between AtSCAR4 and any ABI protein could be found. Several combinations were selected for confirmation in BiFC assays (Table 1). Interactions between these proteins were always found in the cytoplasm without preference to the plasma membrane or the cytoskeleton.

Furthermore, the five SCAR homologues from *A. thaliana* differ in their ability to interact with the HSPC300 homologue BRICK1. Interaction could be detected between HSPC300/BRICK1 and AtSCAR1, 2 and 3, respectively, whereas AtSCAR4 and AtSCAR-LIKE were not able to activate reporter gene expression in yeast (Fig. 6, Table 1). Both examples tested in BiFC were found to interact in the cytoplasm.

We found that BRICK1 homodimerises, and physically interacts with all four ABI proteins. HSPC300/BRICK1 homodimerisation (Fig. 5G,H) and the interaction between HSPC300/BRICK1 and ABI4 have been confirmed in planta by BiFC (Table 1). A physical interaction between HSPC300/BRICK1 and ABI proteins indicates potential cooperativity in a ternary complex formed by HSPC300/BRICK1, ABI and AtSCAR proteins, equivalent to the interactions within the animal complex suggested by the work of Gautreau et al. (Gautreau et al., 2004). We further found that NAP1 also homodimerises and interacts with two ABI proteins (ABI 1 and 2) and with SRA1 (Fig. 6, Table 1). NAP1 might therefore act as an adaptor protein recruiting SRA1 to the SCAR/WAVE complex in a manner similar to that reported for the mammalian NAP1 protein (Gautreau et al., 2004).

Interactions within the ARP2-ARP3 complex and between the SCAR/WAVE complex and components of the ARP2-ARP3 complex

Several anchoring possibilities link subunits of the SCAR/WAVE complex with the ARP2-ARP3 complex. In accordance with published data, the ARP2-ARP3 subunits ARPC3 and ARP3 seem to be central in this respect. We identified interactions between both ARP3 and ARPC3 and AtSCAR3, HSPC300/BRICK1 and ABI1 and 2. ABI4 interacted only with ARP3. Furthermore, AtSCAR1 and AtSCAR2 have been found to interact with ARPC3. AtSCAR4, AtSCAR-LIKE and ABI3 did not bind to components of the ARP2-ARP3 complex (Fig. 6, Table 1). That AtSCAR4 shows no interactions with components of the ARP2-ARP3 complex is surprising as it has been shown that the C-terminus can activate the

Table 1. Summary of protein-interaction tests involving components of the *Arabidopsis* ARP2-ARP3 complex and upstream regulators

	ARP2	ARP3	ARPC1	ARPC2	ARPC3	ARPC4	ARPC5	NAP125	KLK	ABI1	ABI2	ABI3	ABI4	BRICK1	SCAR1	SCAR2	SCAR3	SCAR4	SCAR-like	SPIKE1
ARP2	–/–																			
ARP3	+/–	–/–																		
ARPC1	–/0	–/0	–/0																	
ARPC2	–/–	–/0	–/0	–/0																
ARPC3	–/–	–/0	–/0	–/–	–/–															
ARPC4	–/–	–/0	+/0	+/+	+/+	–/0														
ARPC5	–/0	–/0	–/0	–/0	–/0	+/0	–/0													
NAP125	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0												
KLK	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0	0/0											
ABI1	+/0	+/0	–/0	–/0	+/–	–/0	–/0	+/0	–/0	–/–										
ABI2	–/0	+/0	–/0	–/0	+/0	–/0	–/0	+/0	–/0	–/0	–/0									
ABI3	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0								
ABI4	–/0	+/–	–/0	–/0	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0							
BRICK1	–/–	+/+	–/0	–/0	+/+	–/0	–/0	–/0	–/0	+/0	+/0	+/0	+/+	+/+						
SCAR1	–/–	–/0	–/0	–/0	+/+	–/0	–/0	–/0	–/0	+/0	+/0	–/0	–/0	+/0	–/0					
SCAR2	–/0	–/–	–/0	–/0	+/+	–/0	–/0	–/0	–/0	+/+	+/0	–/0	+/+	+/+	–/0	–/0				
SCAR3	–/0	+/0	–/0	+/+	+/–	–/0	–/0	–/0	–/0	+/–	+/0	+/0	+/+	+/+	–/0	–/0	–/0			
SCAR4	–/0	–/0	–/0	–/0	–/0	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0		
SCAR-like	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0	+/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	
SPIKE1	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/+	+/0	+/0	+/–	–/0	+/–	+/+	+/0	+/0	–/0	+/+
ROP2	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/0	–/+	–/0	–/–	–/0	+/–
ROP2CA	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/0	–/–	–/0	–/–
ROP5	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0	+/0	–/0	+/0	–/0	+/0
ROP5CA	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0
ROP7	–/0	–/0	–/0	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/–	+/+	–/–	–/0	–/0	–/–
ROP7CA	–/0	–/0	–/0	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/–	+/+	–/–	–/–	–/0	–/–
ROP7DN	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/0	–/0	–/0	–/0
ROP8	–/0	–/0	–/0	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0	+/+	+/0	–/0	+/0	+/–
ROP8CA	–/–	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/–	–/–	+/+	+/–	–/0	–/0	–/0
ROP11	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0
ROP11CA	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	–/0	+/0	+/–	–/0	+/0	–/0	+/0

Results from yeast two-hybrid tests are given on the left side of each cell; results of BiFC tests are given on the right side. +, positive result; –, negative result; 0, not determined.

bovine ARP2-ARP3 complex (Frank et al., 2004). Whether this finding is due to our particular experimental setup or whether this reflects that the rest of the AtSCAR4 protein can regulate the binding to the ARP2-ARP3 complex remains to be determined. Interactions of AtSCAR1 and AtSCAR2 with ARPC3, and interactions between HSPC300/BRICK1 and ARP3 and ARPC3, respectively, have been confirmed by BiFC in planta.

We also assessed the interactions between components of the ARP2-ARP3 complex. Based on the two-hybrid system, ARPC4 functions as an integral component as it interacts with ARPC1, ARPC2, ARPC3 and ARPC5. Two of these interactions have exemplarily been confirmed in planta (Fig. 6, Table 1). Similar protein interactions have been detected by yeast two-hybrid analyses in the human ARP2-ARP3 complex (Zhao et al., 2001). Our data indicate additionally direct contact between ARP2 and ARP3. With the exception of a physical interaction of ARPC4 and ARPC5, our data fit with the inter-subunit contacts revealed by the 3D structure of the bovine complex (Robinson et al., 2001), indicating a structural conservation from mammals to plants.

Upstream regulators of the SCAR/WAVE complex

Our protein interaction network reveals a novel interesting link between AtSCAR proteins and ROP GTPases. For the yeast two-hybrid assays we used either the unmodified full-length ROP cDNAs or single point mutations leading to the constitutively active (CA) form or, for the ROP7 protein, the dominant negative (DN) form. The constitutively active form carries the amino acid exchange Glycine 15 to Valine (ROP7CA), Glycine 17 to Valine (ROP2CA, ROP11CA), Glycine 27 to Valine (ROP8CA), and the dominant negative form the Threonine 20 to Asparagine exchange (ROP7DN).

The unmodified ROP5 interacted with AtSCAR1, AtSCAR2 and AtSCAR4; ROP7 interacted in both the wild type and the constitutively active form with AtSCAR2 but did not interact with AtSCAR2 in the dominant negative form; ROP8 interacted in both forms with AtSCAR2 and AtSCAR3, whereas only the unmodified form interacted with AtSCAR1, and constitutively active ROP11 interacted with AtSCAR1, AtSCAR2 and AtSCAR4 (Fig. 6, Table 1). These findings are very interesting because, to our knowledge, no interactions between SCARs and small Rho-like GTPases have been reported previously.

The constitutively active and the dominant negative forms of ROP7 interacted differentially with AtSCAR2 indicating that AtSCAR2 might be a downstream effector of ROP7. Because this question cannot be unambiguously answered with the yeast two-hybrid system, we applied the BiFC technology to investigate the interactions between ROP GTPases and AtSCAR proteins in planta. Upon co-expression of either wild-type ROP7 or the constitutively active form with AtSCAR2 in onion cells and *Arabidopsis* protoplasts, respectively, we observed a strong fluorescence signal localised to the plasma membrane (Fig. 5A-C,E). In contrast, co-expression of the dominant negative form of ROP7 and AtSCAR2 did not result in any detectable yellow fluorescence (Fig. 5D). ROP GTPases cycle between the predominant inactive GDP-bound state in the cytoplasm and a membrane-associated active GTP-bound

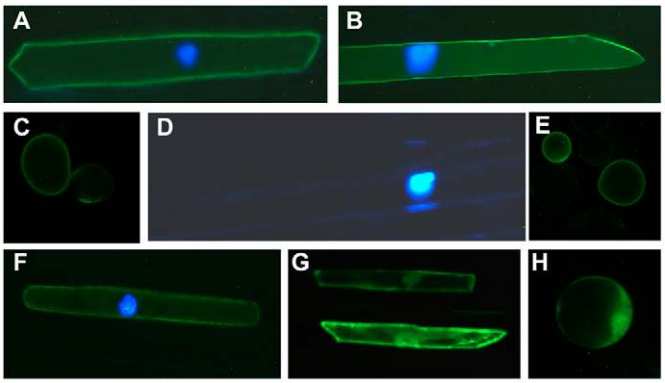

Fig. 5. Protein interactions in planta detected by BiFC. Plasmids encoding fusion constructs with the N- or C-terminal part of YFP (YFP–N or –C), respectively, were co-bombarded into onion cells (**A**,**B**,**D**,**F**,**G**) or were transiently expressed in *Arabidopsis* protoplasts (**C**,**E**,**H**). The nuclear blue fluorescence of co-expressed GL3:CFP served as a transformation control (A,B,D,F). YFP fluorescence indicates direct interaction in planta between AtSCAR2 and ROP7 (A,C), AtSCAR2 and the constant active ROP7CA (B and E). The signal is localised to the plasma membrane. The dominant negative form ROP7DN does not interact with AtSCAR2 (D). Interactions between SPIKE1 and AtSCAR2 (F) and homodimerisation of HSPC300/BRICK1 (G,H) take place in the cytoplasm.

state. Transiently expressed wild-type ROP7 protein should mainly exist in the inactive form in the cytoplasm with a fraction of activated protein at the plasma membrane. The fact that in both the wild type and the constitutively active form we observe the reconstituted fluorescence signal only at the cell periphery indicates that AtSCAR2 interacts specifically with the activated form of ROP7, which is associated with the plasma membrane. Similarly we could detect a BiFC signal specifically at the plasma membrane upon co-expression of ROP2 and ROP8 with AtSCAR2.

Similar to the mammalian SRA1 protein, the *Arabidopsis* SRA1 was previously shown to interact specifically with the activated form of a small Rho-like GTPase (ROP) ROP2 in plants (Basu et al., 2005). We also tested this combination with two different vector systems with both possible bait and prey combinations and with the full length ROPs as well as with ROPs that carry a C-terminal deletion typically used in yeast two-hybrid systems to avoid artefacts due to mislocalisation. In our hands, however, none of the tested ROPs including ROP2, ROP 5, ROP7, ROP8 and ROP11 showed interaction with SRA1.

In order to further assess the upstream regulation of the AtSCAR proteins and AtSCAR-LIKE we included the SPIKE1 protein in our analysis. Because of its sequence similarity to DOCK180 it has been speculated that SPIKE1 is a potential upstream regulator of ROPs (Qiu et al., 2002). We found interactions of SPIKE1 with wild-type forms of ROP2, ROP5 and ROP8, and with the constitutively active form of ROP11. This could support the proposed role of SPIKE1 as a ROP-GEF, but so far, we have not been able to confirm these yeast two-hybrid results with BiFC (Table 1).

Another interesting finding from our systematic protein interaction analyses is a direct interaction between SPIKE1 and all four ABI proteins and four of five AtSCAR proteins. Interactions with ABI1 and with AtSCAR2, respectively, have been confirmed by BiFC (Fig. 5F, Table 1). This finding suggests SPIKE1 is a novel integral component of the *Arabidopsis* SCAR/WAVE complex.

DISCUSSION

The SCAR proteins of *Arabidopsis* and maize are to date the only confirmed activators of the plant ARP2-ARP3 complex. Our results and those of others highlight the importance of isoform AtSCAR2 in the regulation of the ARP2-ARP3 complex during development, yet the phenotype of null alleles is not as severe as those of *ARP2-ARP3* complex mutations, suggesting that other factors contribute towards ARP2-ARP3 complex activation. Mutants of *NAP1*, *SRA1*, and *HSPC300/BRICK1* match the severity of *arp2-arp3* complex mutants, indicating that the genetically redundant partner(s) of AtSCAR2 are also likely to act through the SCAR/WAVE complex. A potential redundancy in plant SCAR/WAVE protein functions has previously been proposed based on the observation that overexpression of dominant negative SCAR leads to an enhanced distorted phenotype (Zhang et al., 2005a). Our data show that no mutant in any other AtSCAR isoform can alone produce a ‘distorted’ phenotype, but a reduction in the gene dosage of AtSCAR4 in an *atscar2* background is sufficient to produce a phenotype with increased severity that mimics the *arp2-arp3* complex mutants. This shows that AtSCAR proteins are indeed essential for the function of ARP2-ARP3 complex activation and do not rely upon a parallel genetic pathway to achieve full activation.

Genetic confirmation of the essential role of plant SCAR homologues in ARP2-ARP3 complex activation highlights the importance of understanding how plant SCAR proteins physically interact with their SCAR/WAVE complex and ARP2-ARP3 complex partners. The current data from animal systems suggest that the SCAR/WAVE complex assembles in a linear series of inter-subunit interactions such that SRA1 binds to NAP1, NAP1 to ABI, ABI to SCAR, and SCAR and ABI bind to HSPC300/BRICK1. Upon activation, the VCA/WA domain of SCAR/WAVE is thought to contact the ARP2-ARP3 complex principally through the ARPC3 subunit (Machesky and Insall, 1998) to stimulate the ARP2-ARP3 complex, although this model is constantly evolving to incorporate new insights into ARP2-ARP3 complex structural biology. In plants key interactions have been verified using a small number of ABI and SCAR isoforms (Basu et al., 2005; Frank et al., 2004) tested in one-to-one interaction assays that have confirmed a minimal number of associations that match the animal model for complex assembly. Our alternative approach has created a yeast two-hybrid interaction matrix that tests the majority of possible interactions between complex components and putative regulators. Confirmation of interactions in planta using the BiFC system has identified a number of novel protein associations not predicted by the animal model.

Genetic and biochemical experiments have identified *Arabidopsis* SCAR2 as a potential activator of the ARP2-ARP3 complex in plants (this work) (Basu et al., 2005; Zhang et al., 2005b). Four plant homologues of the five components of the SCAR regulatory complex have now been associated with phenotypes that either resemble *arp2-arp3* complex mutant alleles (Basu et al., 2004; Brembu et al., 2004; El-Assal et al., 2004a; Saedler et al., 2004b; Zimmermann et al., 2004) or are correlated with disruption of the F-actin cytoskeleton (Frank and Smith, 2002).

Of these components SCAR is the most essential for ARP2-ARP3 regulation; as the conserved SCAR VCA domain makes physical contact with and activates the ARP2-ARP3 complex via an acidic domain and amphipathic helix (Marchand et al., 2001; Pan et al., 2004; Panchal et al., 2003). Consequently, SCAR alone can activate the ARP2-ARP3 complex in vitro (Eden et al., 2002; Machesky et al., 1999).

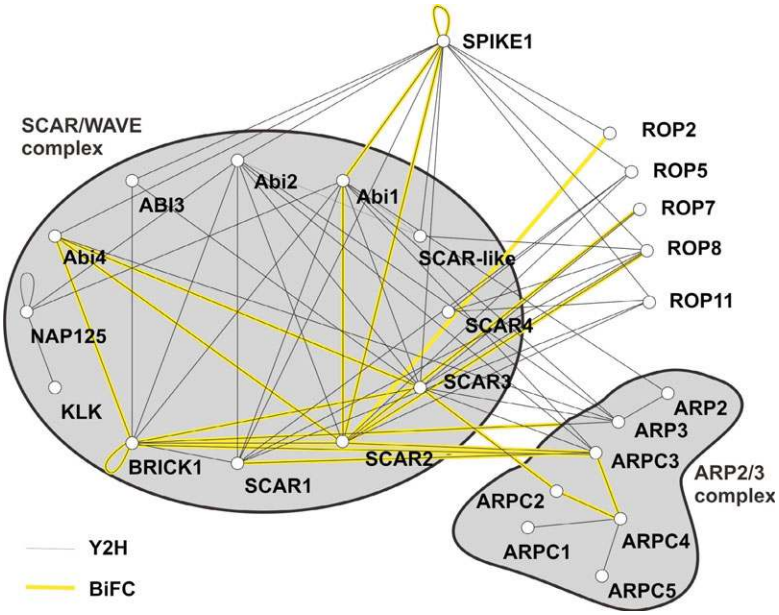

Fig. 6. Protein interaction network of ARP2-ARP3 complex and regulatory components. The subunits of the ARP2-ARP3 complex and upstream regulatory components have been tested for direct interactions using the yeast two-hybrid system (black lines) and Bimolecular Fluorescence Complementation (BiFC, yellow lines). In the yeast two-hybrid system, interactions were detected by growth of yeast cells on triple dropout media containing 3–20 mM 3-AT.

Associating plant SCAR with ARP2-ARP3 regulation is therefore central to understanding the role of NAP1, PIR121, HSPC300 and ABI homologues in plants.

Activating the ARP2-ARP3 complex in plants

It was recently shown that AtSCAR2 binds to *Arabidopsis* ARPC3 in the yeast two-hybrid system (Basu et al., 2005), but data testing alternative ARP2-ARP3- and SCAR/WAVE-complex binding partners were not presented. Our alternative systematic approach revealed two surprising results. First, we identified a specific interaction between AtSCAR3 and ARPC2, which to our knowledge has not been reported to interact with SCAR proteins in other systems. Second, *Arabidopsis* HSPC300/BRICK1 and a subset of ABI proteins bind directly to components of the ARP2-ARP3 complex. The HSPC300/BRICK1-ARPC3 interaction, the HSPC300/BRICK1-ARP3 interaction and the AtSCAR3-ARPC2 interaction were successfully verified in planta by BiFC. The identification of these interactions in the two independent systems suggests that the regulation of the plant ARP2-ARP3 complex by the SCAR/WAVE complex might involve novel binding configurations not previously identified in animal or fungal model species. Plant SCAR proteins dwarf their animal counterparts due to the presence of large plant-specific central domains that vastly increase the distance between the SHD N-terminus and VCA/WA C-terminus. These extra domains could potentially provide novel ARP2-ARP3 complex binding sites, or permit the flexibility required for other SCAR/WAVE complex subunits to contact the ARP2-ARP3 complex directly.

Interactions between the subunits of the putative plant SCAR/WAVE complex

In mammals, the SCAR/WAVE protein family comprises three members, and there are two ABI isoforms, ABI1 and ABI2. So far, no differences in interaction specificities have been found. Investigation of the interactions among these two protein families revealed that all SCAR/WAVE proteins can interact with ABI1 (Stovold et al., 2005). These findings suggest that the regulation of different SCAR/WAVE isoforms is likely at the level of tissue expression or is based upon different affinities to the binding partner, or relies upon additional regulatory components or post-translational modifications (Stovold et al., 2005). Similarly, association of all three members of the mammalian SCAR/WAVE protein family with HSPC300/BRICK1 has been proven (Eden et al., 2002; Gautreau et al., 2004; Stovold et al., 2005). The *Arabidopsis* SCAR and ABI proteins, by contrast, show specific interaction patterns such that they each bind only to a subset of the potential interaction partners. This results in a complex interaction network. In addition to identifying isoform-specific interactions during SCAR/WAVE complex assembly our analysis provides confirmatory evidence for a direct physical interaction between HSPC300/BRICK1 and ABI proteins. For the bovine SCAR/WAVE complex this interaction was predicted by Gautreau and co-workers, as HSPC300/BRICK1 bound to the SRA1-NAP1-ABI complex in the absence of SCAR/WAVE; however, despite being inferred, direct interaction between ABI and HSPC300/BRICK1 was not detected (Gautreau et al., 2004).

Regulation of the plant SCAR/WAVE complex

In mammals, the pentameric SRA1-NAP1-ABI-HSPC300/BRICK1-SCAR complex is activated by small Rho-like GTPases, which in turn are regulated by various upstream regulators including GEFs. Similar to the mammalian SRA1 protein, *Arabidopsis* SRA1 is considered a downstream effector of small Rho-like GTPases in plants because an interaction was found with the activated form of ROP2 (Basu et al., 2004). We could not identify an interaction between *Arabidopsis* SRA1 and any of the ROP proteins in our yeast two-hybrid assays, which may be due to different experimental setups. In our experiments we found, however, interactions between several ROPs and different subsets of the AtSCAR proteins. This raises the possibility that some plant ROPs can regulate AtSCAR activity through direct interactions (see below).

The activity of Rho-like GTPases in animals and yeast is regulated by exchange factors (GEFs). Plant ROP control by GEF proteins was only recently demonstrated (Berken et al., 2005). *Arabidopsis* SPIKE1 protein is considered to act as a GEF because it contains the DOCK domain found in a class of unconventional

guanine nucleotide exchange factors that stimulate the GTPase activity of Rho family proteins in animals (Qiu et al., 2002). As the *spike1* phenotype shares some aspects of *arp2-arp3* complex mutant phenotypes, SPIKE1 is a good candidate regulator of ROP activity in the context of cell morphogenesis. Our yeast two-hybrid data support this concept as SPIKE1 showed interactions with three ROP proteins. These results, however, have to be treated with caution as we could not confirm them in the BiFC system. However, the surprising finding that SPIKE1 interacts with all four ABI proteins and four of five SCAR proteins, and that examples of both interactions can be confirmed by BiFC, raises the interesting possibility that SPIKE1 might act as an integral part of the plant SCAR/WAVE complex. The physical coupling of a ROP GEF to the SCAR/WAVE complex could establish an intriguing signalling feedback loop where the SCAR/WAVE complex ROP effector could influence the status of its own activation through SPIKE1 GEF activity. Alternatively, a close association of the SPIKE1 GEF might be required to regulate small GTPases involved in plant-specific SCAR/WAVE complex interactions (see below).

A potential WASP-like pathway in plants?

In mammalian cells WASP (in contrast to SCAR/WAVE) contains a CRIB domain and consequently has been shown to be a direct effector of the small GTPase Cdc42. With the SRA1-Kette(NAP1)-ABI complex also regulating WASP function (Bogdan et al., 2004; Bogdan and Klambt, 2003; Bogdan et al., 2005), there are two effector proteins for small GTPases (SRA1-Rac1 and WASP-Cdc42) in the regulatory system. To date, no family of ARP2-ARP3 activators other than SCAR have been identified in plants by bioinformatics or experimentally.

Surprisingly, our protein–protein interaction data suggest that *Arabidopsis* SCAR proteins are directly-associated downstream effectors of ROPs. Both yeast two-hybrid data and BiFC demonstrate that AtSCAR2 interacts with ROP7 wild type and constitutive active forms but not the ROP7 dominant negative form. Interaction of AtSCAR2 with active ROP8 was also shown in both systems. Moreover the specific localisation of the ROP interactions at the plasma membrane supports the idea that ROPs can directly recruit SCAR proteins to the cell periphery. These data suggest that in plants, similar to the WASP complex, there might be two entry points for ROP-GTPase regulation. The significance of our finding remains to be determined, as an activating role similar to Cdc42 regulation of WASP seems unlikely as plant SCAR/WAVE proteins appear to be constitutively active and therefore not dependent on the release of an auto-inhibitory domain. Alternatively, active ROP proteins might provide an additional anchor to the plasma membrane for the large plant SCAR proteins. The plant-specific domains of AtSCAR proteins could contain small GTPase binding sites and require further investigation. It is interesting to note that AtSCAR2 is both the most ‘promiscuous’ ROP-binding AtSCAR isoform and is also the AtSCAR isoform shown to play the most prominent role during plant development.

Conclusion

Our data show that AtSCAR2 is an essential AtSCAR isoform required for plant development, and in our assays AtSCAR2 has the ability to interact directly with active small GTPases. Further analysis is required to identify the biological role for this phenomenon, but it should be noted that our systematic approach has identified novel interactions that behave in a more robust manner than some of the perceived ‘canonical’ interactions based upon the animal model of the SCAR/WAVE complex. Genetic analysis of the functional role of each SCAR/WAVE complex member is hindered by the potential functional redundancy between SCAR, ABI and ROP isoforms. Detailed complementation experiments using sophisticated mutant backgrounds combined with technically challenging in vitro reconstitution experiments will eventually be capable of testing the configuration of whole complexes, and be capable of assessing the role of each interaction in plant ARP2-ARP3 complex activation.

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