The Nuclear Actin-Related Protein ARP6 Is a Pleiotropic Developmental Regulator Required for the Maintenance of FLOWERING LOCUS C Expression and Repression of Flowering in Arabidopsis

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Actin-related proteins (ARPs) are found in the nuclei of all eukaryotic cells, but their functions are generally understood only in the context of their presence in various yeast and animal chromatin-modifying complexes. Arabidopsis thaliana ARP6 is a clear homolog of other eukaryotic ARPs, including Saccharomyces cerevisiae ARP6, which was identified as a component of the SWR1 chromatin remodeling complex. We examined the subcellular localization, expression patterns, and loss-of-function phenotypes for this protein and found that ARP6 is localized to the nucleus during interphase but dispersed away from the chromosomes during cell division. ARP6 expression was observed in all vegetative tissues as well as in a subset of reproductive tissues. Null mutations in ARP6 caused numerous defects, including altered development of the leaf, inflorescence, and flower as well as reduced female fertility and early flowering in both long- and short-day photoperiods. The early flowering of arp6 mutants was associated with reduced expression of the central floral repressor gene FLOWERING LOCUS C (FLC) as well as MADS AFFECTING FLOWERING 4 (MAF4) and MAF5. In addition, arp6 mutations suppress the FLC-mediated late flowering of a FRIGIDA-expressing line, indicating that ARP6 is required for the activation of FLC expression to levels that inhibit flowering. These results indicate that ARP6 acts in the nucleus to regulate plant development, and we propose that it does so through modulation of chromatin structure and the control of gene expression.

INTRODUCTION

Members of the actin-related protein (ARP) family display between 17 and 60% amino acid identity with conventional actins and show a range of small insertions and deletions relative to actin and to one another (Frankel and Mooseker, 1996; Machesky and May, 2001). Despite a great diversity in sequence, even the most divergent ARPs are thought to retain the actin fold tertiary structure characteristic of the actins (Kabsch and Holmes, 1995; Robinson et al., 2001). The ARP family was initially described in Saccharomyces cerevisiae, whose genome encodes 10 ARPs. These proteins were named ARP1 through ARP10 based on their degree of similarity to actin, with ARP1 being most similar and ARP10 least similar to actin (Poch and Winsor, 1997). This nomenclature has since been applied to ARPs discovered in other organisms as well. More recent phylogenetic analyses of ARPs from a wide range of organisms suggest that the 10 ARPs of S. cerevisiae are representative of at least eight ancient ARP subfamilies that are conserved throughout the eukaryotic kingdom (Goodson and Hawse, 2002; Kandasamy et al., 2004).

None of the ARPs are known to form the long filamentous polymers characteristic of actin, and in fact the only unifying functional characteristic yet to emerge among the ARPs is their apparently invariant inclusion in large multiprotein complexes. Based on their subcellular localization, the ARPs can be broadly categorized as either cytoplasmic or nuclear. Members of four ARP classes (ARP1, 2, 3, and 10) are consistently found in the cytoplasm of all organisms examined, and these proteins are known to function within complexes that play accessory roles in the actin and tubulin cytoskeletal systems (Schafer and Schroer, 1999; Machesky and May, 2001). The remaining ARPs (ARP4, 5, 6, 7, 8, and 9) are all found in the nucleus of S. cerevisiae and other organisms in which they have been examined (Frankel et al., 1997; Harata et al., 2000; Kandasamy et al., 2003). The functions of the nuclear ARPs are less clearly defined, but like the cytoplasmic ARPs, most are known to be stable components of large protein complexes that often contain more than one ARP and sometimes monomeric actin. All of the nuclear ARPs that have been studied in detail are constituents of either ATP-dependent nucleosome remodeling complexes or histone acetyltransferase complexes, both of which are involved in the modification of chromatin structure and, thus, the regulation of transcription and other DNA transactions (Olave et al., 2002; Shen et al., 2003).

Until recently, our knowledge of ARP6 function lagged behind that of the other nuclear ARPs and was limited to a few qualitative
observations in yeast and Drosophila. In both of these organisms, the protein was shown to be localized to the nucleus, and in Drosophila, it colocalized with heterochromatin protein 1 in pericentric heterochromatin, suggesting a possible role in heterochromatin function (Frankel et al., 1997; Harata et al., 2000). In the past year, two groups have shown that ARP6 is a component of the S. cerevisiae SWR1 chromatin remodeling complex that functions to replace histone H2A with the variant H2A.Z at specific chromosomal locations (Krokan et al., 2003; Mizuguchi et al., 2004). This conserved histone variant acts partly to antagonize the spread of silent heterochromatin into euchromatic regions (Meneghini et al., 2003), but it also has important heterochromatric functions (Dryhurst et al., 2004; Fan et al., 2004). Another recent report indicates that Schizosaccharomyces pombe ARP6 binds to telomeres and is required to maintain the silencing of transgenes inserted into heterochromatic regions in the telomere but not transgenes in the centromere (Ueno et al., 2004). These results indicate that S. pombe ARP6 may play a role in the maintenance of telomeric heterochromatin.

Despite these recent advances in our understanding of ARP6 function in fungi, the role of an ARP6 has not been addressed in the context of the development of a multicellular organism. Here, we report an analysis of the expression patterns, subcellular localization, and loss-of-function phenotypes for Arabidopsis thaliana ARP6, a homolog of ARP6s from fungi and animals. We found that Arabidopsis ARP6 is expressed in most organs and tissues and is localized to the nucleus. Loss of ARP6 function in Arabidopsis leads to defects in the development of the leaf, inflorescence, and flower as well as reduced female fertility and early flowering in both long- and short-day photoperiods. The premature transition from vegetative to reproductive development in arp6 mutants results at least in part from a reduction in the expression of the floral repressor genes FLOWERING LOCUS C (FLC), MADS AFFECTING FLOWERING 4 (MAF4), and MAF5. Taken together, our results are consistent with a role for ARP6 in the chromatin-level control of multiple genes.

RESULTS

Arabidopsis ARP6 Is a Member of the Conserved Eukaryotic ARP6 Class

Based on overall amino acid sequence similarity, the Arabidopsis protein that we have named ARP6 is most closely related to members of the ARP6 class. Arabidopsis ARP6 is 421 amino acids in length and is 25% identical to S. cerevisiae ARP6 and 33% identical to human ARP6. Through phylogenetic analysis, we sought to determine whether Arabidopsis ARP6 represents a true member of the ARP6 subfamily. The amino acid sequences of 23 putative nuclear ARPs from a wide variety of eukaryotes were aligned along with conventional actins, and the resulting alignments were used to generate phylogenies by maximum parsimony, neighbor joining, and UPGMA methods. These methods all produced comparable results and strongly indicated that Arabidopsis ARP6 is indeed a member of the ARP6 class, as illustrated in the neighbor-joining tree presented in Figure 1. In addition, we found that the Oryza sativa genome also encoded a single 429–amino acid ARP6 homolog that was 62% identical to Arabidopsis ARP6, suggesting that the ARP6 protein may be conserved throughout the angiosperms and is therefore likely to play an important role in the biology of higher plants.

ARP6 Is Expressed throughout the Plant

We first investigated the organ- and tissue-level expression patterns of ARP6 through the use of immunoblotting and expression of a transgene carrying the β-glucuronidase (GUS) coding sequence driven by ARP6 5’ and 3’ regulatory sequences. In order to examine the organ-level expression patterns of ARP6, we raised monoclonal antibodies against purified recombinant ARP6 protein in mice. Two independent monoclonal antibodies were isolated (mAbARP6a and mAbARP6b) and both reacted with the 47-kD recombinant ARP6, but they did not react with the closely related Arabidopsis ARP4 or ARP7 (Figure 2). Both antibodies also recognized only a single band of

Figure 1. Relationships between ARP6 and Related Proteins.

The neighbor-joining tree shows representative actin, ARP4, ARP5, and ARP6 sequences from Arabidopsis and other eukaryotes. Each clade is denoted by a vertical bar to the right of the phylogram: At, Arabidopsis thaliana; Bb, Branchiostoma belcheri (lancelet); Ce, Caenorhabditis elegans; Dr, Drosophila melanogaster; Dr, Danio rerio; Gg, Gallus gallus; Hs, Homo sapiens; Os, Oryza sativa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe. Bootstrap values are shown at each branch point. Arabidopsis and rice proteins are shown in bold.
the expected size when tested against plant protein extracts (Figure 3A), indicating that these antibodies are highly specific to ARP6. Protein extracts from 7-d-old seedlings, 15-d-old plants, rosette leaves, cauline leaves, stems, flowers, siliques, seeds, and roots were examined by immunoblotting. We found that ARP6 was expressed at a detectable level in all of these organs but was most abundant in flowers and siliques (Figure 3A).

Examination of plants expressing a GUS transgene under control of the ARP6 regulatory sequences also indicated a wide-ranging distribution of ARP6 expression throughout the plant. The GUS reporter gene was expressed strongly in all vascular tissues of the plant and in all aboveground vegetative tissues. Reporter expression was restricted mainly to the vasculature in young roots, but it was expressed more widely as the root system developed (Figures 3C and 3D). In flowers, GUS expression was observed in the sepals and vascular tissues of the petals and filaments, but it appeared to be restricted within the gynoecium to the stylar tissue (Figures 3E and 3F). However, following fertilization and expansion of the developing fruit, the reporter became active and was expressed strongly in the fruit wall (Figure 3G). The ARP6 regulatory sequences did not appear to be active in the anthers, pollen, stigma, or ovary (Figure 3F).

The results shown in Figure 3 were from a single transgenic line but were representative of multiple, independent transgenic lines expressing the reporter construct. The broad pattern of expression observed by immunoblotting and GUS reporter expression

**Figure 2.** Specificity of Anti-ARP6 Monoclonal Antibodies.

(A) Coomassie blue-stained SDS-polyacrylamide gel showing *Escherichia coli* protein extracts containing recombinant ARP4, ARP6, or ARP7. The position of each ARP is indicated by an arrowhead. (B) and (C) Duplicates of the gel shown in (A) were blotted and probed with the monoclonal antibodies mAbARP6a (B) and mAbARP6b (C).
suggested that ARP6 might be important for the proper growth and development of most plant organs.

**ARP6 Is a Nuclear Protein**

The subcellular localization of Arabidopsis ARP6 was examined by indirect immunofluorescence labeling and expression of an ARP6:green fluorescent protein (GFP) fusion. Immunolocalization of ARP6 in leaf and root tissue with the mAbARP6a antibody showed that the protein was localized mainly to the nucleoplasm of interphase nuclei (Figures 4A to 4D). However, the ARP6 protein was dispersed away from the chromosomes during cell division (see anaphase cell outlined in Figures 4C and 4D). This behavior was observed previously for Drosophila ARP6 (Frankel et al., 1997) as well as for Arabidopsis ARP4 and ARP7 (Kandasamy et al., 2003). Labeling of cells with the mAbARP6b antibody revealed an identical staining of the nucleoplasm (data not shown). As an independent confirmation of these findings, a fusion of GFP to the C terminus of ARP6 was constructed and used to transfet maize (Zea mays) mesophyll protoplasts. This cauliflower mosaic virus (CaMV) 35S promoter-driven fusion protein also accumulated in the nuclei of transfected cells (Figures 4E to 4G), thus confirming the nuclear localization of Arabidopsis ARP6.

**ARP6 Has Photoperiod-Dependent Effects on Leaf Development**

As a means of addressing the functional role of ARP6 in the biology of Arabidopsis, we obtained from the Torrey Mesa Research Institute two T-DNA insertion alleles, which we designated arp6-1 and arp6-2. The arp6-1 allele carried an insertion in exon 1, while the arp6-2 allele had an insertion in exon 4 (Figure 5A). The location of each insertion was confirmed by PCR and sequencing of the junction regions. Immunoblot analysis of whole-plant protein extracts from wild-type, arp6-1, and arp6-2 plants showed that ARP6 protein could only be detected in the wild-type plants and not in either mutant, regardless of which antibody was used (Figure 5B). The exonic insertion sites and lack of detectable protein suggested that both alleles were null. These two mutant alleles also produced indistinguishable phenotypes, as elaborated below.

A striking defect was observed in leaf development in the homozygous arp6 mutants. When grown under long-day conditions, the leaves of arp6-1 plants were dramatically smaller.
than those of the wild type at all developmental stages (Figure 6A). Scanning electron microscopic examination of the adaxial surface of third rosette leaves from 27-d-old wild-type and arp6-1 plants revealed that the average epidermal cell size was the same in both wild-type and arp6-1 leaves (Figures 6C to 6E), indicating that the mutant leaves were composed of fewer total cells rather than a normal number of smaller cells. These data suggest that ARP6 promotes cell proliferation in the leaf during growth under long-day conditions.

By contrast, when plants were grown in short days, the morphology of arp6-1 leaves was dramatically different from that of wild-type plants, but the developmental defect was not the same as that observed under long-day conditions. Short-day-grown arp6-1 plants displayed early juvenile leaves with

Figure 6. Leaf Development in Long- and Short-Day-Grown arp6-1 Plants.

(A) Third, fifth, and seventh rosette leaves of 27-d-old wild-type and arp6-1 plants grown under long-day conditions. One leaf of each stage was taken from three individual plants.

(B) Early (E), middle (M), and late stage (L) leaves of 50-d-old wild-type and arp6-1 plants grown in short days. One leaf of each stage was taken from three individuals.

(C) and (D) Scanning electron micrographs of the adaxial surface of third rosette leaves from 27-d-old wild-type (C) and arp6-1 (D) plants. Insets show a higher magnification view of the same stage leaves. A single cell is outlined in each inset for comparison.

(E) Graph showing the average area (µm²) of adaxial epidermal cells from third rosette leaves of 27-d-old plants grown in long days. Error bars indicate 50.
undulating margins and adult leaves that were deeply serrated along the margins. At all stages of development, the arp6-1 leaves were narrower than the wild type but of comparable length (Figure 6B). Thus, under short-day conditions, arp6-1 mutant leaves were of similar length to the wild type but were deficient in cell proliferation and/or expansion in the leaf-width direction and also had strong defects in margin patterning.

**AR6P Regulates Inflorescence and Flower Development**

We next examined the development of the inflorescence and flowers as well as reproduction in arp6 mutants. Compared with the wild type, the inflorescence of long-day-grown arp6-1 mutants was reduced in primary and secondary growth and exhibited a loss of apical dominance, resulting in a dwarfed, bushy appearance (Figure 7A). Cross sections of the wild-type and arp6-1 primary inflorescences revealed that the mutant inflorescence was approximately one-half the diameter of a wild-type stem. However, the cells composing the mutant stem were of comparable size to those of the wild type (Figures 7B, 7K, and 7L), indicating that, as in the case of long-day-grown arp6-1 leaves, this organ was composed of a smaller number of normally sized cells compared with its wild-type counterpart. Examination of the inflorescence meristems revealed that arp6-1 meristems were greatly reduced in size and were flanked by fewer flower primordia compared with those of the wild type (Figures 7I and 7J). By contrast, arp6-1 plants grown in short days did not exhibit a loss of apical dominance, and their inflorescences were comparable to the wild type in diameter and stature (data not shown).

The flowers borne on arp6-1 inflorescences were also smaller compared with those of the wild type (Figures 7C to 7E). The petals of the mutant flowers generally only opened to an angle of −45° relative to the pedicel, whereas those of wild-type flowers opened to approximately a 90° angle (Figures 7C and 7D). In addition, arp6-1 flowers often had one or more extra petals (Figure 7C). This defect was particularly prevalent when plants were grown under short-day conditions but was generally restricted to the early-arising flowers. In short days, the arp6-1 flowers had an average of 5.4 ± 0.8 petals each (n = 35). Under the same conditions, wild-type flowers always had four petals. Removal of the perianth from wild-type and arp6-1 mutant flowers revealed that the mutant carpels were much smaller than those of the wild type (Figure 7E). In addition, the filaments of arp6-1 mutant stamens were not only shorter than those of the wild type, but their length relative to that of the carpel was also reduced (Figure 7E). Closer examination of the anthers showed that, while those of the wild type were oblong, fully dehiscent, and fully covered with pollen, those of the arp6-1 mutant retained the juvenile heart shape and produced far fewer pollen grains (Figure 7H). Although the pollen grains produced by arp6-1 plants appeared morphologically normal (Figure 7H, inset), the height difference between anthers and stigma in the mutants suggested a possible cause of reduced fertility. Examination of the nearly ripened silique revealed that those of the arp6-1 mutant plants were much shorter, wider, and contained many unfertilized ovules (Figures 7F and 7G), resulting in a dramatic decrease in seed set compared with the wild type. While the average number of seeds per silique for a wild-type plant was 53, the arp6-1 plants only produced an average of 22 seeds per silique when allowed to pollinate naturally (Table 1).

In order to explore the nature of this reduced fertility, we conducted reciprocal crosses between wild-type and arp6-1 plants. We found that the pollen produced by arp6-1 anthers was fully functional because it produced an average of 39 seeds per silique when used to pollinate wild-type stigmas. This was comparable to the result of manual selfing of wild-type flowers, which produced an average of 40 seeds per silique. By contrast, when wild-type pollen was used to pollinate arp6-1 stigmas, the resulting fruits produced on average only 21 seeds per silique (Table 1), indicating that the source of reduced fertility in the mutants was primarily a defect in female reproductive development. In light of the observation that AR6P is expressed strongly in the stylar tissue (Figure 3F), it seems likely that the observed female infertility might be the result of a defect in pollen tube elongation through the transmitting tissue of the style.
ARP6 Is Necessary but Not Sufficient to Repress Flowering in Long and Short Days

In order to assess the role of ARP6 in the transition from vegetative to reproductive development, wild-type and arp6 mutant plants were grown under both long- and short-day conditions, and the time of flowering was recorded as the number of rosette leaves present upon first appearance of flower buds. We found that when plants were grown under long-day conditions, the arp6-1 mutants flowered with an average of only six rosette leaves, whereas wild-type plants had produced 12 rosette leaves at the time of flowering (Figure 8A, Table 2). Thus, arp6-1 plants flowered significantly earlier than the wild type under these conditions. In short days, the arp6-1 plants flowered with 20 rosette leaves, whereas wild-type plants had produced 43 rosette leaves before beginning to flower (Figure 8B, Table 2). We concluded from these results that arp6-1 plants showed photoperiod-independent early flowering but retained some sensitivity to photoperiod because flowering of these mutants was relatively later in short days, although still early compared with the wild type (Table 2). In addition to the observation that both arp6-1 and arp6-2 caused a nearly identical array of phenotypes, a cross between arp6-1 and arp6-2 plants yielded F1 arp6-1/arp6-2 trans-heterozygotes with early flowering and other phenotypes indistinguishable from plants homozygous for either allele (Figure 8A, Table 2). This observation demonstrated that the observed phenotypes were indeed the result of disrupting ARP6 activity.

Several genes that negatively regulate flowering time, particularly transcription factors, have been shown to be not only necessary for the repression of flowering, but also sufficient to repress flowering when overexpressed (Michaels and Amasino, 1999; Scortecci et al., 2001). Thus, we sought to determine whether overexpression of ARP6 would result in late flowering. Multiple transgenic lines carrying ARP6 under control of the CaMV 35S promoter were isolated and shown to accumulate several-fold more ARP6 protein than wild-type plants (Figure 5C). However, none of the transgenic lines examined showed any significant difference in flowering time as compared with the wild type (Figure 8C), indicating that ARP6 is necessary but not sufficient to repress flowering.

Early Flowering of arp6-1 Plants Correlates with Decreased Expression of FLC, MAF4, and MAF5 Floral Repressors

Because the molecular genetic control of flowering time has been extensively studied and the major pathways involved have been elucidated (Mouradov et al., 2002; Putterill et al., 2004), this system represents a tractable entry point for understanding the molecular basis of a major phenotype resulting from the loss of ARP6 function. As such, we investigated changes in gene expression in arp6-1 mutants that might be responsible for the observed early flowering. Using the flowering time pathway diagram depicted in Figure 9A as a guide, we employed semiquantitative RT-PCR analysis to measure the mRNA levels of multiple regulators of flowering time in the shoots of 10-d-old wild-type and arp6-1 plants grown under long-day conditions.

<table>
<thead>
<tr>
<th>Natural Pollination</th>
<th>Manual Pollination</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>arp6-1</td>
</tr>
<tr>
<td>52.71 ± 2.56</td>
<td>21.87 ± 4.94</td>
</tr>
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</table>

Values shown are the mean number ± SD of seeds per silique. These data represent the seed set of at least six siliques for each category. Each set of crosses was repeated at least twice.

Figure 8. Flowering of arp6 Mutants and Overexpression Lines.

(A) Twenty-three-day-old wild-type, arp6-1, arp6-2, and arp6-1/arp6-2 plants grown under long-day conditions. (B) Fifty-day-old wild-type, arp6-1, and arp6-2 plants grown under short-day conditions. (C) Thirty-day-old wild-type and 35S:ARP6 transgenic lines grown in long days. Representatives of three independent transgenic lines are shown.
conditions. At this time point, both wild-type and *arp6*-1 plants had produced only four small rosette leaves; thus, neither had begun the transition from a vegetative to an inflorescence meristem. Therefore, any relevant changes in gene expression in the mutant were likely to reflect the cause of the early transition rather than the effect of such a transition. We measured the mRNA levels of each of the autonomous pathway genes (Simpson, 2004), the photoperiod pathway effector *CONSTANS* (CO) (Putterill et al., 1995), the central floral repressor *FLC* (Michaels and Amasino, 1999), and two of its target genes, *FLOWERING LOCUS T* (*FT*) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*) (Samach et al., 2000). We found that in 10-d-old *arp6*-1 shoots, *FLC* mRNA levels were reduced 3.5-fold compared with the wild type, and the levels of the downstream targets of *FLC*, *FT* and *SOC1*, were upregulated by 2.4- and 1.7-fold, respectively (Figures 9B and 9D). None of the other transcripts assayed showed any significant differences between wild-type and *arp6*-1 plants (Figure 9B).

The observation that the *arp6*-1 mutants flowered earlier than an *flc*-3 null mutant (Michaels and Amasino, 1999) in both long and short days (Table 2) indicated that the *arp6*-1 mutation also affects other flowering time control pathways in addition to its effect on *FLC*. It seemed likely that the *FLC*-independent effects of the *arp6*-1 mutation might be manifested through changes in the expression of other floral repressor genes, such as the *MAF* family of *FLC* paralogs (Parenicova et al., 2003). The *MAF* family consists of five genes (*MAF1*-5) that appear to act independently of *FLC* in the repression of flowering (Ratcliffe et al., 2001, 2003). The mRNA levels of *MAF1*-5 were therefore also assayed by RT-PCR in the shoots of 10-d-old wild-type and *arp6*-1 plants grown under long-day conditions. We found that while the levels of *MAF1*, *MAF2*, and *MAF3* were indistinguishable between wild-type and *arp6*-1 mutants, *MAF4* and *MAF5* transcripts were reduced by 1.8- and 2.3-fold, respectively, in the mutants (Figures 9C and 9D). These reductions are likely to account for at least part of the *FLC*-independent effect of *arp6*-1 mutations on flowering time.

**The *arp6*-1 Mutation Suppresses *FLC*-Mediated Late Flowering**

Plants carrying a strong allele of *FRIGIDA* (*FRI*) are extremely delayed in flowering due to upregulation of the floral repressor *FLC* (Michaels and Amasino, 1999, 2001). In order to genetically test the hypothesis that ARP6 is a positive regulator of *FLC* expression, we crossed the *arp6*-1 mutation into a Columbia line carrying the strong *FRI* allele introgressed from the San Feliu-2 ecotype (*FRI*-Col; Lee and Amasino, 1995) and tested the flowering time of these *FRI*/*arp6*-1 plants under long-day conditions. We found that the *arp6*-1 mutation greatly reduced the flowering time of *FRI*-expressing plants. While plants carrying *FRI* had produced 65 rosette leaves at the time of flowering, the *FRI*/*arp6*-1 plants produced an average of only 12 leaves before flowering (Table 2). These results indicate that ARP6 is required for *FRI* to activate *FLC* to levels that are sufficient to delay flowering. Thus, ARP6 is a positive regulator of *FLC* and acts downstream of *FRI* in the activation of *FLC*.

**DISCUSSION**

The nuclear ARP6s thus far examined are known to be stable components of either ATP-dependent nucleosome remodeling complexes or histone acetyltransferase complexes, both of which function to modulate chromatin structure (Peterson et al., 1998; Galarneau et al., 2000; Olave et al., 2002; Shen et al., 2003; Szerlong et al., 2003; Minoda et al., 2005). Recently, the ARP6 protein in *S. cerevisiae* was identified as a component of the SWR1 chromatin remodeling complex that catalyzes the replacement of nucleosomal histone H2A with the H2A.Z variant, ensuring the proper expression of underlying genes (Krogan et al., 2003; Mizuguchi et al., 2004). In addition, ARP6 has also been implicated, at least circumstantially, in the modulation of chromatin structure in *S. pombe* and *Drosophila* (Frankel et al., 1997; Ueno et al., 2004). In this study, we have shown that *Arabidopsis* ARP6 is a member of this conserved ARP6 subfamily and is a nuclear protein with nearly constitutive expression patterns and multiple roles in plant growth and development. The nuclear localization of *Arabidopsis* ARP6 is consistent with its phylogeny and also supports the notion that, like ARP6 homologs in other species, this protein is likely to be a component of one or more chromatin remodeling complexes.

The broad expression patterns observed for ARP6 suggested that this protein plays fundamental roles in the growth and development of the plant. Indeed, based on our analysis of two null alleles, we have found that ARP6 has a function in the development of nearly every plant organ. A major role for ARP6 that has emerged from this study is the promotion of cell proliferation and the control of organ size, particularly during rapid growth under long-day conditions. This is evidenced by the reduction in size of all aboveground organs in *arp6* mutants and the fact that these organs are not merely composed of smaller cells but rather fewer cells of normal size. This cell proliferation and organ size defect is similar to the phenotype resulting from the loss of *ANTEROGUMENTA* (*ANT*) function (Mizukami and Fischer, 2000), suggesting that ARP6 and ANT may act in a common pathway to promote cell proliferation during organ development.

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**Table 2. Rosette Leaf Number at the Time of Flowering in Wild-Type and Mutant Plants**

<table>
<thead>
<tr>
<th>Light Condition</th>
<th>Wild Type</th>
<th><em>arp6</em>-1</th>
<th><em>flc</em>-3</th>
<th>FRI-Col</th>
<th>FRI*/arp6*-1</th>
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<tr>
<td>Long days</td>
<td>12.06 ± 1.16</td>
<td>6.0 ± 0.44</td>
<td>5.22 ± 0.44</td>
<td>8.27 ± 0.46</td>
<td>65.0 ± 2.2</td>
</tr>
<tr>
<td>Short days</td>
<td>43.1 ± 5.0</td>
<td>19.9 ± 1.9</td>
<td>20.0 ± 1.8</td>
<td>36.42 ± 5.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values shown are the mean number ± SD of rosette leaves at the time of flowering. These data represent at least 12 plants of each genotype, and the experiments were repeated at least twice for each genotype. ND, not determined.
Recent evidence suggests that the effects of ANT on cell proliferation are at least partially dependent on auxin signaling (Hu et al., 2003). The requirement of ARP6 for the maintenance of apical dominance in the inflorescence suggests a role for the protein in the transmission or perception of auxin signaling (Thimann and Skoog, 1934). Thus, it is possible that ARP6 acts in the transduction of auxin signals to ANT in order to promote cell proliferation during organ development.

Although the organ size defect of arp6 mutants is severe during growth under long-day conditions, this defect is not as prevalent under short-day conditions, indicating that photoperiod and/or growth rate have a large impact on ARP6 function with regard to cell proliferation. However, arp6 mutants grown under short-day conditions exhibit narrow leaves with deeply serrated margins, which may reflect local changes in the degree or rate of cell proliferation along the margins of leaf primordia. Cell divisions within these marginal meristems are known to be important to the formation of the leaf blade (Donnelly et al., 1999). Previous studies have also shown that misexpression of cyclin-dependent kinase inhibitors can lead to changes in leaf morphology, including reduced size and increases in serration (Wang et al., 2000; De Veylder et al., 2001).

Figure 9. Expression of Flowering Time Control Genes in arp6-1 Plants.

(A) Diagram summarizing the known pathways that control flowering time in Arabidopsis. Gene activation is indicated by lines with arrowheads, and repression is indicated by lines with bars. The MADS domain–containing transcription factor FLC (box) is a central repressor of the flowering program and acts to negatively regulate the expression of the transcription factors SOC1 and FT. In the absence of repression by FLC, SOC1 and FT promote the expression of APETALA1 and LEAFY (LFY) (oval), which serve to initiate the transition to flowering. Several pathways alter the activity of this central regulatory module either by their effects on FLC or its downstream target genes. The autonomous and vernalization pathways act to repress FLC expression and promote flowering under appropriate conditions, while FRI serves to upregulate FLC expression and inhibit flowering in the absence of an extended cold period. Under long-day conditions, the photoperiod pathway upregulates the expression of FT and SOC1 via the transcription factor CO, promoting flowering. In addition, gibberellin signaling pathways can also activate SOC1 and LFY, contributing to the transition to flowering. The diagram was adapted from Putterill et al. (2004). ARP6 is shown as an activator of FLC expression, as supported by data presented in this figure and Table 2.

(B) Semiquantitative RT-PCR analysis of multiple flowering time control genes was performed using total RNA from 10-d-old wild-type and arp6-1 shoots. Total actin (ACT) transcript levels were also assayed as a control for the amount of input cDNA.

(C) Semiquantitative RT-PCR analysis as in (B) was used to assay the levels of MAF1-MAF5 transcripts.

(D) Graph showing relative expression levels of multiple flowering regulators. For each cDNA sample, the expression level of a given gene was normalized to the actin control for that sample to allow comparison among the three independent experiments. Error bars indicate SD.
Interestingly, ARP6 also appears to play a role as a negative regulator of petal number, particularly during growth under short-day conditions. The extra petal phenotype of arp6 mutants is strikingly similar to the effects of mutations in *EARLY EXTRA PETALS 1* (*EEP1*), which encodes the microRNA *miR164c* (Baker et al., 2005). This microRNA negatively regulates the accumulation of transcripts encoding the CUP SHAPED COTYLEDON 1 (CUC1) and CUC2 transcription factors in early-arising flowers in order to properly specify petal number. The coincidence of these phenotypes in *arp6* and *eep1* mutants suggests that *miR164c* or CUC1 and CUC2 may be subject to regulation by ARP6.

In addition to functions in the control of cell proliferation and/or patterning, ARP6 also controls one of the most important developmental transitions in the life of the plant, namely the transition from vegetative to reproductive development. Mutations in *ARP6* cause photoperiod-independent early flowering due to, at least in part, a reduction in the expression of FLC. Furthermore, these mutations suppress the FLC-mediated late flowering phenotype of plants expressing a strong *FRI* allele. These results indicate that ARP6 acts downstream of at least one pathway that regulates the expression of FLC and is required for the activation of FLC expression to levels that inhibit flowering. From these data, we conclude that the role of ARP6 in FLC regulation is likely to be in maintaining the competence of the gene for high-level expression.

In addition to the regulation of FLC expression, the early flowering of *arp6* mutants must result in part from effects on other flowering pathways because these mutants flower earlier than the *flc-3* null mutant (Michaels and Amasino, 1999) in both long and short days. Likely genes that might account for the FLC-independent effects of the *arp6-1* mutation are *MAF4* and *MAF5*, which were also downregulated in the mutants. Both of these genes are paralogs of *FLC* and are known to act as repressors of flowering under certain conditions (Ratcliffe et al., 2003), although the full nature of their activities and how they integrate with other flowering control pathways remain unknown.

Although ARP6 is clearly necessary for the repression of flowering, overexpression of the gene from the 35S promoter is not sufficient to delay flowering and in fact has no obvious effect on plant development (Figures 5C and 8C). This fact indicates that ARP6 is either subject to regulation in terms of its effects on organ development and flowering time or may be reflective of the functioning of ARP6 within a protein complex, the other members of which are probably limiting. Exactly what signals regulate the assembly or activity of an ARP6-containing complex(es) as well as the nature of such a complex(es) remain to be determined.

It is not yet clear whether chromatin remodeling complexes are generally conserved between yeast and higher organisms, but this notion seems plausible given that many of the components, for example Swi2/Snf2-type ATPases and ARPs, are apparently ubiquitous among eukaryotes (Carlson and Laurent, 1994; Sudarsanam and Winston, 2000; Goodson and Hawse, 2002). As mentioned previously, *S. cerevisiae* ARP6 is a component of the SWR1 histone variant-exchange complex, the core subunit of which is a Swi2/Snf2-type ATPase. A BLAST (Altschul et al., 1997) comparison of the SWR1 ATPase to all Arabidopsis proteins indicates that of the 42 Swi2/Snf2 family proteins encoded by the Arabidopsis genome (see www.chromdb.org), PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (*PIE1*) is the most closely related to SWR1. Interestingly, the phenotypes of *pie1* mutants are strikingly similar to those of *arp6* mutants in terms of leaf and flower development as well as early flowering in long- and short-day photoperiods. In addition, mutations in *pie1* suppress the FLC-mediated late flowering of a *FRI*-expressing line (Noh and Amasino, 2003). These results suggest that PIE1 and ARP6 are likely to act in the same genetic pathways and perhaps the same protein complex or complexes. In addition to ARP6 and PIE1, the Arabidopsis genome also encodes putative homologs of 10 of the 11 other SWR1 complex components (R.B. Deal and R.B. Meagher, unpublished data). Could such a putative SWR1-like complex have a function in *Arabidopsis* similar to its function in yeast? In yeast, the SWR1 complex deposits the histone H2A.Z variant into euchromatic regions (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004), preventing the spread of silent heterochromatin into these regions. Perhaps a plant SWR1-like complex could have an analogous function of depositing an H2A.Z variant(s) into FLC chromatin, ensuring competence for high-level expression of FLC. In the absence of such an activity, FLC levels would remain low even in the presence of activators, resulting in early flowering. Such a scenario would be consistent with the known epigenetic regulation of FLC by multiple chromatin modifying factors (Gendall et al., 2001; He et al., 2003, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004).

In summary, the results presented here show that ARP6 is a nuclear protein with broad expression patterns and equally broad roles in the growth and development of *Arabidopsis*. These roles include leaf, inflorescence, and flower development, and the transition from vegetative to reproductive development. In terms of flowering time control, we have identified ARP6 as a novel positive regulator of FLC expression as well as a possible regulator of the FLC paralogs *MAF4* and *MAF5*. These results are all consistent with the idea that ARP6 regulates plant development through its role in chromatin remodeling and the transcriptional control of multiple genes. Future work in our laboratory will be aimed at isolating ARP6-containing protein complexes and identifying the target genes and activities of such complexes.

**METHODS**

**Phylogenetic Analysis**

Actin and ARP sequences were analyzed from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Homo sapiens* (Hs), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Branchiostoma belcheri* (Bb), *Danio rerio* (Dr), and *Gallus gallus* (Gg). The protein sequences used were as follows: Actins, At ACT2, Os ACT, Dr ACT, Ce ACT, Hs ACT, Sc ACT, Sp ACT; ARP4s, At ARP4, Os ARP4, Dm ARP4, Ce ARP4, Hs ARP4, Sc ARP4, Sp ARP4; ARP5s, At ARP5, Os ARP5, Dr ARP5, Ce ARP5, Hs ARP5, Sc ARP5, Sp ARP5; ARP6s, At ARP6, Os ARP6, Dr ARP6, Ce ARP6, Hs ARP6, Sc ARP6, Sp ARP6, Gg ARP6. Protein sequences used in the analysis were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and were aligned using ClustalX with the default settings (see Supplemental Figure 1 online). Phylogenetic trees were constructed from the aligned sequences using the program
MEGA version 2.1 (Kumar et al., 2001) with the default settings. Multiple methods, including neighbor-joining, maximum parsimony, and UPGMA, were all used to analyze the aligned sequences with comparable results. Five hundred bootstrap trials were run for each phylogeny generated.

**Plant Growth Conditions and Transformation**

All plants were of the Columbia ecotype and were grown on soil or agar in growth chambers at 22°C under fluorescent light for either 16 h (long day) or 8 h (short day) per day. Seeds were planted on wet soil or agar and stored at 4°C for 2 d prior to moving them into the growth chamber for germination. Transformations were performed with *Agrobacterium* tumefaciens strain C58C1 using the vacuum infiltration method (Bechtold and Pelletier, 1998). Transformants were selected by plating on half-strength Murashige and Skoog media (Murashige and Skoog, 1962) containing 50 mg/L hygromycin or 35 mg/L kanamycin. Once germinated, the transformants were transferred to nonselective half-strength Murashige and Skoog media to allow root growth, followed by transfer to soil. BASTA was used at 240 mg/L to select for T-DNA insertion mutants by spraying the seedlings.

**Mutant Alleles and Genotyping**

Both ARP6 mutant alleles used in this study were T-DNA insertion alleles in the Columbia ecotype obtained from the Torrey Mesa Research Institute (http://www.tmri.org). The *arp6*-1 line (Garlic_599_G03) has an insertion in exon 1, and *arp6*-2 (Garlic_236_C07) has an insertion in exon 4. Plants of each line were genotyped using two PCR reactions, one to amplify the wild-type allele and another to amplify the insertion allele. DNA for PCRs was prepared by a rapid alkali method described previously (Klimyuk et al., 1993). For *arp6*-1, the wild-type allele was amplified with primers *arp6*-1-5′(5′-GGTCTCTCTGGTGTACATCA-3′) and *arp6*-1-A(5′-GCCATGAGTTTATAGCTCGGACAAT-3′), and the insertion allele was amplified with LB3 (5′-TAGACCTGATATTCAATACCAATCTCGA-TACAC-3′) and *arp6*-1-A. For *arp6*-2, the wild-type allele was amplified with *arp6*-2-5′(5′-GACCTATCCAGCTCGACATT-3′) and *arp6*-2-A (5′-TAGACCTGCTCTTAAGTGTTGAA-3′), and the insertion allele was amplified with LB3 and *arp6*-2-5′. Both lines were backcrossed to wild-type Columbia at least twice and were shown to carry single T-DNA insertions as evidenced by a 3:1 segregation of BASTA resistance (encoded on the T-DNA) in the progeny of heterozygous individuals. The *frc-3* and *FRI-Col* lines were generously provided by Richard Amasino.

**Plasmid DNA Constructs**

A vector containing the native ARP6 regulatory sequences, including 5′ and 3′ UTRs, was created to drive GUS expression in transgenic plants. The 2040-bp ARP6 promoter and 5′ UTR (−2041 to −1 relative to the start codon) as well as the 400-bp ARP6 3′ UTR and terminator (+1 to +400 relative to the stop codon) were amplified by PCR such that the downstream end of the promoter fragment contained multiple extra restriction sites (*NcoI*, *PstI*, *XhoI*, *EcoRv*, *EcoRI*, and *BamHI*), and the upstream end of the terminator fragment contained an identical sequence. The promoter and terminator fragments were combined by overlap extension PCR, and the resulting promoter-multilinker-terminator fragment was cloned into pBluescript KS+ (Stratagene) via *KpnI* and SacI sites introduced at the ends of the fragment during PCR. This vector was named *P*T<sub>ARP6</sub>. The GUS coding sequence was cloned into *P*T<sub>ARP6</sub> via *NcoI* and *BamHI* to yield *P*T<sub>ARP6</sub>GUS. This expression cassette was then subcloned into the binary vector pCAMBIA1300 via *KpnI* and *SacI* for plant transformation.

A fusion of the GFP coding sequence to the C-terminal end of the ARP6 coding sequence was made in a pUC18-derived vector containing GFP under control of the CaMV 35S promoter and nopaline synthase (NOS) terminator. The ARP6 coding sequence (minus stop codon) was inserted into this vector in frame with GFP via *BspH1* and *StuI* restriction sites to yield pARP6-GFP.

For overexpression, the ARP6 coding sequence was cloned into a pBIN19 binary vector derivative carrying the CaMV 35S promoter and NOS terminator via *KpnI* and SacI sites introduced at the ends of the ARP6 coding sequence by PCR. This construct was named pBIN-P<sub>TARP6</sub>AP6.

**Antibody Production and Immunoblotting**

Monoclonal antibodies against ARP6 were produced at the University of Georgia Monoclonal Antibody Facility. These antibodies were raised in mice by injection of 6×His tagged recombinant ARP6 purified from *Escherichia coli*. Each of five mice was initially injected intraperitoneally with 20 μg of purified protein in complete Freund’s adjuvant. All subsequent injections were given intraperitoneally using 20 μg of protein in incomplete Freund’s adjuvant. One week after the third injection, mouse serum was tested for anti-ARP6 antibodies by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. One mouse showing high titers on ELISA and a strong reaction on immunoblots was chosen for further use. Splenocytes were isolated from this mouse and fused with the myeloma cell line SP2/0. The resulting hybridoma cells were plated over macrophages in 96-well plates and allowed to grow for 10 d. After 10 d, media from wells showing cell growth was tested for anti-ARP6 antibodies by ELISA. Monoclonal cell lines producing antibodies against ARP6 were expanded to flasks to produce large quantities of hybridoma supernatant. After growth in flasks, monoclonal antibodies were isolated from the supernatant by ammonium sulfate precipitation.

Proteins were prepared for immunoblotting by grinding tissue to a fine powder in liquid N<sub>2</sub> followed by resuspension and further grinding of the tissue powder in 1.5 volumes of 2× Laemmli’s sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 30% glycerol, and 1% β-mercaptoethanol). Proteins were separated on 12% SDS-polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore). After blocking for 30 min in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk, blots were probed with primary antibody (1:100 dilution for ARP6 antibodies and 1:10,000 for PEPC antibody; Rockland) diluted in the blocking solution for 1 h. Blots were washed three times for 5 min in TBST followed by probing for 30 min with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences), diluted 1:2000 in blocking solution. Finally, the blots were washed three times for 5 min in TBST, treated with ECL detection reagents (Amersham Biosciences), and placed on Hyperfilm ECL (Amersham Biosciences) for detection of protein bands. For each immunoblot, a duplicate gel was stained with Coomassie Brilliant Blue to confirm equal loading of proteins, and the anti-PEPC antibody was also used to confirm equal loading and transfer of proteins.

**Immunofluorescence Microscopy**

Root and leaf tissues were chemically fixed with 4% paraformaldehyde in PME (50 mM PIPES buffer, pH 7.0, 5 mM EGTA, 1 mM MgSO<sub>4</sub>, and 0.5% casein) containing Roche Complete protease inhibitor cocktail (Roche) for 1 h at room temperature. Following three washes in PBS (150 mM NaCl, 14 mM Na<sub>2</sub>HPO<sub>4</sub>, and 3 mM NaH<sub>2</sub>PO<sub>4</sub>), the tissues were permeabilized and partially dissociated by treatment with 1% Cellulysin (Calbiochem) and 0.1% Pectolyase (Sigma-Aldrich) in PME for 1 h at room temperature. Tissues were dissociated and bound to polylysine-coated slides followed by blocking for 2 h at room temperature in TBST-BSA-GS (TBST plus 5% BSA and 20% goat serum). Primary antibody (mAbARP6A, 1:25 dilution) was applied for 15 h at room temperature followed by three washes in PBS and incubation with a 1:100 dilution of FITC-conjugated goat-anti-mouse IgG secondary antibody (Sigma-Aldrich) for 3 h at room temperature. After
three washes in PBS, slides were incubated with 0.1 μg/mL DAPI (Sigma-Aldrich) in PBS, then washed again in PBS and mounted in 80% glycerol-containing 1 mg/mL p-phenylenediamine (Sigma-Aldrich). Microscopy was performed on a Zeiss fluorescence microscope equipped with Improvision Openlab software.

Maize Protoplast Transformation

Protoplasts were isolated from etiolated maize (Zea mays) leaves by slicing them into 0.5-mm sections followed by vacuum infiltration and incubation in digestion solution (0.6 M mannitol, 20 mM MES, pH 5.7, 1 mM CaCl₂, 0.1% BSA, 1.5% cellulase RS, and 0.3% macerozyme R-10; Yakult Pharmaceuticals) for 4 h with shaking. Cells were filtered through Miracloth (Calbiochem), washed three times in wash solution (0.6 M mannitol, 4 mM MES, pH 5.7, and 20 mM KCl), and finally diluted to 2 × 10⁶ cells/mL. Protoplasts were transfected by adding 20 μg of CaCl₂ gradient-purified pARP6-GFP to 100 μL of protoplasts (2 × 10⁶ cells) in wash solution, followed by addition of 120 μL of polyethylene glycol solution (40% polyethylene glycol [Fluka Chemical], 240 mM mannitol, and 100 mM CaCl₂) and a 7 min incubation at room temperature. The transfected cells were then pelleted and resuspended in 400 μL of wash solution and incubated for 12 h at room temperature. Protoplasts were stained with DAPI to allow visualization of DNA by incubating with 0.1 μg/mL DAPI (Sigma-Aldrich) in PBS for 10 min, then washing twice in PBS. GFP and DAPI fluorescence was visualized with a Zeiss fluorescence microscope equipped with Improvision Openlab software.

RT-PCR

The Qiagen RNeasy plant mini kit was used isolate RNA from 10-d-old wild-type and arp6-1 seedlings (minus roots) grown under long-day conditions. DNA was removed during the purification using RNase-free DNase I (Qiagen) according to the manufacturer’s instructions. A total of 3 μg of total RNA was reverse transcribed using the Thermoscript RT-PCR kit (Invitrogen) according to the manufacturer’s instructions, and first-strand cDNA was quantified using Pico Green reagent (Molecular Probes). For each PCR, 5 ng of cDNA was used as template in a reaction consisting of 10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, 0.5 μM each primer, and 0.05 units/μL Promega Taq DNA polymerase. The thermal profile for all reactions was as follows: initial denaturation at 94°C for 3 min followed by a variable number of cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 1 min. Initial reactions were run for 40 cycles, and 5-μL aliquots of each reaction were taken after 25, 30, 35, and 40 cycles in order to establish the linear range of amplification for each reaction. Subsequent reactions were run between 25 and 35 cycles, depending on the kinetics of the particular reaction. All primer sequences used can be found in Supplemental Table 1 online. Quantification of PCR products was performed by analyzing the digitized gel image with NIH Image 1.63 software. The amount of each PCR product from a given cDNA preparation was normalized to the amount of actin product obtained from that cDNA in order to allow for comparisons between experiments. These experiments were repeated at least twice on each of three independent sets of total RNA samples.

Histology Techniques and Microscopy

Histochemical staining of transgenic plants carrying the P/TARe6-GUS construct was performed by incubating tissue in GUS staining solution (50 mM sodium phosphate buffer, pH 7, 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1 mg/mL X-gluc [Gold Biotechnologies], and 0.5% Triton X-100) at 37°C for up to 12 h. Tissues were then stored in 70% ethanol for preservation and to remove chlorophyll. Stained tissues were observed though a Leica dissecting microscope (Leica Microsystems) equipped with a Hamamatsu CCD camera.

Inflorescence stem sections were stained with phloroglucinol (Sigma-Aldrich) to visualize lignin. A 2% phloroglucinol solution was prepared in 95% ethanol, and sections were incubated in this solution for 2 min at room temperature followed by incubation in 50% HCl to produce the colored stain. Stained tissues were observed though a Leica dissecting microscope equipped with a Hamamatsu CCD camera.

For microtome sectioning, inflorescence stems were cut just below the first internode and fixed in 4% glutaraldehyde for 2 h, followed by embedding in Leica Historesin according to the manufacturer’s instructions. Sections of 10-μm thickness were taken on a rotary microtome, and tissues were stained with 0.5% toluidine blue.

Leaves were prepared for scanning electron microscopy by fixation in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) for 3 h at room temperature followed by incubation in 1% OsO₄ for 2 h at room temperature. Samples were then washed in 25 mM sodium phosphate buffer, pH 7, and dehydrated through a graded ethanol series (30, 50, 70, 95, and 100%) for at least 30 min per step. The samples were then critical point dried, mounted, and sputter-coated with a mixture of gold and palladium. Inflorescence tips were prepared for electron microscopy by removing the older flowers, freezing in liquid N₂ slush, and sputter coating with gold in a Gatan cryoprep chamber (Gatan). Leaves and inflorescence meristems were viewed with a LEO 982 field emission scanning electron microscope (LEO Electron Microscopy).

Accession Numbers

Sequence data from this article can be found in the GenBank database (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: AtACT2, At3g18780; OsACT, XP_469569; DmACT, AAA28314; CeACT, CAA34718; Hs ACT, BAD96752; ScACT, NP_116614; SpACT, BAA12315; AtARP4, At1g18450; OsARP4, XP_479987; DmARP4, NP_611209; CeARP4, AA980947; HsARP4, CAB66543; ScARP4, NP_012454; SpARP4, CAB66436; AtARP5, At3g12380; OsARP5, NP_909224; DmARP5, AAF55504; HsARP5, AAH38402; ScARP5, CAA95933; SpARP5, CAB44762; AtARP6, At3g33520; OsARP6, BAD81174; DmARP6, NP_511165; CeARP6, AAC47513; HsARP6, BAD96679; ScARP6, NP_013186; SpARP6, CAA19116; BbARP6, AAQ83895; DrARP6, AAH45961; GgARP6, NP_989968.

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