The Putative Arabidopsis Arp2/3 Complex Controls Leaf Cell Morphogenesis[1][w]

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The evolutionarily conserved Arp2/3 complex has been shown to activate actin nucleation and branching in several eukaryotes, but its biological functions are not well understood in multicellular organisms. The model plant Arabidopsis provides many advantages for genetic dissection of the function of this conserved actin-nucleating machinery, yet the existence of this complex in plants has not been determined. We have identified Arabidopsis genes encoding homologs of all of the seven Arp2/3 subunits. The function of the putative Arabidopsis Arp2/3 complex has been studied using four homozygous T-DNA insertion mutants for *ARP2*, *ARP3*, and *ARPC5/p16*. All four mutants display identical defects in the development of jigsaw-shaped epidermal pavement cells and branched trichomes in the leaf. These loss-of-function mutations cause mislocalization of diffuse cortical F-actin to the neck region and inhibit lobe extension in pavement cells. The mutant trichomes resemble those treated with the actin-depolymerizing drug cytochalasin D, exhibiting stunted branches but dramatically enlarged stalks due to depolarized growth suggesting defects in the formation of a fine actin network. Our data demonstrate that the putative Arabidopsis Arp2/3 complex controls cell morphogenesis through its roles in cell polarity establishment and polar cell expansion. Furthermore, our data suggest a novel function for the putative Arp2/3 complex in the modulation of the spatial distribution of cortical F-actin and provide evidence that the putative Arp2/3 complex may activate the polymerization of some types of actin filaments in specific cell types.

Actin nucleation is critical for the reorganization and dynamics of the actin cytoskeleton. Two actin nucleation factors have been identified in yeast and animals. Formins nucleate actin polymerization required for actin cable formation in yeast (Evangelista et al., 2002; Sagot et al., 2002). The Arp2/3 complex activates the polymerization of branched F-actin and promotes actin nucleation from the side of an existing actin filament, leading to the formation of a branched actin network (Mullins et al., 1998; Svitkina and Borisy, 1999; Blanchoin et al., 2000; Cooper et al., 2001; Higgs and Pollard, 2001; Robinson et al., 2001). The Arp2/3 complex consists of seven subunits (ARP2, ARP3, ARPC1/p41, ARPC2/p31, ARPC3/p21, ARPC4/p20, and ARPC5/p16; Higgs and Pollard, 2001). ARP2 and ARP3 are actin-related proteins. The structural analysis of the activated Arp2/3 complex implicates Arp2 and Arp3 as the actin nucleation sites by forming a heterodimer that resembles an actin dimer (Cooper et al., 2001; Robinson et al., 2001). The localization of the Arp2/3 complex is concentrated in the actin patches in yeast cells and at the leading edge of migrating animal cells (Welch et al., 1997a; Morrell et al., 1999; Schaerer-Brodbeck and Riezman, 2000a, 2000b; Higgs and Pollard, 2001). As a multifunctional organizer, the cellular roles of the Arp2/3 complex involve the control of actin polymerization nucleation for many cell functions such as cell movement, cell division, endocytosis, vesicle trafficking, and bacterial invasion (Welch et al., 1997b; Machesky and Gould, 1999; Schaerer-Brodbeck and Riezman, 2000a, 2000b; Higgs and Pollard, 2001). The Arp2/3 complex was discovered in *Amoeba* sp. cells (Machesky et al., 1994), but its function has been primarily studied genetically in the single-celled yeast and biochemically in mammalian cell culture. In complex multicellular organisms, the biological function of the Arp2/3 complex has been studied only in fruitfly (*Drosophila melanogaster*). A mutation of one Arp2/3 complex subunit, ARPC1, causes spindle fusion because of pseudocleavage furrow disruption in syncytical fruitfly embryos (Hudson and Cooley, 2002; Stevenson et al., 2002). Arabidopsis, with numerous advantages for genetic studies including the availability of various collections of T-DNA insertion mutants, provides a good model system to study the function of the Arp2/3 complex in higher eukaryotes.

The actin cytoskeleton is an important component of plant cell growth and development. Pharmacological and genetic studies suggest its role in a variety of cellular processes such as organelle movement, guard cell movement, cell polarity development, po-

larized cell growth, and cell division (Volkmann and Baluska, 1999; Belanger and Quatrano, 2000; Vantard and Blanchoin, 2002). Using live markers such as green fluorescent protein (GFP; Kost et al., 1998) fused to the actin-binding domain of the mouse talin and phalloidin staining in fixed cells, different types of actin structures have been observed in plant cells (Szymanski et al., 1999; Hepler et al., 2001; Frank and Smith, 2002). Actin cables, presumably composed of unbranched actin filaments, are detected in all living plant cells, whereas dynamic F-actin, composed of diffuse F-actin, is seen at the front of tip-growing pollen tubes and root hairs (Fu et al., 2001; Jones et al., 2002). Diffuse F-actin is also associated with the expanding parts of diffusely growing cells (Fu et al., 2002). The formation of both types of diffuse F-actin is dependent upon the Rho related GTPase from plant (ROP) subfamily of Rho GTPases, the plant relative of animal Rac/Cdc42 subfamilies of Rho GTPases known to signal the activation of the Arp2/3 complex (Fu et al., 2001, 2002; Higgs and Pollard, 2001; Jones et al., 2002). The assembly, reorganization, and dynamics of specific actin structures are regulated by intracellular and environmental signals in plants (McCurdy et al., 2001). Many of the conserved actin-binding proteins that regulate actin dynamics and organization have been identified and studied such as ADF, profilin, fimbrin, and actin motor proteins (Kovar et al., 2000; Ramachandran et al., 2000; Dong et al., 2001; McCurdy et al., 2001; McKinney et al., 2001). However, the molecular mechanism for actin nucleation in plants remains unknown.

The possible existence of the Arp2/3 complex in plants was suggested by the cloning of the *AtARP2* gene encoding the ARP2 homolog (Klahre and Chua, 1999). We have now identified Arabidopsis genes encoding six additional putative subunits of the Arp2/3 complex. These genes are ubiquitously expressed in various tissues. T-DNA insertions into any of the Arp2/3 subunit genes examined induced cell shape changes in epidermal pavement cells and trichomes in the leaf. The defects were associated with mislocalization of diffuse cortical F-actin and reduction of a fine actin network in both pavement cells and trichomes. This work provides genetic evidence showing a role for the Arp2/3 complex in cell morphogenesis in higher eukaryotes.

RESULTS

The Arabidopsis Genome Encodes All of the Putative Subunits of the Arp2/3 Complex

Conducting a blast search with the yeast Arp2/3 complex subunits, we identified six new putative Arp2/3 complex subunits from the Arabidopsis genome sequence. The coding sequences of Arabidopsis ARP3, ARPC1/p41, and ARPC3/p21 were predicted by The Arabidopsis Information Resource database, and the coding sequences of ARPC2/p34, ARPC4/p20, and ARPC5/p16 were predicted based on their homologous gene organizations in yeast. The coding sequences of the six subunits were confirmed through cloning and sequencing of cDNAs by reverse transcriptase (RT)-PCR using primers (supplemental data; they can be viewed at http://www.plantphysiol.org). Table I shows a comparison of the Arabidopsis homologs with those of human, *Dictyostelium* sp., fruitfly, budding yeast, and fission yeast (*Schizosaccharomyces pombe*). *AtARP3*, like *AtARP2*, shares about 50% identity with Arabidopsis *actins* in amino acid sequence (data not shown). All of the Arabidopsis *Arp2/3* complex subunits except for ARPC1/p41 are encoded by a single gene. ARPC1/p41 is encoded by two genes arranged in reverse orientation and in close proximity. The two Arabidopsis *ARPC1/p41* genes share very high nucleotide sequence identity both in the coding and promoter region. Protein motif analysis revealed seven WD-40 repeats in both *ARPC1/p41* sequences. ARPC1/p41, ARPC3/p21, and ARPC4/p20 share considerable amino acid identity with their counterparts in other organisms. As in other organisms, ARPC2/p34 and ARPC5/p16 are the most diverged of the seven subunits. RT-PCR analysis shows that transcripts for all seven subunits are expressed in all tissues examined (Fig. 1). All of the subunits have low expression levels, but with relatively high expression in inflorescences. Among these seven subunits, ARPC2/p34 and ARPC4/p20 have the lowest expression.

Loss of Function Mutants for the Putative Arabidopsis Arp2/3 Subunits

To investigate the function of Arp2/3 subunits, we obtained several mutants for Arabidopsis Arp2/3

Table I. *Comparison of Arabidopsis Arp2/3 complex subunits with their counterparts in other organisms*

	Human	Fruitfly	*Dictyostelium* sp.	Fission Yeast	Budding Yeast
ARP3	56[a]	55	57	53	51
ARPC1/p41	41	37	43	35	33
ARPC2/p34	26	26	33	26	28
ARPC3/p21	47	42	41	43	39
ARPC4/p20	65	61	68	56	59
ARPC5/p16	32	35	31	25	23

[a] The number indicates percentage of identity of predicted amino acid sequences of Arabidopsis Arp2/3 subunits compared with those from other species.
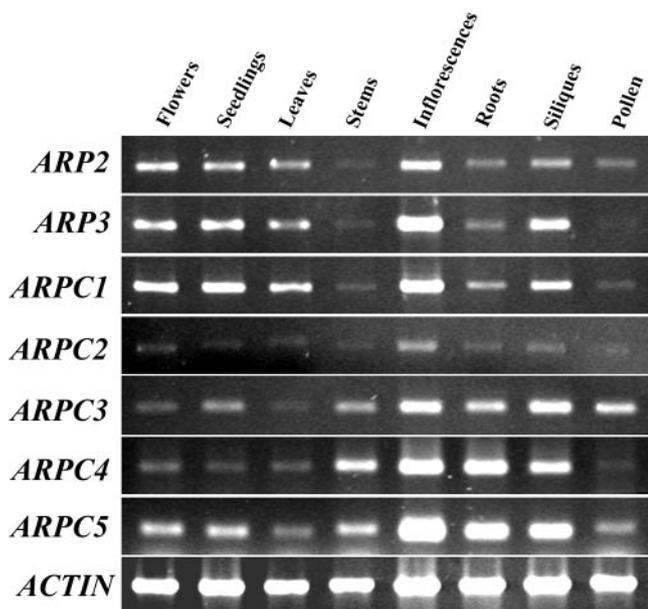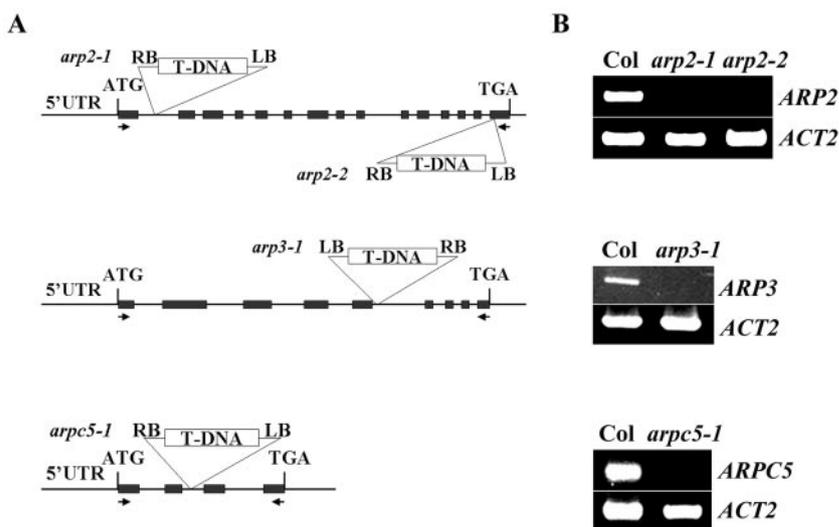
Figure 1. RT-PCR analysis of the transcript expression of the Arabidopsis Arp2/3 subunit genes in various tissues. RNA was isolated from organs of Arabidopsis COL-0 WT plants grown in light and used for RT-PCR analysis as described in the text. A 25-cycle PCR reaction was carried out for all genes except for ARPC4/p20, for which 30 cycles were used. *ACTIN2* was used as a control except for pollen RT-PCR that involves the use of the *ACTIN3* gene.

subunits from the SALK Arabidopsis T-DNA mutant collection (Fig. 2). *arp2-1* and *arp2-2* were isolated with a T-DNA insertion in the first intron and the last exon, respectively. *arpc5-1/p16-1* was isolated with a T-DNA insertion in the second intron, and *arp3-1* contains a T-DNA insertion in the fifth intron. RT-PCR analysis revealed that none of these four mutant lines had detectable transcripts for their respective genes (Fig. 2). We consider them to be strong and possibly null alleles. Homozygous mutant plants with a single T-DNA insertion were obtained for all

four mutants. By visual examination of these mutants under normal growth conditions in soil or on agar plates, we did not observe obvious changes in morphology as a whole plant in darkness or light. This is surprising, given the importance of actin nucleation in the regulation of actin organization as well as the essential role for the Arp2/3 complex in yeast.

The Putative Arabidopsis Arp2/3 Complex Is Required for Proper Leaf Epidermal Cell Morphogenesis

We next investigated whether the mutants are altered in cell shape formation, which has been linked to diffuse cortical F-actin (Szymanski et al., 1999; Fu et al., 2001, 2002; Frank and Smith, 2002; Jones et al., 2002; Frank et al., 2003). We first examined tip growing cells, root hairs, and pollen tubes. Surprisingly, neither the growth of pollen tubes nor root hairs was dramatically affected in all four mutants grown under normal conditions (data not shown). We then examined a possible effect of the Arp2/3 mutations on pavement cells, because their development has been linked to cortical F-actin as well. Mature pavement cells on the adaxial side of a wild-type (WT) leaf are jigsaw puzzle shaped with a striking outgrowth of lobes, giving a wavy cell outline. In all four mutants, including *arp2-1, arp2-2, arp3-1,* and *arpc5-1,* the outline of pavement cells was smoother and much less wavy (Fig. 3A). Quantitative analysis showed that the neck width was not affected, whereas the lobe height was dramatically reduced (Fig. 3, B–D).

We next examined the effects of mutation in the Arp2/3 complex on trichome morphogenesis. We did this because F-actin has been shown to play an important role in morphogenesis of this epidermal cell type (Mathur et al., 1999; Szymanski et al., 1999; Schwab et al., 2003). As shown in Figure 4, we observed a very striking defect in trichome morphogen-

Figure 2. Characterization of the Arp2/3 subunit mutants. A, The location of the T-DNA insertions in *arp2-1, arp2-2, arp3-1,* and *arpc5-1/ p16-1* are shown on the maps of *AtARP2, AtARP3,* and *AtARPC5/p16* genes. Arrows indicate the location of primers used for RT-PCR analysis of transcripts. Exons are boxed and introns and untranscribed flanking sequences are shown as lines. B, RT-PCR analysis of transcript levels for *arp2-1, arp2-2, arp3-1,* and *arpc5/ p16-1* mutants. Total RNA was extracted from inflorescences of each homozygous mutant plant and WT plants. The full-length coding sequence was amplified for each primer set.
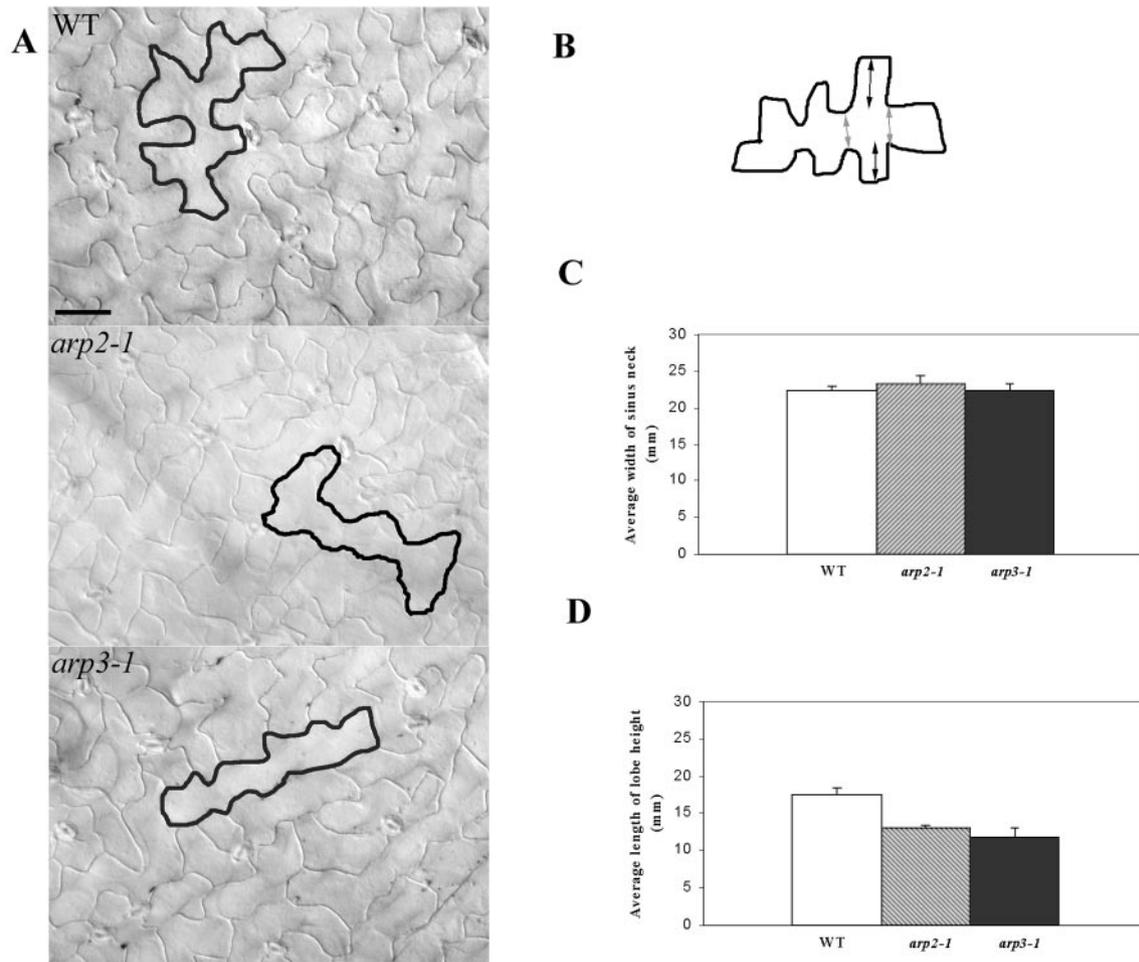
Figure 3. Characterization of leaf pavement cell shapes in *arp2-1* and *arp3-1* plants. A, Leaf pavement cell shapes. The fourth rosette leaves from 2-week-old plants were cleared, and differential interference contrast images were taken from the middle region of the cleared leaves. Leaves from WT plants and all mutant plants are of similar sizes. Tracing was used to highlight the cell shape of a typical pavement cell. Bars = 30 μm. B, A schematic diagram of a leaf pavement cell illustrates how the lobe height and neck width were measured as shown in C and D. The distance indicated by dark arrows represents the lobe height. The distance indicated by light arrows represents neck width. C, Comparison of the average width of the neck region between WT and mutant pavement cells. Measurements in both C and D were carried out using the fourth leaf of a 2-week-old plant, and 150 epidermal cells were used for each mutant line as well as WT. Statistical test (*t* test) shows no significant difference in neck width between WT and mutant pavement cells. D, Comparison of the average length of lobe heights between WT and mutant pavement cells. Statistical test (*t* test) shows that the length of lobe height is significantly reduced in the mutant pavement cell compared with the WT pavement cell.

esis in the mutant plants (Fig. 4). WT Arabidopsis trichomes typically have three branches. Mutants including *arp2-1*, *arp2-2*, *arp3-1*, and *arpc5-1* have very severe and identical trichome defects. Trichomes in these mutants had stunted branch outgrowth and unrestricted stalk expansion. Despite the striking defects in the trichome shape, trichome branch initiation was normal in all four mutants. The WT trichomes on the stem are usually without branches or only with two branches. The trichomes on the mutant stems clearly showed cell shape defects featured as a dramatic increase in radial expansion and irregular trichome shapes. The mutant trichome phenotype is very similar to that caused by treatment with actin-disrupting drugs such as cytochalasin D or

by mutations in the *DISTORTED* genes (Mathur et al., 1999; Schwab et al., 2003). This phenotype can be attributed to the combined defects in cell polarity development in stalks and polar growth in branches. Because branches are formed, the polarity establishment for branch formation is not affected.

The fact that *arp2-1*, *arp2-2*, *arp3-1*, and *arpc5-1* show almost identical phenotypes in leaf epidermal cell morphogenesis strongly suggests that the observed phenotype is due to a defect in the activity of the putative Arp2/3 complex. In fact, we have just obtained a T-DNA insertion mutant for *ARPC4/p20* from the SALK collection that shows an identical phenotype to the four mutants (S. Li, unpublished data). Furthermore, a second T-DNA insertion mu-
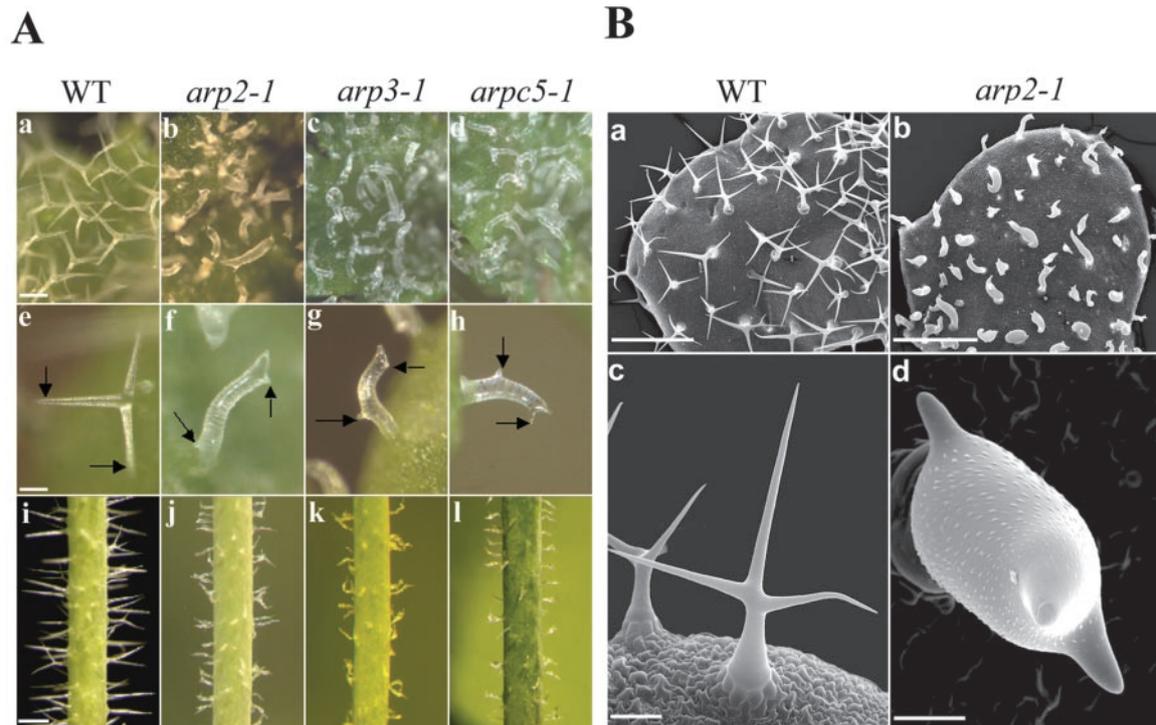
Figure 4. Trichome phenotypes of *arp2-1*, *arp3-1*, and *arpc5-1* mutants. A, Phenotype analyses of *arp2-1*, *arp3-1*, and *arpc5-1* mutant trichomes using light microscopy. a to d, Trichomes on a young leaf from WT and mutant plants. e to h, Single trichomes with branches from WT and mutant plants. Arrows point to the branches. i to l, Trichomes on stems from WT and mutant plants. a, e, and i are from WT plants. b, f, and j are from *arp2-1* plants. c, g, and k are from *arp3-1* plants. d, h, and l are from *arpc5-1* plants. Arrows in e to h point to the branches. Bar in a (100 μm) is the same for b through d. Bar in e (50 μm) is the same for f through h. Bar in i (500 μm) is the same for j through l. B, Scanning electron microscopic analyses of *arp2-1* trichomes. a and b show mature trichomes on a young leaf from WT and *arp2-1* plants, respectively. c and d show single trichomes with the typical three branches from WT and arp2-1 plants, respectively. Bar in a and b = 500 μm. Bar in c = 100 μm. Bar in d = 20 μm.

tant for ARP3 obtained from a different T-DNA insertion population showed the same phenotype as *arp3-1*. Therefore, we conclude that the putative ARP2/3 complex plays a critical role in the control of cell morphogenesis in Arabidopsis leaf epidermal pavement cells and trichomes. In the root epidermis, root hairs are slightly wavy, but other epidermal cells in the root appear to be normal in shape and size (data not shown).

The Spatial Distribution and Formation of Cortical F-Actin Is Altered in the Arp2/3 Subunit Mutants

To understand the mechanism by which the putative Arabidopsis Arp2/3 complex modulates cell morphogenesis, we investigated the actin cytoskeleton structure in these Arp2/3 subunit mutants. To visualize F-actin, an actin-binding domain of the mouse talin tagged with GFP (GFP-mTalin) was transiently expressed in leaf epidermal cells of mutant *arp2-1* and *arp3-1* as well as WT plants (Fu et al., 2002). In WT plants, the young pavement cells capable of expanding in all directions characteristically contain strong diffuse F-actin throughout the entire cell periphery, and a few actin cables localize to the

cell cortex and the cytoplasm. At this stage, no obvious F-actin structural change is detected in the pavement cells of either *arp2-1* or *arp3-1* mutants (Fig. 5A). When the WT pavement cells develop expanding lobes, patches of strong diffuse F-actin are found at the tips of the extending lobes. These actin patches are rarely observed in the nonexpanding neck region of WT pavement cells. In both *arp2-1* and *arp3-1* mutants, the lobes are initiated, but their growth is inhibited. Interestingly, the patches of strong cortical diffuse F-actin typically associated with lobes were reduced. Instead, cortical diffuse F-actin was more evenly distributed throughout the cell periphery. A quantitative analysis was performed to measure the percentage of association of strong diffuse F-actin with lobes in stage II cells from WT and mutant plants. In the mutant plants, the percentage of association of strong diffuse F-actin with lobes is significantly reduced compared with that of WT plants. Consequently, in the mutant cells with extending lobes, cortical diffuse F-actin was frequently found in the neck region. At this stage, actin cables are not extensive and seem to be randomly distributed both in WT and mutant pavement cells. In mature WT leaf pavement cells, F-actin exists mainly as extensive
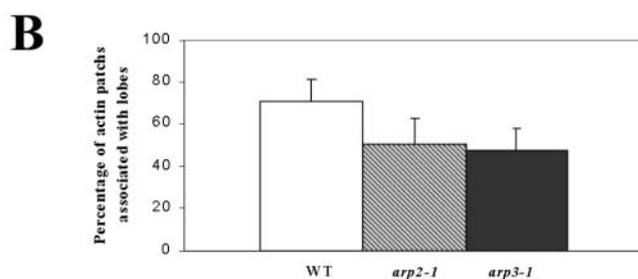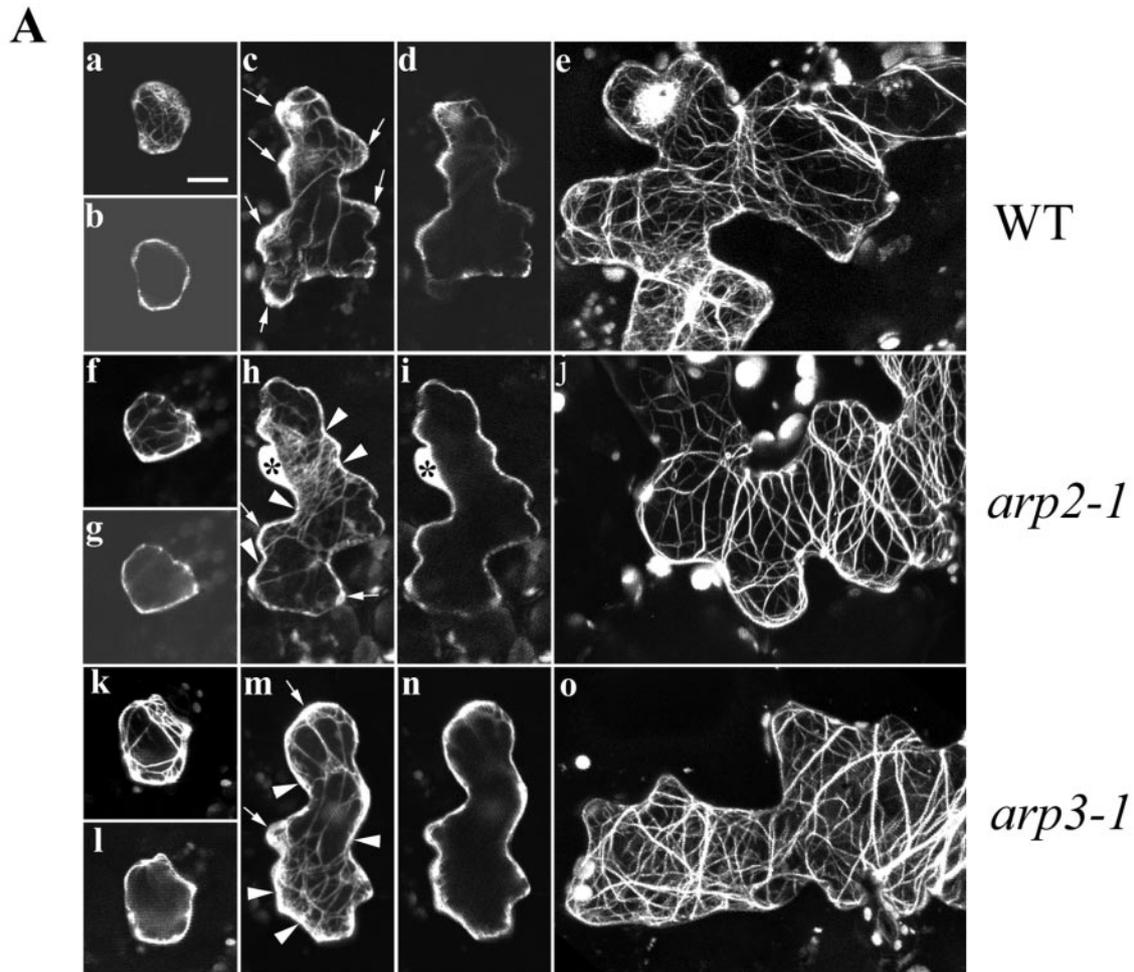
Figure 5. Analysis of actin organization in leaf pavement cells of *arp2-1* and *arp3-1* plants. A, F-actin organization in leaf pavement cells from *arp2-1* and *arp3-1* plants visualized by GFP-mTalin at different developmental stages. Leaves from 10-d-old plants were bombarded with GFP-mTalin construct, and cells expressing GFP-mTalin were imaged using confocal microscopy as described in the text. Pavement cells at three developmental stages are shown: b, g, and k, Stage I cells before lobe initiation; c, h, and m, stage II cells forming lobes; and e, j, and o, stage III cells with fully extended lobes. More than 60 cells were imaged for each stage of cell in every genotype. Shown are cells with representative actin organization. a to e, Leaf pavement cells of WT plants. f to j, Leaf epidermal cells of *arp3-1* plants. k to o, Leaf epidermal cells of *arp2-1* plants. a, c, e, f, h, j, k, m, and o show three-dimensional images projected from laser scanning. b, d, g, i, l, and n are single medial sections of a, c, f, h, k, and m, respectively. Arrows in c, h, and m point to the diffuse F-actin patches associated with growing lobes. Arrowheads in h and m point to diffuse F-actin associated with neck regions. Asterisks in h and i indicate the nucleus of the cell. Bar = 15 μm for all images. B, Quantitative analysis of association of diffuse cortical F-actin with the tip of expanding lobes in pavement cells. Measurements were done on 30 stage II cells by visual examination. The percentage of strong diffuse cortical F-actin associated with lobes for WT, *arp2-1*, and *arp3-1* pavement cells are 70.83 ± 10.57, 50.22 ± 12.08, and 47.08 ± 10.91, respectively. Statistical analyses (*t* test) showed that the percentage of strong diffuse F-actin associated with lobes of both mutant pavement cells is significantly reduced ($n = 30$, $P \leq 0.05$).

long actin cables that appear to be oriented randomly. In the mature pavement cells of *arp2-1* and *arp3-1* mutants, the actin cables were also quite extensive but tended to be oriented perpendicularly to the long axis of the cell. These observations suggest that mutations in the Arp2/3 subunit genes affect the spatial distribution, but not the assembly, of either cortical diffuse F-actin or actin bundles in Arabidopsis leaf pavement cells.

Actin organization in the trichomes of *distorted* mutants has been extensively characterized (Mathur et al., 1999; Szymanski et al., 1999; Schwab et al., 2003). To assess whether the Arabidopsis Arp2/3 complex affects the same pathway as the *DISTORTED* genes, we examined F-actin in the *arp2-1* and *arp3-1* mutant trichomes. Young WT trichomes contain network-like fine actin filaments that are oriented randomly and diffuse cortical F-actin in growing branches as well as the stalk. Thick actin cables are rarely detected in these cells. A network of fine actin bundles is found in the stalk of WT mature trichomes, whereas the mature branches usually contain longitudinal actin cables (Fig. 6). In both *arp2-1* and *arp3-1* mutants, actin structural defects were detected in the young as well as in the mature trichomes. Thick actin cables occurred in young mutant trichomes, whereas both fine actin network and diffuse F-actin were greatly reduced in their swollen stalks and stunted branches. In the mature mutant trichome, the severity of the F-actin structure alteration was also correlated with the deformation of the trichome. When the trichome shape was severely deformed with very limited growth of branches, transverse actin bundles were observed in both the stalk and the branches, analogous to the actin cytoskeleton in the mature pavement cells of the mutants. All of these actin cytoskeleton structure defects observed in Arp2/3 subunit mutants are similar to the description of *DISTORTED* mutants (Mathur et al., 1999; Schwab et al., 2003). Previous work has shown that disruption of the actin cytoskeleton by cytochalasin D induces the same cell shape changes in trichomes, suggesting that it is the loss of a certain actin population such as the fine actin network and diffuse actin that are responsible for the trichome phenotypes.

DISCUSSION

In this work, we have identified all subunits of the putative Arabidopsis Arp2/3 complex and investigated the function of three subunit genes using T-DNA insertion mutants. This work represents an important step of the description and functional analysis of a conserved actin-nucleating machinery in the plant kingdom. Importantly, we have demonstrated a critical role for the putative Arabidopsis Arp2/3 complex in cell polarity development and cell morphogenesis. Similar functional roles for this complex have been reported in yeast but not in any multicellular organisms. Interestingly, our results suggest that the putative Arabidopsis Arp2/3 complex modulates polar cell expansion probably through localizing diffuse cortical actin to the site of growth, a role previously unknown for the Arp2/3 complex in other organisms. However, this complex appears to control cell polarity development in trichomes through activating the formation of a network of cortical fine actin bundles. Similarly, this complex is known to activate the formation of cortical actin patches in yeast and a dense actin network in the leading edge of migrating *Dictyostelium* sp. and animal cells.

Genetic studies in yeast have shown that the Arp2/3 complex is an essential actin nucleation machinery (Winter et al., 1999). Deletion of any subunit gene causes defects in either actin patch formation or actin patch movement, leading to lethality. However, none of the homozygous T-DNA mutants for the Arabidopsis Arp2/3 subunits affect the viability of the plants or cause dramatic defects in organ development. This situation is somewhat similar to the observations in multicellular animals. The Arp2/3 complex has been identified in human and various animal model systems (fruitfly, *Caenorhabditis elegans*, and *Dictyostelium* sp.). The loss-of-function mutations in fruitfly ARPC1 and ARP3 genes only affect a subset of actin rearrangements and cause defects in ring canal expansion during oogenesis (Hudson and Cooley, 2002), whereas RNAi suppression of any of the Arp2/3 subunit genes in *C. elegans* only affect ventral closure (Sawa et al., 2003). Interestingly, none of our arp2/3 subunit mutants show dramatic developmental defects. It is possible that loss of Arp2, Arp3, or ARPC5 gene alone does not completely disrupt the activity of the complex. It has been shown that the human Arp2/3 complex subunits p34 and p16 are not essential for the activity of the complex in a reconstitution assay system (Gournier et al., 2001). It will be interesting to see whether a phenotype related to actin cytoskeleton dynamics is more severe in a mutant in which more than one Arabidopsis Arp2/3 subunit units are knocked out.

In animal cell systems, it has been proposed that a predominant role of this actin-nucleating complex is to promote the polymerization of branched actin filaments and the formation of a highly dense branched actin network in the leading edge that allows membrane protrusion and cell migration. Indeed, genetic studies in fly and worm have shown that the Arp2/3 complex is important for cell migration (Hudson and Cooley, 2002; Sawa et al., 2003). Therefore, our findings about the function of the Arp2/3 complex in cell morphogenesis in pavement cells and trichomes are interesting. Shape formation in these cells is dependent upon the spatial pattern of deposition and remodeling of cell walls, which bear the turgor pressure that drives cell expansion. This raises an interesting question: whether the plant Arp2/3 com-
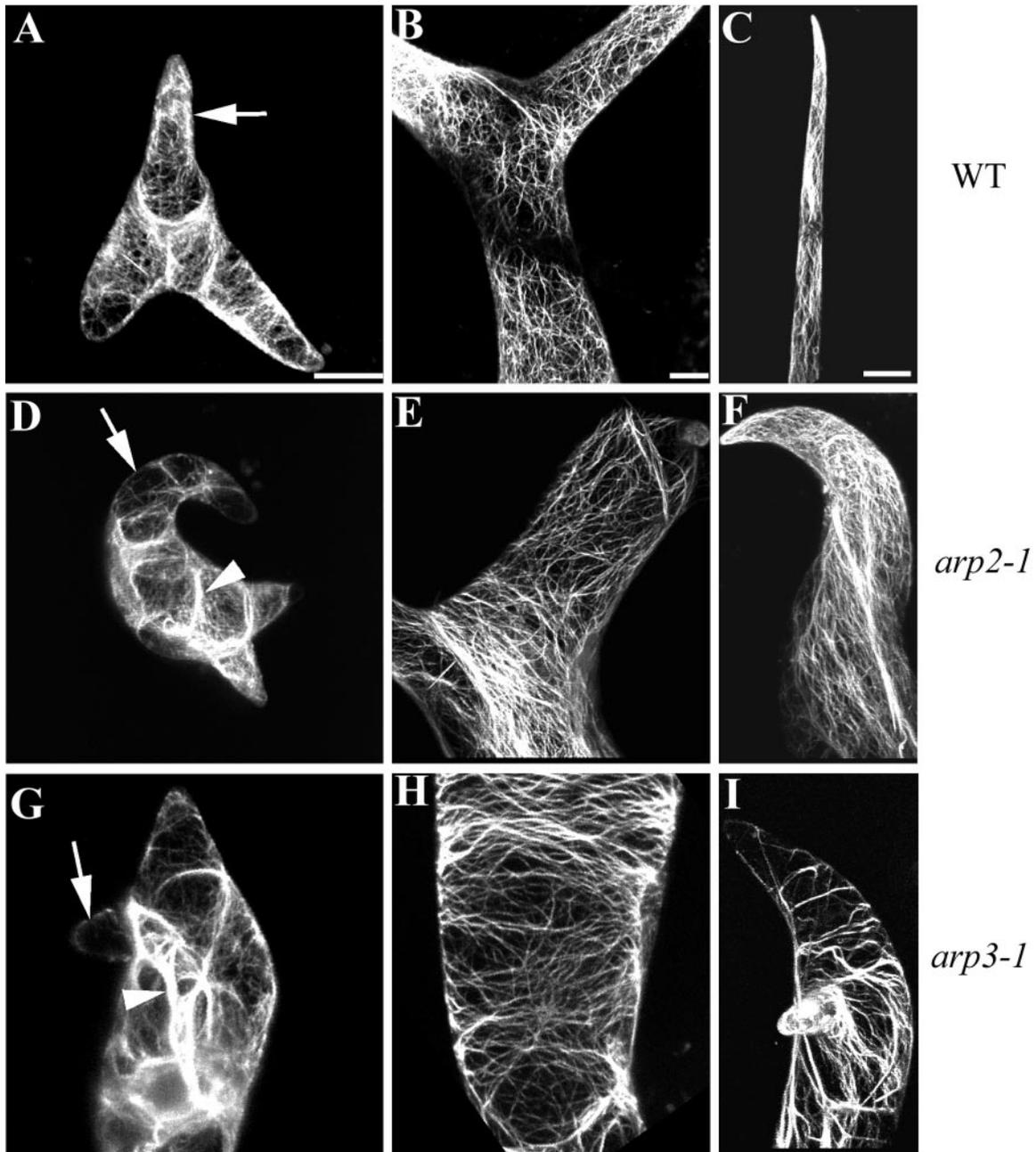
Figure 6. Actin organization in trichomes of *arp3-1* and *arp2-1* plants. F-actin was visualized as described in Figure 5. All images shown are projections. Bar in A is for A, D, and G. Bar in B is for B, E, and H. Bar in C is for C, F, and I. Bar = 20 μm. A, D, and G, Young trichomes from a WT, *arp2-1*, and *arp3-1* plants, respectively. Arrow in A points to diffuse cortical F-actin signal in a rapid growing branch. Arrows in D and G indicate the loss of fine F-actin in the mutant branches. Arrowheads in D and G point to thick actin cables. B, E, and H, Stalks of mature trichomes from WT, *arp2-1*, and *arp3-1* plants, respectively. C, F, and I, Branches of mature trichomes from WT, *arp2-1*, and *arp3-1* plant, respectively.

plex has a distinctly different cellular and biochemical function from the animal counterpart, or whether shape formation in leaf epidermal cells such as pavement cells and trichomes necessitates a more complex model than previously posed, one in which cells can accommodate targeted expansion requiring the Arp2/3 complex-mediated actin polymerization or remodeling.

The defects in the expansion of pavement cell lobes and trichome branches observed in all mutants examined define a role for the putative Arp2/3 complex in the modulation of polar cell expansion, which is crucial for cell morphogenesis in plants. How might the Arp2/3 complex control polar cell expansion? In leaf pavement cells, lobe expansion is thought to require a population of ROP GTPase-

dependent diffuse F-actin localized to the tip of lobes, which might function to deliver cell wall and cell membrane materials to the active growing site via post-Golgi vesicles. In the Arp2/3 subunit mutant plants, lobes are formed, but their extension is severely inhibited. This is consistent with our observation that the diffuse cortical F-actin, although decreased in amount due to a more random distribution, is found in the lobes of the mutant pavement cells. The ectopic distribution of the diffuse cortical F-actin in the neck region in the mutant did not lead to increased expansion in that region, most likely due to the presence of a mechanism to suppress cell expansion here, such as transverse cortical microtubules (Mathur and Hulskamp, 2002). We propose that the random distribution of the diffuse cortical F-actin is the direct result of the defect in the Arp2/3 complex activity and that it is not due to cell shape changes per se. Evidence to support this is the fact that DN-rop2 expression also inhibits lobe extension but does not cause redistribution of the cortical F-actin (Fu et al., 2002). From these observations, we conclude that at least one function of the Arp2/3 complex is to modulate the localization of diffuse cortical F-actin in plant cells.

During trichome branch formation, a diffuse form of F-actin is also apparent near the tip of the branch in WT plants and is reduced in the stunted mutant branches. This reduction could be due to its random distribution, as occurs in pavement cells, or to its reduced assembly. The dramatic isotropic growth in the mutant trichome stalks makes it difficult to distinguish between these possibilities or to determine whether the change in the actin cytoskeleton is the result or the cause of cell shape changes. In maize (*Zea mays*) leaf pavement cells, mutations in *BRK1*, which encodes a homolog of HSPC300 that regulates the Arp2/3 complex in mammalian cells, eliminate lobes and associated diffuse cortical actin (Frank et al., 2003). This raises the possibility that the Arp2/3 complex may be required for the assembly of a fine actin network in some plant cells. Additionally or alternatively, the Arabidopsis Arp2/3 complex could regulate polar cell expansion through other mechanisms, e.g. targeting of post-Golgi vesicles or movement of other endomembrane structures or organelles (which ultimately affect polar secretion). Arp2/3 complex-mediated actin polymerization has been shown to regulate the trafficking of vesicles (Rozelle et al., 2000; Fucini et al., 2002; Luna et al., 2002; Stamnes, 2002) and pathogen movement in animal cells (Welch et al., 1997b). In budding yeast, *arc35-1*, a mutation in the ARPC2 subunit, affects calmodulin localization to the active growing sites where rapid endocytosis and exocytosis occur. Overexpression of calmodulin in the *arc35-1* cells suppresses the defects both in the actin and microtubule cytoskeleton. Given that so many proteins need to be specifically targeted to their destination, the Arp2/3 complex may receive various signals to activate actin polymerization during multiple steps of vesicle trafficking to confer the specificity of vesicle targeting.

The isotropic growth in the trichome stalk of the Arp2/3 subunit mutants is very interesting because it suggests a role for the Arp2/3 complex in the control of cell polarity development. This role is similar to one of the roles of the yeast Arp2/3 complex (Morrell et al., 1999). Mutations in several Arp2/3 subunits cause defects in the establishment of cell polarity in the mother cell during cell division, leading to the unrestricted isotropic growth of the mother cell. This defect is thought to be due to the abolishment of actin patch-mediated endocytosis, which is essential for cell polarity establishment. Interestingly, we found that the swollen mutant stalk lacks the extensive cortical network of fine actin bundles normally found in WT trichome stalks, but contains some thick actin bundles. Complete disruption of F-actin by cytochalasin D phenocopies the Arp2/3 subunit mutants (Mathur et al., 1999), suggesting that it is the loss of some actin population(s) that is responsible for the depolarization of the stalk. The formation of thick actin bundles is probably an indirect effect due to the increased G-actin pool from the loss of a fine actin network. Thus, these observations imply that the Arabidopsis Arp2/3 complex controls the establishment of cell polarity by regulating the assembly of a fine actin network.

An interesting question would be: How can this actin network control cell polarity, through endocytosis as in yeast or by other mechanisms? It was proposed that the phenotype of *distorted* mutants is a result of disrupted directional vesicular trafficking (Mathur et al., 1999). Directed vesicle targeting could be controlled by the fine actin network or directly by the Arp2/3 complex-mediated actin polymerization. Interestingly, a recent study on the *distorted* mutants combined with cytochalasin treatments suggest that F-actin may regulate the orientation of cortical microtubules, which could directly control cell polarity (Mathur et al., 1999). Given the conserved functional role for the Arp2/3 complex in cell polarity (Mellman and Warren, 2000), it is important and interesting to determine the mechanism by which the Arabidopsis Arp2/3 complex regulates this fundamental process.

Another interesting question that needs future attention is how the Arabidopsis Arp2/3 complex is regulated. The Wiskott-Aldrich Syndrome domains family of proteins, the effectors of Cdc42 and the activators of the Arp2/3 complex, have not been found in the Arabidopsis genome. Furthermore, ROP GTPases do not seem to activate the formation of diffuse cortical F-actin through the Arp2/3 complex in pavement cells, and the DN-rop2 expression does not produce trichome defects, although it eliminates diffuse cortical F-actin in pavement cells (Fu et al., 2002). Additionally, *arp2-1* and *arp3-1* mutant plants have minor defects in root hair and pollen tube

growth (S. Li, unpublished data), processes that show tip growth dependence on actin and ROP GT-Pases (Fu et al., 2001; Jones et al., 2002). These observations suggest that additional actin polymerization mechanism(s) exist in plants. In yeast and animals, various Arp2/3 activators that are structurally divergent have been identified besides the Cdc42/Wiskott-Aldrich Syndrome domains (Higgs and Pollard, 2001). Therefore, the plant Arp2/3 complex is likely to be regulated by novel mechanisms. The identification of the plant activators of the Arp2/3 complex will be of tremendous interest in the future. Development of an in vitro assay for the plant Arp2/3 complex will be essential for the identification of its activators.

MATERIALS AND METHODS

Database Search and Bioinformatics

To identify additional putative Arabidopsis Arp2/3 subunits, the yeast Arp2/3 subunit cDNAs (Winter et al., 1997) were used as queries for a BLAST search of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) and The Arabidopsis Information Resource database (http://www.arabidopsis.org). From this search, we identified putatively annotated coding sequences for genes of *ARP3*, *ARPC1*, and *ARPC3*. The coding sequences for *ARPC2*, *ARPC4*, and *ARPC5* were predicted based on the gene organizations of their counterparts in yeast. The accession numbers for ARP3 ARPC1, ARPC2, ARPC3, ARPC4, and ARPC5 are: At1g13180, At2g30910/At2g31300, At1g30825, At1g60430, At4g14140, and At4g01710, respectively. The amino acid alignment was conducted by ClustalW program (http://www.ebi.ac.uk/clustalw).

DNA Manipulation and Plasmid Construction

To confirm that these six annotated Arp2/3 subunit sequences are indeed expressed, we amplified cDNAs for each subunit from a flower cDNA library (a gift from Hong Ma [Pennsylvania State University, University Park, PA]) using PCR primers covering the predicted Arp2/3 subunit-coding sequences (see supplemental data). PCR fragments of each were then subcloned into an AT cloning vector pGEM-T (Invitrogen, Carlsbad, CA). The resulting six clones were then sequenced.

T-DNA Mutant Identification and Reverse Transcription and PCR Analysis

Putative T-DNA lines were ordered from the Arabidopsis Biological Resource Center. The accession numbers for *arp2-1*, *arp2-2*, *arp3-1*, and *arpc5-1/p16-1* are SALK_03448, SALK_077920, SALK_010045, and SALK_123936, respectively. For *arp2-1*, *arp2-2*, and *arpc5-1/p16-1*, T-DNA insertions were verified by PCR reactions using T-DNA LB primers and gene-specific primers. Homozygotes of each line were identified by RT-PCR using RNA extracted from inflorescences.

For the analysis of the transcription of each subunit, total RNA was isolated from different Arabidopsis tissues using the TRIZOL Reagent (Invitrogen). For silique and stem RNAs, two additional chloroform extractions were performed. Reverse transcription and PCR amplification were carried out as described previously. For the *ARPC4* gene, 30 cycles of PCR amplification were used. For other Arp2/3 subunit genes, 25 cycles of PCR amplification (94°C for 45s, at 54°C for 45s, and at 72°C for 1 min 30s) were carried out using primers shown in Table I. As PCR amplification and loading controls, the same template cDNA was amplified using primers for the constitutive *actin-2* gene. The *actin-3* gene was used as a control in pollen RT-PCR. Twenty microliters of each PCR product was loaded on an 0.8% (w/v) agarose gel to visualize the amplified subunit cDNAs. Five microliters of actin PCR product was loaded as a control.

Light Microscopy Analysis of Leaf Epidermal Cell Shapes and Trichome Phenotypes

All experiments described in this study involve Arabidopsis ecotype Col-0. Arabidopsis plants were grown in growth chambers at 25°C under a light regime of 8 h of darkness and 16 h of light. The morphology of leaf pavement cells from WT and mutant plants was analyzed using cleared intact leaves as described previously (Fu et al., 2002). The fourth rosette leaf from 2-week-old plants was immersed in 5% (w/v) NaOH, boiled for 1 min, washed with distilled water, and incubated in bleach until it became clear. Cleared leaves were observed with an inverted microscope (Eclipse TE300, Nikon, Tokyo) equipped with a cooled CCD camera (C4742–95, Hamamatsu, Hamamatsu City, Japan). The measurement of neck width and lobe height of pavement cells was conducted using Metamorph 4.5 software (Universal Imaging, West Chester, PA). Trichome phenotypes were analyzed using a M2 Bio Epi-fluorescence microscope (Zeiss, Thornwood, NY) with a digital camera.

Scanning Electron Microscopy

Ten-day-old seedlings were fixed in 37% (w/v) formaldehyde, glacial acetic acid, 70% (v/v) ethanol (5:5:90, v/v) solution for 48 h and dehydrated in an ethanol series (70%, 75%, 80%, 85%, 90%, 95%, and 100%, v/v). After critical point drying and coating, samples were observed with a Philips XL30-FEG scanning electron microscope (FEI Company, Hillsboro, OR). Images were taken and processed using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA)

Particle Bombardment-Mediated Transient Expression in Arabidopsis Leaves

Rosette leaves collected from 10-d-old plants were used for transient expression using a particle bombardment procedure described previously (Fu et al., 2002). All plasmid DNAs were amplified in the *Escherichia coli* strain Top 10 and purified using Qiagen plasmid Midi or Mini Kits according to the manufacturer's instructions (Qiagen USA, Valencia, CA). Routinely, 0.5-mg gold particles were coated with 0.5 μg of pBI221:GFP-mTalin DNA. Bombarded leaves were kept on damp filter paper before observation under the confocal microscope as described below.

Visualization of F-Actin and Confocal Microscopy

To visualize F-actin in the leaf pavement cells and trichomes, GFP-mTalin was expressed transiently as described above. Sixteen to 24 h after bombardment, leaf pavement cells or trichomes expressing GFP-mTalin were observed using a confocal microscope (MRC 600, Bio-Rad Laboratories, Hercules, CA) as described previously (Fu et al., 2001). Optical sections (0.5 μm) were used to collect a three-dimensional projection. Confocal images were analyzed using Metamorph 4.5 software and processed using Adobe Photoshop 5.5. For the quantification of association of strong diffuse F-actin with lobes, confocal projection images of actin cytoskeleton in stage II pavement cells from both WT and both mutant plants were randomly mixed together. The counting was based on visual examination. After sorting, the percentage of diffuse F-actin associated with lobes was assigned to individual samples.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all parts of the material. Obtaining any permission will be the responsibility of the requestor.

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