BRICK1/HSPC300 functions with SCAR and the ARP2/3 complex to regulate epidermal cell shape in Arabidopsis

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The Arp2/3 complex, a highly conserved nucleator of F-actin polymerization, is essential for a variety of eukaryotic cellular processes, including epidermal cell morphogenesis in Arabidopsis thaliana. Efficient nucleation of actin filaments by the Arp2/3 complex requires the presence of an activator such as a member of the Scar/WAVE family. In mammalian cells, a multiprotein complex consisting of WAVE, PIR121/Sra-1, Nap1, Abi-2 and HSPC300 mediates responsiveness of WAVE to upstream regulators such as Rac. Essential roles in WAVE complex assembly or function have been demonstrated for PIR121/Sra-1, Nap1 and Abi-2, but the significance of HSPC300 in this complex is unclear. Plant homologs of all mammalian WAVE complex components have been identified, including HSPC300, the mammalian homolog of maize BRICK1 (BRK1). We show that, like mutations disrupting the Arabidopsis homologs of PIR121/Sra-1, Nap1 and Scar/WAVE, mutations in the Arabidopsis BRK1 gene result in trichome and pavement cell morphology defects (and associated alterations in the F-actin cytoskeleton of expanding cells) similar to those caused by mutations disrupting the ARP2/3 complex itself. Analysis of double mutants provides genetic evidence that BRK1 functions in a pathway with the ARP2/3 complex. BRK1 is required for accumulation of SCAR1 protein in vivo, potentially explaining the apparently essential role of BRK1 in ARP2/3 complex function.

KEY WORDS: WAVE/Scar, Arp2/3 complex, HSPC300, BRICK1, Trichomes, Pavement cells

INTRODUCTION

Nucleation of actin filaments by the Arp2/3 complex, composed of actin-related proteins Arp2 and Arp3 and five additional subunits called ArpC1-ArpC5, is crucial for a variety of eukaryotic cellular processes. For example, in migrating animal cells, Arp2/3 complex-dependent actin polymerization at the leading edge generates a protrusive force that is crucial for directed cell migration (Pollard and Borisy, 2003). In S. cerevisiae, Arp2/3 complex-dependent actin polymerization drives the internalization and inward motility of endocytic vesicles (Engqvist-Goldstein and Drubin, 2003; Chang et al., 2003). In plants, mutations in genes encoding four different subunits of a putative ARP2/3 complex (ARP2, ARP3, ARPC2 and ARPC5) cause epidermal cell shape defects. The most conspicuous defect is a ‘distorted’ trichome (epidermal hair) shape that has been associated with alterations in the organization and/or density of F-actin in expanding trichomes (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; El-Assal et al., 2004a; Saedler et al., 2004).

Activators of the Arp2/3 complex, such as members of the Scar/WAVE family, greatly increase the efficiency of actin filament nucleation by the Arp2/3 complex in vitro and in vivo (Welch and Mullins, 2002). In recent years, significant advances have been made in understanding the regulation of Scar/WAVE activity in animal cells. WAVE1 and WAVE2 proteins isolated from mammalian cell extracts were both found in a complex with four additional proteins: PIR121/Sra-1, Nap1, Abi and HSPC300 (Eden et al., 2002; Gautreau et al., 2004); WAVE3 has also been shown to associate with Abi and HSPC300 in vivo (Stovold et al., 2005). PIR121/Sra-1 and Nap1 have been implicated in responsiveness of the Scar/WAVE complex to the upstream regulators Rac and Nck, respectively (Eden et al., 2002). Abi enhances WAVE activity in vitro (Innocenti et al., 2004) and also plays a key role in complex assembly (Gautreau et al., 2004). Genetic analyses in a variety of animal systems have confirmed crucial roles for PIR121/Sra-1, Nap1 and Abi in regulation of Scar/WAVE-dependent actin polymerization in vivo (Blagg and Insall, 2004; Vartiainen and Machesky, 2004).

The significance of HSPC300 in the Scar/WAVE complex is less clear than that of other subunits. A very small protein that is highly conserved in multicellular eukaryotes, HSPC300 has been shown to bind directly to WAVE2 in vitro (Gautreau et al., 2004), to interact in vivo with the N-terminal Scar homology domains (SHDs) of Scar/WAVE 1, 2 and 3, and to co-localize with all three WAVE proteins in vivo (Stovold et al., 2005). HSPC300 was present in WAVE complexes isolated from HeLa cell extracts, but unlike all other subunits, the majority of HSPC300 was found in a cytoplasmic pool not associated with the complex (Gautreau et al., 2004). Moreover, mammalian WAVE, PIR121/Sra-1, Nap1 and Abi expressed in insect cells formed a complex that stimulated Arp2/3-dependent actin polymerization in vitro, and the activity of this complex was not affected by addition of HSPC300 (Innocenti et al., 2004). RNAi-mediated knockdown of HSPC300 in Drosophila cultured cells resulted in a reduction of cortical F-actin and alterations in cell morphology that were similar to, but much weaker than those resulting from RNAi-mediated knockdown of Scar, PIR121/Sra-1, Nap1 and Abi (Kunda et al., 2003). Mutations in the maize brick1 gene, which encodes the maize homolog of HSPC300, cause a complete loss of pavement cell lobe formation, minor defects in epidermal hair morphology and occasional defects in the polarization of asymmetrically dividing subsidiary mother cells (Frank and Smith, 2002; Gallagher and Smith, 2000). These defects...
were associated with loss of localized cortical F-actin enrichments, pointing to a role for BRK1 in promoting actin polymerization. However, the relationship of maize BRK1 to the ARP2/3 complex is not clear.

A family of four proteins distantly related to Scar/WAVE at their N and C termini (SCAR1–4) has been identified in Arabidopsis (Deeks et al., 2004; Brembu et al., 2004). C-terminal domains of SCAR2, SCAR3 and SCAR4 have been shown to activate the bovine Arp2/3 complex in vitro, indicating that these are bona fide WAVE/Scar homologs (Frank et al., 2004; Basu et al., 2005). Homologs of the other four mammalian WAVE/Scar complex subunits (Nap1, PIR121/Sra-1, Abi and HSPC300) are also present in Arabidopsis (Szymanski, 2005). Like the corresponding mammalian proteins, the SHDs of Arabidopsis SCARs bind to BRK1/HSPC300 and an Abi-like protein ABIL1 (Frank et al., 2004; Basu et al., 2005; Zhang et al., 2005); binding interactions have also been observed between SRA1 and NAP1 (Bus et al., 2004; El-Assal et al., 2004b) and between SRA1 and ABIL1 (Bus et al., 2005). Thus, although not yet directly demonstrated, it is very likely that a complex equivalent to the mammalian Scar/WAVE complex exists in Arabidopsis. Moreover, genetic evidence strongly supports the conclusion that this complex plays an essential role in activation of the ARP2/3 complex in Arabidopsis. ‘Distorted’ trichome mutants gnarled (grl) and pirogi/klunker (pirkl), with phenotypes very similar to those of ARP2/3 complex subunit mutants, are mutations in the Arabidopsis homologs of Nap-1 and Pir21/Sra-1, respectively (El-Assal et al., 2004b; Basu et al., 2004; Deeks et al., 2004; Brembu et al., 2004; Zimmermann et al., 2004a; Li et al., 2004). In addition, dis3/tib1 mutations disrupting Arabidopsis SCAR2 produce a milder version of the same phenotype (Bus et al., 2005; Zhang et al., 2005). Furthermore, analyses of double mutants lacking both an ARP2/3 complex subunit and SCAR2 or NAP1 provide strong genetic evidence that both of these proteins function in a pathway with the ARP2/3 complex (Deeks et al., 2004; Basu et al., 2005).

We present an in vivo functional analysis of Arabidopsis BRK1. Our observations indicate that BRK1 functions in a pathway with the ARP2/3 complex and suggest that BRK1 plays an essential role in its function. Further observations demonstrate a requirement for BRK1 in stabilization of SCAR protein in vivo, providing an explanation for the apparent dependence of the ARP2/3 complex on BRK1.

### MATERIALS AND METHODS

#### Plant stocks, genotyping, and growth conditions

The Seattle TILLING Project (Colbert et al., 2001) (http://tilling.fhcrc.org:9366/) identified two premature stop codon mutations at the locations shown in Fig. 1A in the BRK1 gene (At2g22640) in ABRC stocks CS86554 and CS93199 (Columbia background; homoygous for erecta mutation er105). To genotype for these mutations, PCR products amplified with primers BRK1-01 and BRK1-02 (see Table 1) were digested with MseI (CS86554=brk1-1) or Hpy188III (CS93199=brk1-2). brk1-1 mutants used for the experiments presented in Figs 6 and 7 had been backcrossed twice to Columbia and were homozygous wild type at the ERECTA locus. brk1 mutant plants used for other experiments shown had either not been backcrossed or backcrossed once to Columbia, but the trichome morphology phenotype was confirmed to remain after two backcrosses. ABRC stocks SALK 079920 and SALK 123936 (Alonso et al., 2003) (Columbia background) with T-DNA insertions in ARP3 (DIS1), NAP1 (GRL) and SRA1 (PIR/KLK), respectively, were identified from their previously characterized phenotypes. FLAgbi/FST line 123F07 (Samson et al., 2002) carrying a T-DNA insertion in the SCAR1 gene was obtained from INRA-Versailles. The wild-type SCAR1 allele was amplified with SCAR1-02 and SCAR1-03, and the mutant scar1 allele with T-DNA-2 and SCAR-2. Sterilized seeds were plated on 1× MS salts with 0.05% 2-(4-morpholino)-ethane sulfonic acid (MES) and 0.8% agar. For the experiments presented in Figs 3, 4 and Fig. 8 (in the supplementary material), 2-week-old seedlings were transplanted to soil and grown for at least 3 additional weeks. Plants were grown on a 16 hour light/8 hour dark cycle at 20-22°C.

#### RNA analysis

Total RNA for RT-PCR and northern blot analysis was isolated using TRIzol RNA analysis. SCAR1 mRNA. To detect \(/\)tubulin cDNA clone isolated by Marks et al., 2002) carrying a T-DNA insertion in ARP3 (DIS1), NAP1 (GRL) and SRA1 (PIR/KLK), respectively, were identified from their previously characterized phenotypes. FLAgbi/FST line 123F07 (Samson et al., 2002) carrying a T-DNA insertion in the SCAR1 gene was obtained from INRA-Versailles. The wild-type SCAR1 allele was amplified with SCAR1-02 and SCAR1-03, and the mutant scar1 allele with T-DNA-2 and SCAR-2. Sterilized seeds were plated on 1× MS salts with 0.05% 2-(4-morpholino)-ethane sulfonic acid (MES) and 0.8% agar. For the experiments presented in Figs 3, 4 and Fig. 8 (in the supplementary material), 2-week-old seedlings were transplanted to soil and grown for at least 3 additional weeks. Plants were grown on a 16 hour light/8 hour dark cycle at 20-22°C.

#### Production of BRK1p::BRK1-T7 transgenic plants

A fragment comprising the BRK1-coding region minus the stop codon and 1.4 kb upstream sequence was amplified from Columbia genomic DNA with primers BRK1-07 and BRK1-08, and cloned into pET28a (Novagen) upstream of the T7 tag. BRK1p::BRK1-T7 was then amplified from this
construct with primers BRK1-08 and T7 (Table 1), and cloned into pEZRK-LCY (a gift of David Ehrhardt, Carnegie Institution, Department of Plant Biology), replacing the CaMV 35S promoter and YFP-coding region with BRK1p::BRK1-T7. The 3′ UTR was then amplified from genomic DNA with primers UTR-01 and UTR-02, and cloned downstream of the T7 tag. This construct was introduced into Columbia wild type and brk1-2 mutants via Agrobacterium-mediated transformation using the floral dip method (Clough and Bent, 1998).

**Visualization and analysis of F-actin**

Immunofluorescent labeling of F-actin in expanding trichomes was carried out on intact 10- to 14-day-old seedlings as described by Zhang et al. (Zhang et al., 2005), except that cell wall permeabilization was achieved by incubating in 0.5% Pectolyase Y23 (ICN Biomedicals) for 80 minutes, and Alexa Fluor 488-conjugated anti-mouse Ig (Molecular Probes) was used to visualize antibody labeling. Leaf primordia were excised and mounted in Vectorshield (Vector Laboratories) for confocal microscopy. Fluorescence was excited with a 488 nm line from an argon laser and visualized using a Nikon TE-200U microscope equipped with a 60X 1.2 NA immersion objective, a Yokogawa Nikopw spinning disc confocal head, a Chroma HQ525/50 band pass emission filter, and a Coolsnap HQ cooled CCD camera controlled by MetaMorph software (Universal Imaging).

For imaging of F-actin in expanding cotyledon pavement cells, 3- to 4-day-old seedlings grown on MS-agar plates were bombarded with 1 mg of 1 μm gold particles (Bio-Rad) coated with 200 ng of 35S-GFP-mTALIN plasmid described by Fu et al. (Fu et al., 2001). Particle coating and bombardment using a Bio-Rad PDS-1000 helium biolistic system was carried out according to the manufacturer’s instructions at a helium pressure of 1100 psi. Eighteen to 24 hours later, cotyledon pavement cells exhibiting intermediate levels of GFP-mTALIN fluorescence located in the upper two-thirds of the cotyledon (excluding the extreme edges) with areas between 900 and 8000 μm² were imaged by confocal microscopy as described above except that a 20 X objective was used. For quantitative analysis of cortical F-actin distribution, the linescan tool of Metamorph was used to measure fluorescence intensities around the entire perimeter of each cell and also around the peripheries of emerging lobes (defined here as areas of the cell surface exhibiting outward curvature). Linescan data were transferred to Excel files and used to calculate the proportion of the cell periphery exhibiting fluorescence intensities in the lowest quarter (‘dim’), next to lowest quarter (‘intermediate’) and upper half (‘bright’) of the fluorescence intensity range for each cell. In addition, the proportion of ‘bright’ peripheral fluorescence associated with lobes was calculated.

**Trichome and pavement cell shape analyses**

To visualize trichome shapes, mature rosette leaves were attached to stubs and imaged without further processing using an FEI Quanta 600 Environmental Scanning Electron Microscope at a low pressure setting of 133 Pa. For trichome branch length analysis, rosette leaves were fixed and cleared with chloral hydrate as described previously (Hamada et al., 2000) and visualized using DIC optics on a Nikon E600 microscope with a 4X objective. Images were captured using NIH ImageJ version 1.32j software with a DAGE MTI CCD72 camera coupled to a Scion LG-3 framegrabber. Branch lengths were then measured using ImageJ.

For imaging of cotyledon pavement cell shapes, fully expanded cotyledons were removed from 12-14 day-old seedlings grown aseptically on MS-agar plates. Cotyledons were mounted under a coverslip in 0.01% g/ml propidium iodide, and adaxial surfaces imaged by confocal microscopy. Fluorescence was excited with a 488 nm line from an argon laser and visualized using a Nikon TE-200U microscope equipped with a 60X 1.2 NA immersion objective, a Yokogawa Nikopw spinning disc confocal head, a Chroma HQ525/50 band pass emission filter and 10X objective were used. For each cotyledon analyzed, a set of images was collected spanning the entire width of the cotyledon along a straight line perpendicular to the tip-base axis approximately halfway from base to tip, but excluding the extreme edges where cells are more lobed than they are elsewhere in the cotyledon. The freehand selection tool in ImageJ version 1.32j was used measure the perimeter and area of every cell in each image collected. For cells with areas from 10,000 to 30,000 μm² (the majority) a ‘form factor’ was calculated as (area)/(4×perimeter) – a unitless number reflecting the degree of convolution of the cell periphery (Russ, 2002).

**SCAR1 protein analysis**

Polyclonal chicken antibodies were raised and affinity purified against an internal fragment of SCAR1 corresponding to amino acids 302-463 at GenWay Biotech (San Diego, CA). Shoot tips from 3-week-old seedlings were homogenized in extraction buffer [TBS with 5 mM 2-mercaptoethanol, 0.05% Tween, 10% sucrose and 1/100 plant protease inhibitor cocktail (Sigma)] using an Omni TH homogenizer at 4°C. Following centrifugation at 700 g then 16,000 g, 1 μl of anti-SCAR1 was added to the supernatant. Following incubation at 4°C for 30 minutes with end-over-end rotation, 10 μl of goat anti-chicken IgY antibody-conjugated microbeads (Genway) were added, and samples were incubated with rotation at 4°C for another hour. After two washes in extraction buffer, bead-bound proteins were removed by boiling in SDS loading buffer and electrophoresed on NuPage Novex Bis-Tris 4-12% acrylamide gels (Invitrogen) using MES-SDS running buffer, and transferred to polyvinylidene fluoride membrane (Millipore). Western blotting was carried out as described by Harlow and Lane (Harlow and Lane, 1998) using anti-SCAR1 antibody diluted to 0.1 μg/ml and horseradish peroxidase-conjugated goat anti-chicken antibody (GenWay) diluted 1:10,000.

**RESULTS**

**Loss or overexpression of Arabidopsis BRK1 causes trichome morphology defects similar to those in ARP2/3 complex mutants**

The Arabidopsis thaliana genome contains a single gene homologous to maize brk1. The 9.5 kDa protein encoded by this gene is 76% identical to maize BRK1 (Fig. 1A). Similar levels of Arabidopsis BRK1 mRNA were found by semi-quantitative RT-PCR analysis of expanding and fully expanded cotyledons and rosette leaves as well as expanding silicles; lower expression levels were detected in flower buds and root tips (Fig. 1B). Moreover, a survey of Arabidopsis microarray hybridization results using the ‘Digital Northern’ tool at Genevestigator (https://www.genevestigator.ethz.ch/) (Zimmerman et al., 2004b) indicated similar levels of BRK1 mRNA in all major organs and tissue types, both mature and immature (data not shown). Thus, like maize brk1 (Frank and Smith, 2002), Arabidopsis BRK1 is widely expressed in all major organs and tissue types, including leaves, stems, flowers, and siliques. The Arabidopsis BRK1 mRNA is expressed throughout the Arabidopsis embryo, but is most abundant in the shoot apical meristem and flower buds. The Arabidopsis BRK1 mRNA is expressed throughout the Arabidopsis embryo, but is most abundant in the shoot apical meristem and flower buds. The Arabidopsis BRK1 mRNA is expressed throughout the Arabidopsis embryo, but is most abundant in the shoot apical meristem and flower buds. The Arabidopsis BRK1 mRNA is expressed throughout the Arabidopsis embryo, but is most abundant in the shoot apical meristem and flower buds. Therefore, Arabidopsis BRK1 is a potential target for selective gene silencing in plants.

**Fig. 1. Sequence and expression of Arabidopsis BRICK1.**
(A) Alignment of maize and Arabidopsis BRICK1 protein sequences. Dark and light shading highlight identical and similar residues, respectively. The Arabidopsis BRK1 protein sequence is supported by the sequence of full-length cDNA clone RAFL04-19-H22 (GenBank Accession Numbers AV821991 and AV782595). Asterisks indicate the locations of premature stop codons in mutant alleles brk1-1 and brk1-2. (B) RT-PCR analysis illustrating expression of Arabidopsis BRK1 and ubiquitin in all tissues analyzed (FB, flower buds; RT, root tips; ES, expanding silicles; EC, expanding cotyledons; MC, mature cotyledons, EL, expanding leaves; ML, mature leaves).

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**DEVELOPMENT**

**Arabidopsis BRK1 and cell morphogenesis**

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expressed, suggesting a global function. Two mutations introducing premature stop codons in the first exon of Arabidopsis BRK1 were identified by TILLING (Colbert et al., 2001); these mutations truncate the BRK1 protein at the locations shown in Fig. 1A. All aspects of the phenotype reported here were observed in plants homozygous for either of these mutations. Moreover, a transgene comprising the Arabidopsis BRK1 gene expressed from its native promoter was able to rescue all the epidermal cell morphology defects in brk1-2/brk1-2 mutants described below. Thus, we can conclude that these phenotypes resulted from mutations in the BRK1 gene.

Like arp2 (wurm) and arpc5 (crk) mutants, 2-week-old brk1 mutant seedlings appeared very similar in overall morphology to wild type (Fig. 2A-D). However, brk1 mutants have trichome morphology defects similar to those previously described for ARP2/3 complex and SCAR complex subunit mutants. As illustrated in Fig. 3 for brk1 (B,F) and arp2 (C,G), the branches of mutant trichomes fail to initiate or are initiated but fail to elongate normally, whereas the stalks are swollen and sometimes excessively elongated compared with wild type. Aberrant elongation of the trichome stalk between branches sometimes displaces aborted branches (Fig. 3F,G, arrows). Trichome branch lengths are reduced in brk1 mutants to the same extent as in arp2 and arpc3 mutants, and more so than in arpc5 mutants (Fig. 3I,J). Similar to previously reported findings that branches of sral (pir/klk) and napl (gri) trichomes are longer than those of arp2 (dis2) mutants (Basu et al., 2004; El-Assal et al., 2004b), we found that sral and napl trichome branches are longer than those of brk1, arp2 and arpc3 mutants, and are similar to those of arpc5 mutants (Fig. 3I,J). Thus, with respect to trichome branch length, the phenotype of brk1 mutants is equivalent to that of the most severe ARP2/3 complex subunit mutants examined, and is more severe than that of other SCAR complex subunit mutants.

Like trichome branch elongation, tip growth of root hairs and pollen tubes is well known to depend crucially on actin polymerization (Hepler et al., 2001). Nevertheless, tip growth has been reported to be affected very little (Mathur et al., 2003a; Mathur et al., 2003b; Li et al., 2003) or not at all (e.g. Le et al., 2003; Brembu et al., 2004; El-Assal et al., 2004a) in ARP2/3 complex and SCAR complex subunit mutants. When brk1-2 heterozygotes were self pollinated, the frequency of brk1-2 mutants in the next generation was found not to be significantly different from 25% (79 brk mutants/360 progeny, 0.1<P<0.5 by χ² analysis). Moreover, no difference was observed between root hairs of wild type, brk1-1, arp2, arpc3, arpc5, napl and sral (see Fig. S1 in the supplementary material). Thus, tip growth of both root hairs and pollen tubes appears to be unaffected in Arabidopsis brk1 mutants, as previously reported for maize brk mutants (Frank and Smith, 2002; Frank et al., 2003). Apparently, other actin nucleators besides the ARP2/3 complex are sufficient to stimulate the actin polymerization that is essential for tip growth.

A genomic fragment comprising the BRK1 gene (fused at the C terminus to a T7 epitope tag) together with ~1.4 kb of 5’ and 200 bp of 3’ sequence was introduced into the Columbia wild-type background. Surprisingly, in eight out of 16 independent T2 transgenic lines analyzed, some plants exhibited trichome morphology defects similar to those of brk1 loss-of-function mutants (Fig. 4A, white arrowheads), although abnormal trichomes were always accompanied by normal trichomes (Fig. 4A, black arrowhead). Northern blot analysis showed a minor increase in BRK1 mRNA levels (endogenous and transgenic combined) in BRK1p::BRK1-T7 transgenic lines with abnormal trichomes compared with wild-type plants (Fig. 4B), ruling out co-suppression as the cause of the phenotype. Moreover, our observation that the same construct was able to complement the phenotypes of brk1-2 homozygous mutants demonstrates that the C-terminal T7 tag does not interfere with BRK1 function. Together, these data suggest that the occasional appearance of aberrantly shaped trichomes in wild type plants carrying the BRK1p::BRK1-T7 transgene is due to slightly elevated levels of functionally normal BRK1 protein. Presumably, the transgene restores wild-type trichome morphology in a brk1 mutant background because transgenic BRK1 protein levels in this background do not significantly exceed the levels of endogenous BRK1 protein normally produced in wild-type plants.

**Similar alterations in F-actin organization are observed in expanding trichomes of Arabidopsis brk1 and arp2 mutants**

If BRK1 functions in ARP2/3 complex activation, then brk1 and ARP2/3 complex subunit mutations would be expected to have similar effects on the F-actin cytoskeleton. We directly compared F-actin in expanding trichomes of wild-type plants to that in arp2 and brk1 mutant trichomes following formaldehyde fixation and immunofluorescent labeling of F-actin. As previously reported in studies where aldehyde fixation and phalloidin or antibody labeling were used to visualize F-actin (e.g. Szymanski et al., 1999), we observed arrays of cytoplasmic F-actin bundles at all stages of wild type trichome expansion examined that were of relatively uniform thickness and were fairly well aligned with each other along the long axes of the trichome branches (Fig. 5A-C). As previously shown (Zhang et al., 2005), close examination of the surfaces of elongating wild-type trichome branches also revealed a very fine network of cortical F-actin filaments that are aligned transversely to the axis of the trichome branch, perpendicular to the overall alignment of cytoplasmic F-actin bundles (gray arrowheads, Fig. 5D).

In expanding trichomes of both arp2 and brk1-1 mutants, we observed alterations in F-actin organization similar to those previously described for arp2 mutants when aldehyde fixation and antibody labeling were used to visualize F-actin (Le et al., 2003). At all stages of arp2 and brk1-1 mutant trichome expansion examined, actin filament bundles permeated the cytoplasm of branches and

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**Fig. 2. Mutant and wild-type seedlings at 2 weeks post germination.** (A) Columbia wild type; (B) arp2 (wurm) mutant; (C) arpc5 (crk) mutant; (D) brk1-1 mutant; (E) brk1-1,arp2 double mutant; (F) brk1-2,arpc5 double mutant.
filament bundles were observed in 5E-J). Shortly after initiation of trichome branches, misaligned actin overall density of cytoplasmic F-actin compared with wild type (Fig. 4G, H). Relatively little overall alignment was observed within the network of cytoplasmic F-actin bundles by the stage illustrated in Fig. 5G (arp2) and 5J (brk1). Thus, an overall loss of alignment within the network of cytoplasmic F-actin bundles was associated with the swelling of mutant trichomes. Notably, the fine network of transversely aligned cortical F-actin observed in wild type was also present in aberrantly expanding trichome branches of brk1-1 and arp2 mutants (gray arrowheads, Fig. 5K, L, respectively).

**brk1 mutations reduce pavement cell lobe formation more than ARP2/3 complex or other SCAR complex mutants**

In brk1 mutants of maize, epidermal pavement cell lobes are completely eliminated. Moreover, some Arabidopsis ARP2/3 complex and SCAR complex subunit mutants have been reported to reduce pavement cell lobe formation (Le et al., 2003; Li et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; Brembu et al., 2004). Therefore, we were interested to determine the effects of Arabidopsis brk1 mutations on pavement cell lobe formation. pavement cells of wild type cotyledons and rosette leaves have irregularly-shaped but well-defined lobes that interlock with those of neighboring cells (Fig. 6A and see Fig. S2A in the supplementary material, respectively). Reduced pavement cell lobing was observed in brk1 mutant cotyledons, as well as in ARP2/3 complex and other SCAR complex subunit mutants (Fig. 6 and data not shown). To quantify these phenotypes, we calculated for each genotype a mean “form factor,” which measures the relationship between cell perimeter and area with higher numbers indicating a higher degree of lobing (Russ, 2002) (see Materials and methods for details). As shown in Fig. 6H, cotyledon pavement cell form factors were significantly decreased (P<0.01) in brk1, arp2, arp3, arp5, nap1 and nap1 mutants relative to wild type (Fig. 6H). As shown earlier for trichome branch lengths (Fig. 3I), form factors are reduced less in arp5 mutants than in arp2 or arp3 mutants (Fig. 6H). Moreover, as described previously for rosette leaves (Brembu et al., 2004), we found a greater reduction in pavement cell form factors for nap1 cotyledons than sra1 cotyledons (Fig. 6H). Although sra1 and nap1 trichome branch lengths are similar to those of arp5 mutants (Fig. 3I), pavement cell form factors for sra1 and nap1 are comparable with those of the more severe ARP2/3 complex subunit mutants arp2 and arp3 (Fig. 6H). Interestingly, lobe formation was reduced significantly more in brk1 mutants than in any of the other mutants (P<0.01). This phenotype could be clearly attributed to loss of BRK1 function as it was observed in both brk1-1 and brk1-2 mutants, and was fully complemented by the BRK1p::BRK1 transgene (Fig. 6H). Analysis of rosette leaves also showed a significantly greater reduction (P<0.01) of pavement cell form factors in brk1 mutants than in arp2 or arp5 mutants (see Fig. S2 in the supplementary material).

Examination of cotyledons also revealed occasional intercellular gaps in brk1 mutants as well as in all other mutants examined (Table 2, Fig. 6). These gaps were most often adjacent to stomata (white arrowheads, Fig. 6C, E, G) but were sometimes seen in other locations (white arrows, Fig. 6E). Intercellular gaps in cotyledons and other tissues have been observed previously in ARP2/3 complex and SCAR complex subunit mutants (e.g. Le et al., 2003; Mathur et al., 2003a; Mathur et al., 2003b; El-Assal et al., 2004a; El-Assal et
Fig. 4. Analysis of BRK1p::BRK1-T7/His transgenic plants.
(A) Scanning electron micrograph showing aberrant (white arrowheads) and normal (black arrowhead) trichome morphologies observed in some BRK1p::BRK1-T7/His transgenic plants. Scale bar: 100 μm.
(B) Northern blot comparing BRK1 RNA levels in four BRK1p::BRK1-T7 transgenic lines with abnormal trichomes with four non-transgenic Columbia samples (blot was probed with a full-length BRK1 cDNA, which does not distinguish between endogenous and transgene mRNAs). BRK1:β-tubulin mRNA ratios were higher in all four transgenic samples than in the wild-type samples.

al., 2004b; Basu et al., 2004; Basu et al., 2005; Zhang et al., 2005), and have in most cases been interpreted to reflect a reduction in intercellular adhesion.

Cortical F-actin distribution is altered in brk1 mutant pavement cells
The F-actin cytoskeleton was examined in expanding cotyledon pavement cells to investigate whether reduced lobe formation in brk1 mutants is associated with depletion of cortical F-actin, as found previously for brk1 mutants of maize (Frank and Smith, 2002). In our hands, cortical F-actin in expanding pavement cells was not consistently well preserved by fixation. Moreover, when all pavement cells are labeled, it is difficult to distinguish the cortical actin of one cell from that of its neighbors. Therefore, we visualized actin filaments in individual, living pavement cells from 4-5 day old cotyledons transiently expressing GFP fused to an actin binding fragment of mouse talin following particle bombardment (Fu et al., 2002; Fu et al., 2005). GFP-talin labeled F-actin was examined in cotyledon pavement cells with areas of ~900 to 8000 μm², spanning a growth phase in which lobes are forming (Fig. 7). In wild-type cells, we observed cytoplasmic and subcortical F-actin cables along with fine cortical F-actin, with areas of bright cortical actin restricted to a relatively small proportion of the cell surface (Fig. 7A-C). Quantitative analysis (see Materials and methods for details) showed that in wild-type cells, on average, ~8% of the cell periphery exhibited ‘bright’ fluorescence (values in the upper half of the intensity range), 25% exhibited ‘intermediate’ fluorescence (values from 25 to 50% of the intensity range) and 66% exhibited ‘dim’ fluorescence (values in the bottom 25% of the intensity range) (Fig. 7G). As previously described for expanding rosette leaf pavement cells expressing GFP-talin (Fu et al., 2002; Fu et al., 2005), areas of the cell surface with bright cortical F-actin often (Fig. 7A-C, white arrowheads), though not always (Fig. 7C, gray arrowheads), corresponded to sites where lobes appeared to be emerging. When fluorescence intensities for the entire cell periphery were compared with those at sites on the cell surface exhibiting outward curvature, 60% of ‘bright’ cortical F-actin was found to be associated with apparent sites of lobe outgrowth (Fig. 7G). Although the majority of lobes did not exhibit bright cortical F-actin, these may have been lobes that were not actively growing at the time of observation.

Expanding brk1-1 cotyledon pavement cells were generally less lobed than wild-type cells of similar size, but their actin cytoskeletons appeared similar to those of wild-type cells, with no obvious reduction in F-actin density either in the cytoplasm or cortex (Fig. 7D-F). However, cortical F-actin in brk1 cells appeared to be somewhat more broadly distributed. Quantitative analysis showed that this difference was small but statistically significant (P<0.05), with areas occupied by bright and intermediate fluorescence increased to 12% and 30% of the cell periphery, respectively, and areas occupied by dim fluorescence decreased to 58% (Fig. 7G). Notably, the proportion of bright cortical F-actin located at sites of apparent lobe outgrowth was reduced from 60% in wild type to 38% in brk1 cotyledons (Fig. 7G). Similar results were obtained when F-actin in expanding rosette leaf pavement cells was labeled via transient expression of GFP fused to an actin-binding domain of Arabidopsis fimbrin (Wang et al., 2004) (see Fig. S3 in the supplementary material). Thus, similar to previous findings for arpc2 and arpc3 mutants (Li et al., 2003), we found that cortical F-actin was more broadly distributed and local F-actin enrichments were less likely to be associated with emerging lobes in both cotyledon and rosette leaf pavement cells of brk1 mutants compared with wild type.

Analysis of double mutants indicates that BRK1 functions in a pathway with the ARP2/3 complex
If BRK1 functions to activate the ARP2/3 complex, then brk1 mutations would be expected to have no phenotypic effects in the absence of ARP2/3 complex function. That is, the phenotypes of brk1;arp2 and brk1;arpc5 double mutants would be no more severe than those of arpc5 and brk1 single mutants. We found the morphologies of 2-week-old brk1;arp2 and brk1;arpc5 double mutant seedlings to be very similar to those of brk1, arpc5 and brk1 single mutants (Fig. 2). Moreover, the appearance and the branch lengths of brk1;arp2 and brk1;arpc5 double mutant trichomes were not significantly different from those of the single mutants (Fig. 3). As described earlier, pavement cell lobing was reduced more in brk1 mutants than in arpc5 or arpc2 mutants, but the degree of lobing in brk1;arp2 and brk1;arpc5 double mutants was not significantly different from that of brk1 single mutants (Fig. 6F,G,H for cotyledons; see Fig. S2E,F,G in the supplementary material for rosette leaves). Thus, analysis of brk1;arp2 and brk1;arpc5 double mutants indicates that BRK1 acts in a pathway with the ARP2/3 complex.

BRK1 protects SCAR1 from degradation in vivo
In Dictyostelium, cells lacking PIR121/Sra-1 function (Blagg et al., 2003) as well as in Drosophila cultured cells with RNAi-mediated inhibition of PIR121/Sra-1, Nap1 or Abi function (Kunda et al., 2003; Rogers et al., 2003), Scar/WAVE protein levels are greatly reduced compared with wild type. Thus, in addition to the roles these proteins play in complex assembly and reception of regulatory input,
they also protect Scar/WAVE from degradation in vivo. As shown in Fig. 8 (lane 1, arrowhead), an antibody raised against SCAR1 immunoprecipitates a protein from Arabidopsis seedling extracts that is close to the expected molecular mass of SCAR1 (92 kDa). This protein co-migrates with SCAR1 protein produced via in vitro transcription/translation (not shown), and is undetectable in plants homozygous for a T-DNA insertion in the SCAR1 gene (lane 2), confirming its identity as SCAR1. As shown in Fig. 8 (lane 3), levels of this protein are dramatically reduced in extracts from brk1-1 mutants. Thus, as previously described for other Scar/WAVE complex subunits in animal cells, BRK1 appears to protect SCAR1 from degradation in vivo.

**DISCUSSION**

Previous work has demonstrated an essential function for the maize brick1 (brk1) gene in the formation of lobes on the margins of leaf epidermal cells, and in the formation of localized cortical F-actin enrichments associated with lobe outgrowth (Frank and Smith, 2002). The mammalian homolog of BRK1, HSPC300, is a component of a complex regulating the activity of the Arp2/3 complex activator, Scar/WAVE (Blagg and Insall, 2004). Homologs of all components of the mammalian Scar/WAVE complex are present in Arabidopsis and play essential roles in actin-dependent aspects of epidermal cell morphogenesis (Szymanski, 2005). Together, these and other findings suggest that BRK1, along with other components of a SCAR complex, function to stimulate ARP2/3 complex-dependent actin polymerization in plant cells. In this study, we have taken a genetic approach to investigate this hypothesis, taking advantage of mutations in the Arabidopsis BRK1 gene and genes encoding ARP2/3 complex and other SCAR complex subunits. Our findings support the conclusion that BRK1 is essential for activation of the ARP2/3 complex in vivo, and shed new light on its role.

Consistent with the idea that BRK1 plays an essential role in activation of the ARP2/3 complex, Arabidopsis brk1 mutants display distorted trichome shapes, reduced pavement cell lobing, and other phenotypes characteristic of ARP2/3 complex and other SCAR complex subunit mutants. Surprisingly, however, lobe formation is reduced more in brk1 mutants than in any of these other mutants. This finding raises the possibility that BRK1 may have ARP2/3 complex-independent function(s) that contribute to pavement cell lobing. Alternatively, it is possible that ARP2/3 complex function is not completely eliminated in any of the ARP2/3 complex subunit mutants we analyzed, but is completely eliminated in brk1 mutants. Indeed, although the arp2, arp3 and arpc5 alleles used in our study are all RNA null alleles (Le et al., 2003; Li et al., 2003), analysis of yeast arp2, arp3 and arpc5 null mutants in S. cerevisiae showed that in some genetic backgrounds and under some growth conditions, partially assembled Arp2/3 complexes remained and Arp2/3 complex function was not completely abolished (Winter et al., 1999). Strong genetic evidence that BRK1 acts in a pathway with the putative ARP2/3 complex is provided by the finding that trichome and pavement cell morphology defects are no more severe in brk1;arp2 and brk1;arpc5 double mutants than they are in the corresponding single mutants.
Unexpectedly, expression of an epitope-tagged version of BRK1 from its native promoter in a wild-type background caused occasional trichome morphology defects similar to those observed in brk1 loss-of-function mutants, perhaps owing to elevated levels of functionally normal BRK1 protein. BRK1 was shown to bind in vitro to Arabidopsis SCAR proteins (Frank et al., 2004; Zhang et al., 2005). Similarly, HSPC300 binds to WAVE proteins and may also bind directly to Abi (Gautreau et al., 2004; Stovold et al., 2005). Thus, binding of excess BRK1 to Arabidopsis SCARs and/or Abi-like proteins may make them unavailable for formation of functional SCAR complexes, thereby reducing the levels of active SCAR. Alternatively, disruption of trichome morphogenesis by excess BRK1 protein might be due to an ARP2/3 complex-independent function of BRK1, if it has such a function.

Just as brk1 and ARP2/3 complex subunit mutations produce similar changes in cell morphology, these mutations also have similar effects on the F-actin cytoskeletons of expanding cells. Similar to previous findings for ARP2/3 complex and SCAR complex subunit mutants when aldehyde fixation and phalloidin or antibody labeling were used to visualize F-actin (Szymanski, 2005), we observed that aberrant trichome expansion in brk1 and arp2 mutants was associated with a loss of alignment within the network of cytoplasmic F-actin cables. Given the well-established role of cytoplasmic F-actin in intracellular transport of Golgi stacks (Nebenführ et al., 1999) and secretory vesicles (at least in tip growing cells) (Hepler et al., 2001), aberrant morphogenesis of brk1 and arp2 mutant trichomes could be due to mistargeting of Golgi and/or secretory vesicles by disorganized actin cables. Indeed, Golgi motility was shown to be impaired in

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Columbia wild type</th>
<th>brk1-1</th>
<th>arp2 (wurm)</th>
<th>arp3 (dis1)</th>
<th>arp3 (crk)</th>
<th>sra1 (pir)</th>
<th>nap1 (grl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of gaps per mm²</td>
<td>0.0±0.0 (n=5)</td>
<td>8.5±6.4 (n=7)</td>
<td>5.1±3.8 (n=3)</td>
<td>8.6±2.0 (n=6)</td>
<td>6.4±2.0 (n=5)</td>
<td>2.9±2.7 (n=4)</td>
<td>5.0±2.5 (n=4)</td>
</tr>
</tbody>
</table>
Error bars shown are s.e.m. intermediate actin, 25-50% of range; dim actin, bottom 25% of range. However, a variety of alternative explanations are also possible (Szymanski, 2005; Smith and Oppenheimer, 2005). Similar to previous results for expanding trichomes of arpc5 (erk) mutants (Mathur et al., 2003b). However, a variety of alternative explanations are also possible (Szymanski, 2005; Smith and Oppenheimer, 2005). Similar to previous results for expanding trichomes of arpc5 (erk) mutants (Li et al., 2003), we also found that in expanding brk1-1 pavement cells, cortical F-actin was more broadly distributed and F-actin enrichments were less likely to be associated with apparent sites of lobe outgrowth. Localized cortical F-actin polymerization has been proposed to promote lobe outgrowth in both maize and Arabidopsis pavement cells by an unknown mechanism (Frank and Smith, 2002; Fu et al., 2002; Fu et al., 2005). Thus, the altered distribution of cortical F-actin observed in expanding brk1 pavement cells could potentially explain the reduced lobe formation seen in these mutants. Determining the intracellular sites of ARPP23 complex-dependent actin polymerization in expanding trichomes and pavement cells would most probably lead to a better understanding of its role in promoting normal patterns of epidermal cell expansion.

As no striking reduction in cortical F-actin density was observed in expanding brk1 epidermal cells, we find no clear evidence that BRK1 stimulates cortical F-actin polymerization in expanding epidermal cells of Arabidopsis, as found previously for maize BRK1 (Frank and Smith, 2002; Frank et al., 2003) and as the Arp2/3 complex does in S. cerevisiae and migrating animal cells (Pollard and Borisy, 2003). Loss of ARP2/3 complex activity in brk1 mutants may somehow alter the activity or distribution of other actin nucleators to cause the changes in F-actin organization/distribution observed in brk1 mutant cells, but such nucleators remain to be identified. In expanding pavement cells of Arabidopsis, cortical F-actin is markedly depleted in the absence of the putative SCAR complex. However, another ROP-binding protein, RIC4, appears to play a more crucial role in mediating ROP-dependent cortical actin polymerization in expanding pavement cells (Fu et al., 2005). RIC4 may itself be a novel actin nucleator, or may activate another nucleator such as a member of the formin family.

Analysis of Dicystostelium and Drosophila cells with reduced PIR121/Sra-1, Nap1 and Abi function has shown that in addition to their roles in regulation of Scar/WAVE activity, all three proteins also protect Scar/WAVE from degradation in vivo (Blagg et al., 2003; Rogers et al., 2003; Kunda et al., 2003). Presumably, degradation of Scar/WAVE is due to reduced stability of the complex in the absence of any of these three subunits. Similarly, we found that SCAR1 protein levels are dramatically reduced in brk1 mutants, indicating that BRK1 functions to protect SCAR1 from degradation in vivo and providing further indirect evidence for the existence of a complex containing both SCAR1 and BRK1. If all four Arabidopsis SCAR proteins are degraded in brk1 mutants [including SCAR2, which unlike SCAR1 has been shown to play a role in trichome morphogenesis (Busu et al., 2005; Zhang et al., 2005)], this would explain why BRK1 appears to be essential for ARPP23 complex activity in vivo. Indeed, based on our findings, it may be that the only function of BRK1 is stabilization of the putative SCAR complex in vivo. Alternatively, like PIR121/Sra-1, Nap1 and Abi, BRK1 may have additional functions in regulation of complex assembly or activity, but further work will be required to determine what these functions might be.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/6/1091/DC1
References