THE ROLE OF ACTIN-RELATED PROTEIN 6 (ARP6) IN THE EPIGENETIC REGULATION OF *ARABIDOPSIS* DEVELOPMENT

by

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ABSTRACT

The actin-related proteins (ARPs) are an evolutionarily conserved group of proteins that share between 17-60% amino acid identity with cytoskeletal actins. Phylogenetic analysis of ARPs from a wide range of organisms indicates that several distinct subclasses of these proteins are generally shared among all eukaryotes. The ARPs that are most closely related to actin are found in the cytoplasm and have been implicated in a number of cytoskeletal functions, while the more divergent ARPs are nuclear proteins, most of which are known to be stable components of various chromatin modifying complexes. In *Saccharomyces cerevisiae*, ARP6 is a component of the SWR1 chromatin remodeling complex, which catalyzes the replacement of histone H2A with the H2A.Z variant at specific chromosomal loci. In this study we sought to examine the role of *Arabidopsis* ARP6 in plant growth and development, and to elucidate its underlying molecular functions. We found that *Arabidopsis* ARP6 was localized to the nucleus and was expressed in all vegetative tissues as well a subset of reproductive tissues. Null mutations in *ARP6* resulted in a multitude of 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*), and the *FLC*-like genes *MADS AFFECTING FLOWERING 4* (*MAF4*) and *MAF5*. Furthermore, we found that mutations in *PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1* (*PIE1*), a homolog of the yeast SWR1 ATPase subunit, produced developmental phenotypes similar to those of *arp6* plants, and both mutants showed misregulation of a common set of genes. Using H2A.Z-specific antibodies we demonstrated that ARP6 and PIE1 were both required for the deposition of H2A.Z at the *FLC*, *MAF4*, and *MAF5* loci, supporting the existence of a SWR1-like complex in plants and indicating that H2A.Z is normally required to promote high-level expression of these genes. Collectively, these results indicate that ARP6 controls several fundamental aspects of *Arabidopsis* development by promoting the deposition of H2A.Z into chromatin and thereby regulating the expression of multiple developmentally important genes.

INDEX WORDS: *Arabidopsis thaliana*, development, flowering, Actin-related protein, histone variant, H2A.Z, chromatin, epigenetics

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CHAPTER I:

INTRODUCTION AND LITERATURE REVIEW

Actin and the Actin-Related Protein Family

The actin superfamily of ATPases is an ancient and diverse group of proteins whose origin apparently predates the divergence of the prokaryotic and eukaryotic lineages (van den Ent et al. 2001). The ranks of this family include the prokaryotic cell-cycle proteins FtsA and MreB, the heat-shock cognate 70 proteins, sugar kinases, actin, and the actin-related proteins. Although many members of the actin superfamily show no obvious amino acid sequence similarity to one another, all share a common tertiary structure centered around a motif known as the actin fold (Kabsch and Holmes 1995). This motif consists of two major domains of similar alpha helix/beta sheet structure connected by a hinge region to form a horseshoe shaped molecule with a large central cleft as exemplified in Figure 1.1. It is within this central cleft that nucleotide binding and hydrolysis take place, and these activities are manifested as conformational changes within the molecule. The prevailing theme within the actin superfamily is the coupling of nucleotide binding and hydrolysis to conformational changes that alter the functional characteristics of the protein. Thus, despite a great functional diversity, a common core structure unites the members of the actin superfamily. Differences in function arise from extra sequence elements outside the core fold, as well as polymorphism within the core helices and in the loops joining beta strands.

Of all the actin superfamily members, actin itself has been most intensively studied and is known to be involved in a multitude of cellular processes including cytoplasmic organization,

cell shape generation, intracellular trafficking, cytokinesis, and cellular motility (Schmidt and Hall 1998). Each of these functions is based on the ability of actin monomers to polymerize into filaments, forming a network known as the actin cytoskeleton. The formation and disassembly of these filaments is a highly dynamic process that is regulated by a large number of factors including divalent cations, actin-binding proteins (e.g., profilin, ADF/cofilin, ARP2/3 complex, capping protein), and the ATPase activity of the actin molecule itself (Carlier et al. 1994; Schmidt and Hall 1998). Given the vital nature of the processes that actin supports, it is not surprising that it has been found in all eukaryotes examined. In fact, many organisms possess multiple isoforms of actin, each with presumably specialized functions and/or tissue specificity.

In recent years it has become apparent that in addition to the actin family, eukaryotes also encode numerous proteins that are related to actin but are not similar enough to be classified as true actins. These so-called actin-related proteins (ARPs) share only between 17-60% amino acid identity with cytoskeletal actins, which is significantly less homology than is seen between any two actins. An analysis of the *S. cerevisiae* genome revealed ten ARP genes (Poch and Winsor 1997), which were named *ARP1* through *ARP10* on the basis of their protein sequence similarity to conventional actin, with ARP1 being most similar and ARP10 least similar to actin. Sequence alignments of actin and the ARPs show large blocks of similarity throughout the length of the proteins, and for this reason it is thought that even the most divergent ARPs will retain the actin fold structure. Indeed, the crystal structures of ARP2 and ARP3 revealed that these proteins are highly similar in structure to actin (Robinson et al. 2001), and another recent report demonstrates that ARP2 is able to hydrolyze ATP to release inorganic phosphate, as does actin (Le Clainche et al. 2003).

Despite the similarities, distinct differences exist between actins and the ARPs, as well as among the ARPs themselves. ARPs are generally longer than the roughly 377 amino acids of conventional actin, owing to various insertions that, in many cases, are thought to form surface loops on the molecules in which they reside. In the case of yeast ARP4, one such inserted domain has been shown to mediate interactions with other proteins (Harata et al. 1999b). In addition to inserted regions, several ARPs are missing small segments or even entire sub-domains found in actin. It is the combination of these physical differences that is likely to be the basis for the functional diversity seen among the ARPs, and the reason why they do not behave like actin. None of the ARPs form the long filaments characteristic of actin, and in fact only a few are directly involved in cytoskeletal functions. ARPs 1 and 10 are part of the 11-member dynactin complex that mediates vesicle trafficking along microtubules, while ARP2 and ARP3, along with 5 other proteins, form a complex that regulates the nucleation and branching of actin filaments. The remaining ARPs (ARP4 – ARP9) are found in the nucleus of *S. cerevisiae*, and a large body of evidence indicates that most, if not all, of these proteins function in the modulation of chromatin structure (Olave et al. 2002).

A recent phylogenetic analysis of actin-related proteins from many different organisms suggests that the ten ARPs of *S. cerevisiae* are representative of 10 ARP subfamilies that are generally conserved throughout the eukaryotic kingdom (Goodson and Hawse 2002). However, some organisms lack one or more of these ARP subclasses and may encode ARPs that do not fall into any of the 10 conserved subclasses. The *Arabidopsis* genome encodes 9 ARPs that fall into six of the ten conserved ARP subclasses (Goodson and Hawse 2002; McKinney et al. 2002). Single representatives of classes 2, 3, 5, 6, and 8 are present, while two representatives of class 4 are found (Figure 2). One of these ARP4s is typical of those found in other eukaryotes,

consisting of 441 amino acids, while the other appears to be derived from a duplication of less than half of the larger *ARP4* gene. In addition to previously identified ARP classes, the *Arabidopsis* genome also encodes two ARPs, AtARP7 and AtARP8, which do not clearly group with any known ARP class. Homologs of these two proteins are also present in the genome of *Oryza sativa*, suggesting that they may represent plant-specific ARP classes. Noticeably absent from the *Arabidopsis* genome are clearly identifiable representatives of classes 1, 7, 9, and 10 (Kandasamy et al. 2004).

Functions of the Actin-Related Proteins

The cellular functions of the ARPs, particularly those found in the cytoplasm, have been the subject of intense investigation since their discovery in the last decade. ARPs 1 and 10 are components of the dynactin complex, which was first identified as an activator of dynein-mediated vesicle motility (Gill et al. 1991). In addition to the ARPs, dynactin contains 9 other proteins including the conventional actin binding protein, CapZ. ARP1 is apparently unique among the ARPs in its ability to form short actin-like filaments, an ability that is critical to dynactin function. Within the complex, ARP1 forms a filament of approximately 10 monomers that is capped at one end by CapZ and at the other end by a subcomplex containing the p62, p27 and ARP10 subunits. The capped ARP1 filament is thought to interact with the spectrin network surrounding a vesicle or organelle (Vallee et al. 1995), and this complex is then linked to dynein through a dimer of the p150 subunit which binds ARP1, microtubules, and dynein. This molecular linkage allows vesicles, organelles, or other cellular structures to be transported along microtubules by the dynein motor protein. In addition to vesicle and organelle motility, the dynactin complex also plays a role in the assembly and orientation of the mitotic spindle,

kinetochore movement, and nuclear migration (Banks and Heald 2001; Machesky and May 2001; Dujardin and Vallee 2002). As mentioned previously, the *Arabidopsis* genome lacks ARPs 1 and 10, as well as dynein and most other components of the dynactin complex. The same appears to be true of other plants for which substantial sequence data exists, suggesting that the functions normally ascribed to dynactin are performed by other systems in plants (Lawrence et al. 2001).

The ARP2/3 complex was first discovered in *Acanthamoeba castellani* and has since been found in all eukaryotes examined, including plants. This complex consists of ARP2, ARP3, and five other proteins each present as a single copy in the complex. The ARP2/3 complex functions to promote the nucleation of new actin filaments as well as branches on the sides of existing filaments (Mullins and Pollard 1999), and is therefore an essential component of the actin cytoskeletal network. The importance of the ARP2/3 complex is underscored by the fact that both ARP2 and ARP3 are essential for viability in *S. cerevisiae* (Schwob and Martin 1992; Huang et al. 1996). The activity of the ARP2/3 complex is regulated mainly by WASP (Wiskott-Aldrich Syndrome Protein) family proteins, which act to stimulate the filament nucleating function of the complex. These WASP proteins act downstream of Rho-family GTPases, and therefore may serve to relay signals to the ARP2/3 complex, which in turn translates these signals into the proper temporal and spatial organization of the actin cytoskeleton. Several recent reports have demonstrated that the ARP2/3 complex in *Arabidopsis* controls cell polarity and cell shape through the modulation of cortical actin filament density (Le et al. 2003; Li et al. 2003; Mathur et al. 2003a; Mathur et al. 2003b). The fact that *Arabidopsis AtARP3* complements an *arp3Δ* mutant of *S. cerevisiae* (Le et al. 2003), and the human homolog of the p16 subunit can

substitute for Arabidopsis p16 (Mathur et al. 2003b) suggests that the molecular function of the complex may be conserved throughout the eukaryotes.

Unlike ARPs 1, 2, 3, and 10 which are involved in cytoskeletal processes, the remaining ARPs (ARP4 – ARP9) are found in the nucleus of *S. cerevisiae* and other organisms where they have been examined (Olave et al. 2002). 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 type of ARP. All of the nuclear ARPs that have been studied in detail are constituents of either ATP-dependent nucleosome remodeling complexes (NuRCs) or histone acetyl transferase (HAT) complexes, both of which are involved in the modification of chromatin structure and thus the regulation of gene expression. The ATP-dependent NuRCs all contain a DNA-dependent ATPase subunit and use the energy of ATP hydrolysis to drive the repositioning of nucleosomes on DNA, whereas the HAT complexes transfer an acetyl group onto histone N-terminal tails, thereby altering chromatin structure indirectly. Surprisingly, many of these complexes also include monomeric actin in addition to one or more ARPs. While little is known about the functions of the ARPs or actin within these complexes, it has been suggested that they may act to control the assembly and/or activity of the complexes, possibly by virtue of their putative ability to shift between distinct conformational states upon nucleotide binding and/or hydrolysis (Boyer and Peterson 2000). Another possibility is that the ARPs serve to localize chromatin remodeling complexes to specific sites through their interactions with histones or other chromatin associated proteins.

In *S. cerevisiae ARP4* is an essential gene and the protein it encodes is a component of the NuA4 HAT complex as well as the INO80 ATP-dependent NuRC (Harata et al. 1994; Galarneau et al. 2000; Shen et al. 2000). The 1.3 MDa NuA4 complex contains 11 subunits, including

ARP4 and actin, and is known to acetylate primarily histone H4. In temperature-sensitive *arp4* mutants the NuA4 complex is absent (Galarneau et al. 2000), indicating that ARP4 is required for the assembly or stability of the complex. These same mutants display defects in the transcription of certain target genes at the restrictive temperature, coincident with changes in the normal chromatin structure around those genes (Jiang and Stillman 1996; Harata et al. 2002). In addition, ARP4 has been shown to bind all four core histones in vitro (Harata et al. 1999a). Thus, ARP4 could provide a targeting function for chromatin modifying complexes by interacting with histones and with other components of the complex. Beyond yeast, ARP4 is also found in three different ATP-dependent NuRCs and one HAT complex in mammals, at least one remodeling complex in *Drosophila* (Olave et al. 2002), and is known to be a nuclear protein with complex developmental functions in *Arabidopsis* as well (Kandasamy et al. 2003; Kandasamy et al. 2005).

In addition to ARP4, the yeast INO80 complex also contains ARP5, ARP8, monomeric actin, and seven other subunits. Loss-of-function mutations in the INO80 ATPase subunit lead to defects in the transcription of genes involved in inositol biosynthesis as well as hypersensitivity to DNA damaging agents (Ebbert et al. 1999; Shen et al. 2000), suggesting a role for the INO80 complex not only in transcriptional control but also in DNA damage repair. Deletion mutants of *ARP5* and *ARP8* also display the *ino80*$^{-}$ phenotype, suggesting that these proteins are critical to the function of the complex. INO80 complexes purified from *arp5Δ* or *arp8Δ* strains still retain most subunits but are deficient in INO80 ATPase activity, DNA binding, and nucleosome remodeling functions. In addition, the complex purified from *arp8Δ* cells also lacks ARP4 and actin, indicating that ARP8 is needed to recruit these components to the complex (Shen et al. 2003). The finding that ARP8 also binds histones H3 and H4 in vitro (Shen et al. 2003) suggests

that in addition to stimulating the ATPase activity of the INO80 subunit, the ARPs may also serve as the point of contact between the complex and chromatin. An INO80-like complex was also recently discovered in mammals (Jin et al. 2005) and is expected to be conserved in other organisms, including plants (Meagher et al. 2005).

The yeast SWI/SNF and RSC complexes are two related ATP-dependent NuRCs containing 10 and 15 subunits, respectively, including ARP7 and ARP9. Unlike the complexes described above, neither SWI/SNF nor RSC contain monomeric actin. The SWI/SNF complex was identified independently in screens for genes involved in mating-type switching and sucrose fermentation, and the RSC complex was later isolated based on homology to SWI/SNF complex components. Strains lacking ARP7 or ARP9 show typical *swi/snf*⁻ phenotypes, indicating that these proteins play an essential role in the function of SWI/SNF. In addition to the *swi/snf*⁻ phenotype, mutations in *ARP7* or *ARP9* also lead to other transcriptional defects, implicating RSC in transcriptional activation as well (Cairns et al. 1998). Surprisingly, RSC complexes isolated from *arp7Δ/arp9Δ* cells are fully intact and retain the ability to remodel nucleosomes in vitro. However, a screen for suppressors of *arp7* and *arp9* mutations revealed the transcription factor NHP6, which interacts physically with RSC and enhances the activity of the complex in vitro (Szerlong et al. 2003). This finding suggests that ARP7 and ARP9 serve to connect the RSC complex to interacting proteins or other complexes, allowing functionality *in vivo*. Complexes homologous to SWI/SNF and RSC likely exist in organisms other than yeast but they almost certainly do not exist in the same form in plants, which lack clear homologs of ARP7 and ARP9. However, plants encode two unique ARPs as well as a many SWI/SNF-type ATPases, and therefore may also contain novel types of chromatin remodeling complexes (Meagher et al. 2005).

While little is yet known regarding the biochemical functions of the ARPs or actin in NuRC and HAT complexes, a number of important themes have emerged. All of the ARPs studied in detail are known to be stable components of large multi-protein complexes, often containing more than one ARP and in several cases monomeric actin. Within these complexes the ARPs and actin may be required for (a) assembly or stability of the complex, (b) stimulation of core ATPase activity, or (c) connecting the complex to other proteins or protein complexes.

Current Understanding of Actin-Related Protein 6 (ARP6)

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 co-localized with heterochromatin protein 1 (HP1) in pericentric heterochromatin, suggesting a possible role in heterochromatin function (Frankel et al. 1997; Harata et al. 2000). However, several groups have now shown that ARP6 is a component of the 13 member *S. cerevisiae* SWR1 chromatin remodeling complex. This complex is similar in composition to other ATP-dependent nucleosome remodeling complexes but has the unique ability to catalyze the replacement of histone H2A with the variant H2A.Z at specific chromosomal locations (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). The SWR1 complex is also functionally conserved in *Drosophila* and humans, where it is known as Tip60 or SRCAP, respectively. However, in these organisms the complex appears to be a fusion of SWR1 with the NuA4 histone acetyltransferase complex, and as such it is capable of both histone acetylation and H2A.Z exchange (Cai et al. 2005). Within the yeast SWR1 complex, ARP6 recruits several other critical subunits, one of which interacts directly with H2A.Z, and it is also required for binding of the complex to

nucleosomes (Wu et al. 2005). Thus, ARP6 is essential for the activity of the complex and therefore the deposition of H2A.Z into chromatin.

The fundamental repeating unit of chromatin, known as the nucleosome, consists of roughly 150 bp of DNA wrapped in two superhelical turns around an octameric protein particle composed of two copies of each of the four core histones: H2A, H2B, H3, and H4. These histone proteins are encoded by multiple gene copies and are produced in large quantities to accommodate the nascent genome during DNA replication. In addition to these bulk histones, eukaryotic genomes also encode variant forms of histones H3 and H2A that are deposited independently of DNA replication and serve to functionally specialize or differentiate particular chromatin regions. In yeast, the H2A.Z variant plays important roles in both promoting transcription and antagonizing the spread of heterochromatin into euchromatic regions (Santisteban et al. 2000; Adam et al. 2001; Larochelle and Gaudreau 2003; Meneghini et al. 2003). Several genome-wide studies of H2A.Z occupancy have shown that this variant localizes to the promoters of most euchromatic yeast genes, and its presence is required for the optimal transcriptional activation of many of these genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005). H2A.Z has also been observed at the 5′ ends of several active genes in human and chicken cell lines (Bruce et al. 2005; Farris et al. 2005) suggesting that, similar to yeast H2A.Z, it is also involved in transcriptional activation. In contrast, cytological and genetic studies suggest that in metazoans H2A.Z also plays a role in heterochromatin formation and/or maintenance (Rangasamy et al. 2003; Swaminathan et al. 2005). However, null mutations in mouse and *Drosophila* H2A.Z result in embryonic lethality (Faast et al. 2001; van Daal and Elgin 1992), making it difficult to study the role of H2A.Z in the developmental program of these organisms. However, the flowering plant *Arabidopsis thaliana*, with its

completely sequenced genome and ability to tolerate many mutations that are lethal to metazoans, offers a unique system to study the functions of H2A.Z and its deposition machinery in the epigenetic control of multicellular development. In order to accomplish this goal, we have employed a reverse genetic strategy to analyze the developmental and molecular functions of the *Arabidopsis* homolog of ARP6, a putative component of the plant H2A.Z deposition apparatus.

The chapters that follow are a series of manuscripts describing our findings on the functions of ARP6 in *Arabidopsis thaliana*. In Chapter II we describe our dissection of the developmental functions of ARP6 through the analysis of *arp6* mutants. Chapter III details how we used our knowledge of the developmental functions of ARP6 as a means to probe how the protein acts at the molecular level to regulate development. In the final chapter we attempt to tie together all of the data and present the significance of these findings in the broader context of developmental and molecular genetics.

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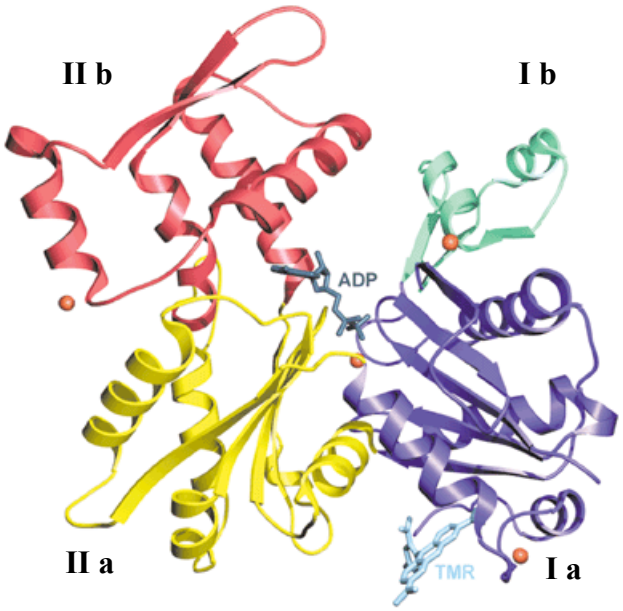

Figure 1.1 The Crystal Structure of Actin

The crystal structure of rabbit skeletal muscle actin is shown. The structure can be divided into two halves (I and II), and further into four subdomains (denoted by numbering and coloration). The nucleotide-binding pocket (containing ADP) is formed at the interface of the four subdomains. Red spheres indicate Ca^{2+} ions and TMR represents a tetramethylrhodamine-5-maleimide molecule linked to Cys^{374}. Reprinted with permission from Otterbein *et al*. (2001) *Science* **293**: 708-711. Copyright 2001 American Association for the Advancement of Science.

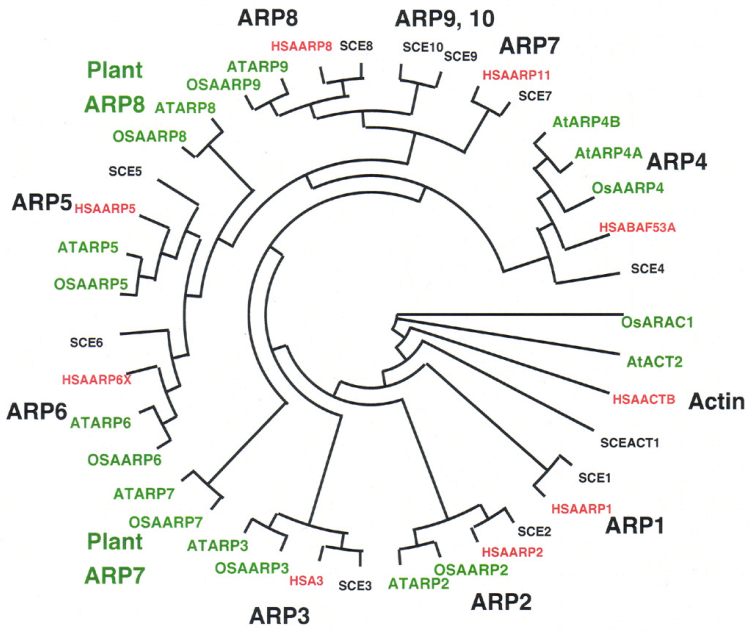

Figure 1.2 Phylogeny of Actin and the Actin-Related Proteins (ARPs)

The phylogenetic tree shows representative actin and ARP sequences from budding yeast (SCE), Human (HSA), *Arabidopsis* (AT), and Rice (OSA). ARP class designations for each clade are shown in bold along the outer edge of the phylogram. Plant ARP7 and Plant ARP8 denote putative plant-specific ARP classes.

CHAPTER II:

THE NUCLEAR ACTIN-RELATED PROTEIN ARP6 IS A PLEIOTROPIC DEVELOPMENTAL REGULATOR REQUIRED FOR THE MAINTENANCE OF *FLOWERING LOCUS C* (*FLC*) EXPRESSION AND REPRESSION OF FLOWERING IN *ARABIDOPSIS*[1]

[1] Deal, R.B., Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. 2005. *Plant Cell*. 17: 2633-46

ABSTRACT

Actin-related proteins (ARPs) are found in the nuclei of all eukaryotic cells, but their functions are generally understood only on the basis of their presence in various yeast and animal chromatin-modifying complexes. *Arabidopsis thaliana* ARP6 is a clear ortholog of ARP6s from other eukaryotes including *S. cerevisiae* ARP6, which was identified as a component of the SWR1 chromatin remodeling complex. In order to address the function of ARP6 in *Arabidopsis* we have examined the subcellular localization, expression patterns and loss-of-function phenotypes for this protein. We found that *Arabidopsis* ARP6 was localized to the nucleus during interphase but dispersed away from the chromosomes during cell division. ARP6 expression was observed in all vegetative tissues, and in a subset of reproductive tissues as well. Null mutations in *ARP6* resulted in a multitude of 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* (*FRI*) expressing line, indicating that ARP6 is required for the activation of *FLC* expression to levels that inhibit flowering. Together these results indicate that ARP6 acts in the nucleus to regulate multiple aspects of plant development, and we propose that it does so through a role in the 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 ten 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 multi-protein 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 co-localized with heterochromatin protein 1 (HP1) 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 (Krogan 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 heterochromatic functions (Dryhurst et al. 2004; Fan et al. 2004). Another recent report indicates that *S. 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 never 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 *FLC*, *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 identity, 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 twenty-three 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 2.1. In addition, we found that the *Oryza sativa* genome also encoded a single 429 amino acid ARP6 ortholog that was 62% identical to

Arabidopsis ARP6, suggesting that the ARP6 protein may be conserved throughout the angiosperms and is therefore likely to have 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 2.2). Both antibodies also recognized only a single band of the expected size when tested against plant protein extracts (Figure 2.3A), indicating that these antibodies are highly specific to ARP6. Protein extracts from 7-day-old seedlings, 15-day-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 2.3A).

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 (Figure 2.3C and D). 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 (Figure 2.3E

and F). However, following fertilization and expansion of the developing fruit, the reporter became active and was expressed strongly in the fruit wall (Figure 2.3G). The *ARP6* regulatory sequences did not appear to be active in the anthers, pollen, stigma, or ovary (Figure 2.3F). The results shown in Figure 2.3 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 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:GFP fusion protein. 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 (Figure 2.4A-D). However, the ARP6 protein was dispersed away from the chromosomes during cell division (Figure 2.4C and D). This behavior was observed previously for *Drosophila* ARP6 (Frankel et al. 1997) as well as *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 the green fluorescent protein (GFP) to the C-terminus of ARP6 was constructed and used to transfect maize mesophyll protoplasts. This *CaMV 35S* promoter-driven fusion protein also accumulated in the nuclei of transfected cells (Figure 2.4E-G), 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 2.5A). 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 2.5B). 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 wild type at all developmental stages (Figure 2.6A). Scanning electron microscopic examination of the adaxial surface of 3rd rosette leaves from 27-day-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 (Figure 2.6C-E), 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.

In 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 undulating margins and adult leaves that were deeply serrated along the margins. At all stages of development the *arp6-1* leaves were narrower than wild type but of

comparable length (Figure 2.6B). Thus, under short-day conditions *arp6-1* mutant leaves were of similar length to wild type but were deficient in cell proliferation and/or expansion in the leaf-width direction, and also had strong defects in margin patterning.

ARP6 regulates inflorescence and flower development

We next examined the development of the inflorescence and flowers, as well as reproduction in *arp6* mutants. Compared to 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 2.7A). Cross sections of the wild type and *arp6-1* primary inflorescences revealed that the mutant inflorescence was roughly one-half the diameter of a wild type stem. However, the cells composing the mutant stem were of comparable size to those of wild type (Figure 2.7B and I) 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 to its wild type counterpart. In contrast, *arp6-1* plants grown in short days did not exhibit a loss of apical dominance, and their inflorescences were comparable to wild type in diameter and stature (data not shown).

The flowers borne on *arp6-1* inflorescences were also smaller compared to those of the wild type (Figure 2.7C-E). The petals of the mutant flowers generally only opened to an angle of approximately 45° relative to the pedicel, whereas those of wild type flowers opened to nearly a 90° angle (Figure 2.7C and D). In addition, *arp6-1* flowers often had one or more extra petals (Figure 2.7C). 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 4 petals. Removal of the perianth from wild type and *arp6-1* mutant flowers revealed that the mutant carpels were much smaller than those of wild-type (Figure 2.7E). In addition, the filaments of *arp6-1* mutant stamens were not only shorter than those of wild type, but their length relative to that of the carpel was also reduced (Figure 2.7E). 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 2.7H). Although the pollen grains produced by *arp6-1* plants appeared morphologically normal (Figure 2.7H, inset), the height difference between anthers and stigma in the mutants suggested a possible cause of reduced fertility. Examination of the nearly ripened siliques revealed that those of the *arp6-1* mutant plants were much shorter, wider, and contained many unfertilized ovules (Figure 2.7F and G), resulting in a dramatic decrease in seed set compared to 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 2.1).

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. In contrast, when wild type pollen was used to pollinate *arp6-1* stigmas, the resulting fruits produced on average only 21 seeds per silique (Table 2.1), indicating that the source of reduced fertility in the mutants was primarily a defect in female reproductive development. In light of the observation that *ARP6* is expressed strongly in

the stylar tissue (Figure 2.3F), 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 a*rp6-1* mutants flowered with an average of only 6 rosette leaves, whereas wild type plants had produced 12 rosette leaves at the time of flowering (Figure 2.8A and Table 2.2). Thus, *arp6-1* plants flowered significantly earlier than 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 2.8B and Table 2.2). 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 to wild type (Table 2.2). 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 F_1 *arp6-1*/*arp6-2* trans-heterozygotes with early flowering and other phenotypes indistinguishable from plants homozygous for either allele (Figure 2.8A, Table 2.2). 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 to wild type (Figure 2.8C), 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; Simpson and Dean 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 semi-quantitative RT-PCR analysis to measure the mRNA levels of multiple regulators of flowering time in the shoots of 10-day-old wild type and *arp6-1* plants grown under long-day 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-day-old *arp6-1* shoots, *FLC* mRNA levels were reduced 3.5 fold compared to wild type, and the levels of the downstream targets of *FLC*, *FT* and *SOC1*, were upregulated by 2.4- and 1.7-fold, respectively (Figure 2.9B and D). None of the other transcripts assayed showed any significant differences between wild-type and *arp6-1* plants (Figure 2.9B).

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 2.2) 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 *MADS AFFECTING FLOWERING* (*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; Ratcliffe et al. 2003). The mRNA levels of *MAF1-MAF5* were therefore also assayed by RT-PCR in the shoots of 10-day-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 (Figure 2.9C and D). 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; Michaels and Amasino 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 2.2), and had a 10-fold lower level of *FLC* (Figure S1) than did the *FRI* line. 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 ARPs 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; Szerlong et al. 2003; Minoda et al. 2005; Shen et al. 2003). 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 orthologs 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 such as in 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 *AINTEGUMENTA* (*ANT*) function (Mizukami and Fischer 2000), suggesting that ARP6 and ANT may act in a common pathway to promote cell proliferation during organ development. 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 the 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).

Interestingly, ARP6 also appears to have 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 coinicidence of these phenotypes in *arp6* and *eep1* mutants suggests that miRNA164c 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, at least in part, to 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 remains 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 (Figure 2.5C, Figure 2.8C). 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) remains to be determined.

It is not yet clear whether homologous chromatin remodeling complexes are conserved between yeast and higher organisms, but this notion seem 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 suggests 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 clear orthologs of at least four other SWR1 complex components (Deal and Meagher, unpublished observations). 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 (He and Amasino 2005).

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, male and female reproductive 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 actin-related protein sequences were analyzed from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Drosophila melaongaster* (Dm), *Caenhorabditis 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:** AtACT2, At3g18780; OsACT, XP_469569; DmACT, AAA28314; CeACT, CAA34718; HsACT, BAD96752; ScACT, NP_116614; SpACT, BAA12315; **ARP4s:** AtARP4, At1g18450; OsARP4, XP_479987; DmARP4, NP_611209; CeARP4, AAF60947; HsARP4, CAB66543; ScARP4, NP_012454; SpARP4, CAB66436; **ARP5s:** AtARP5, At3g12380; OsARP5, NP_909224; DmARP5, AAF55504; HsARP5, AAH38402; ScARP5, CAA95933; SpARP5, CAB44762; **ARP6s:** AtARP6, At3g33520; OsARP6, BAD81174; DmARP6, NP_511165;

CeARP6, AAC47513; HsARP6, BAD96679; ScARP6, NP_013186; SpARP6, CAA19116; BbARP6, AAQ83895; DrARP6, AAH45961; GgARP6, NP_989968. Protein sequences used in the analysis were obtained from NCBI (www.ncbi.nlm.nih.gov) and were aligned using ClustalX with the default settings. 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.

Plant growth conditions and transformation

All plants were of the Columbia ecotype and were grown on soil or agar in growth chambers at 22º C under fluorescent light for either 16 hr (long day) or 9 hr (short day) per day. Seeds were planted on wet soil or agar and stored at 4º C for 2 days prior to moving them into the growth chamber for germination. Transformations were carried out with *Agrobacterium tumefaciens* strain C58C1 using the vacuum infiltration method (Bechtold and Pelletier 1998). Transformants were selected by plating on 1/2 strength MS media (Murashige and Skoog 1962) containing 50 mg/L hygromycin or 35 mg/L kanamycin. Once germinated, the transformants were transferred to non-selective 1/2 strength MS 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 by 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-S (5′- GTTCTTCCTGATGGTGTTACACATA-3′) and arp6-1-A (5′-GGCATGAGTTTATAGCTCGGACAAT-3′), and the insertion allele was amplified with LB3 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′) and arp6-1-A. For *arp6-2*, the wild type allele was amplified with arp6-2-S (5′-GACGTTATTCCAGCCTGCAGATTTA-3′) and arp6-2-A (5′-TACAGTCTCTCCTTAAGTTGTGGAA-3′), and the insertion allele was amplified with LB3 and arp6-2-S. 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 *flc-3* and *FRI*-Col lines were generously provided by Dr. Richard Amasino.

Plasmid DNA constructs

A vector containing the native *ARP6* regulatory sequences, including 5′ and 3′ UTRs, was created to drive *β-glucuronidase* 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 (*Nco*I, *Pst*I, *Xho*I, *Eco*RV, *Eco*RI, *Bam*HI) 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, La Jolla, CA) via *Kpn*I and *Sac*I sites introduced at the ends of the fragment during PCR. This vector was named P/T_{ARP6} . The *β-glucuronidase* coding sequence was cloned into P/T_{ARP6} via *Nco*I and *Bam*HI to yield P/T_{ARP6}*:GUS*. This expression cassette was then subcloned into the binary vector pCAMBIA1300 via *Kpn*I and *Sac*I 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 *Cauliflower Mosaic Virus* (*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 *Bsp*HI and *Stu*I 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 *Kpn*I and *Sac*I sites introduced at the ends of the *ARP6* coding sequence by PCR. This construct was named pBIN-P_{35S}*:ARP6*.

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 6x-His tagged recombinant ARP6 purified from *E. coli*. Each of 5 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 immunoblot 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 days. After 10 days, 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_2 followed by resuspension and further grinding of the tissue powder in 1.5 vol of 2x Laemmli's sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 30% glycerol, 1% β-mercaptoethanol). Proteins were separated on 12% SDS-polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA). After blocking for 30 min in TBST (10 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% non-fat dry milk, blots were probed with primary antibody (1:100 dilution for ARP6 antibodies, 1:10,000 for PEPC antibody (Rockland, Gilbertsville, PA) diluted in the blocking solution, for 1-2 hr. 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, Piscataway, NJ), 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.

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 $MgSO_4$, 0.5% casein) containing Roche Complete protease inhibitor cocktail (Roche, Mannheim, Germany) for 1 hr at room temperature. Following three washes in PBS (150 mM NaCl, 14 mM Na_2HPO_4, 3 mM NaH_2PO_4), the tissues were permeabilized and partially dissociated by treatment with 1% Cellulysin (Calbiochem, San Diego, CA) and 0.1% Pectolyase (Sigma, St. Louis, MO) in PME for 1 hr at room temperature. Tissues were dissociated and bound to poly-lysine coated slides followed by blocking for 2 hr 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 hr 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, St. Louis, MO) for 3 hr at room temperature. After three washes in PBS, slides were incubated with 0.1μg/mL DAPI (Sigma) in PBS, then washed again in PBS and mounted in 80% glycerol containing 1mg/mL *p*-phenylenediamine (Sigma). Microscopy was performed on a Zeiss fluorescence microscope (Zeiss, Jena, Germany) equipped with Improvision Openlab software (Improvision, Lexington, MA).

Maize protoplast transformation

Protoplasts were isolated from etiolated Maize 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 $CaCl_2$, 0.1% BSA, 1.5% cellulase RS and 0.3% macerozyme R-10 [Yakult Pharmaceuticals, Tokyo, Japan]) for 4 hr with shaking. Cells were filtered through Miracloth (Calbiochem), washed three times in wash solution (0.6 M mannitol, 4 mM MES pH=5.7, 20

mM KCL) and finally diluted to 2 x 10^6 cells /mL. Protoplasts were transfected by adding 20 μg of CsCl gradient-purified pARP6-GFP to 100 μL of protoplasts (2 x 10^5 cells) in wash solution, followed by addition of 120 μL of polyethylene glycol solution (40% PEG [Fluka Chemical, Milwaukee, WI], 240 mM mannitol, 100 mM $CaCl_2$) 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 hr at room temperature. Protoplasts were stained with DAPI to allow visualization of DNA by incubating with 0.1μg/mL DAPI (Sigma) in PBS for 10 min, then washing twice in PBS. GFP and DAPI fluorescence was visualized with a Zeiss fluorescence microscope (Zeiss) equipped with Improvision Openlab software (Improvision).

RT-PCR

The Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA) was used isolate RNA from 10-day-old wild type and *arp6-1* seedlings (minus roots) grown under long-day conditions. DNA was removed during the purification by 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, Carlsbad, CA) according to the manufacturer's instructions, and first-strand cDNA was quantified using the Pico Green Reagent (Molecular Probes, Eugene, OR). 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 $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, and 0.05 u/μL Promega Taq DNA polymerase (Promega, Madison, WI). 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 sec, 45˚ C for 40 sec, 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 Table 2.3. 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/T$_{ARP6}$: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, St. Louis, MO], 0.5% Triton X-100) at 37˚ C for up to 12 hr. Tissues were then stored in 70% ethanol for preservation and to remove chlorophyll. Stained tissues were observed though a Leica dissecting microscope (Leica Microsystems, Bannockburn, IL) equipped with a Hamamatsu CCD camera (Hamamatsu Corporation, Japan).

Inflorescence stem sections were stained with phloroglucinol (Sigma) 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 (Leica Microsystems) equipped with a Hamamatsu CCD camera (Hamamatsu Corporation).

For microtome sectioning, inflorescence stems were cut just below the first internode and fixed in 4% glutaraldehyde for 2 hr, followed by embedding in Leica Historesin (Leica

Microsystems) 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, 3.7% formaldehyde) for 3 hr at room temperature followed by incubation in 1% OsO_4 for 2 hr 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%, 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. Leaves were viewed with a LEO 982 field emission scanning electron microscope (LEO Electron Microscopy, Thornwood, NY).

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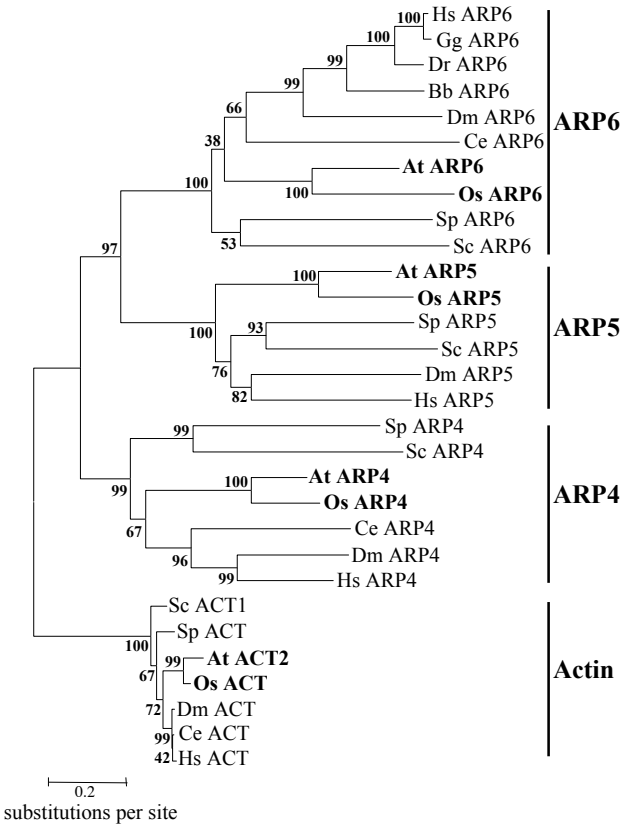

Figure 2.1 Relationships Between ARP6 and Related Proteins

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, *Caenhorabditis elegans*; Dm, *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.

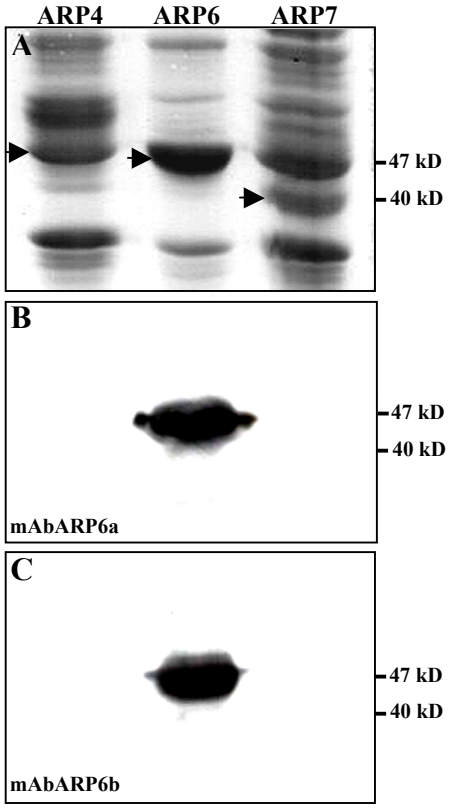

Figure 2.2 Specificity of Anti-ARP6 Monoclonal Antibodies

(A) Coomassie blue-stained SDS-polyacrylamide gel showing *E. coli* protein extracts containing recombinant ARP4, ARP6, or ARP7. The position of each ARP is indicated by an arrowhead. Duplicates of the gel shown in (A) were blotted and probed with the monoclonal antibodies mAbARP6a (**B**) and mAbARP6b (**C**).

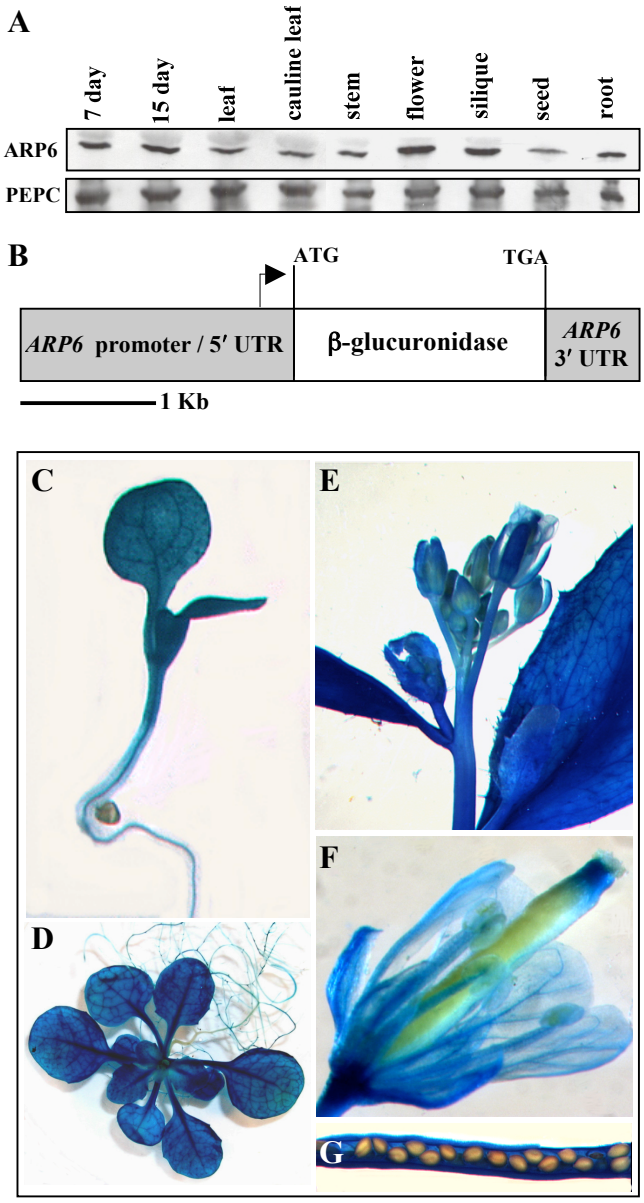

Figure 2.3 Organ- and Tissue-level Expression Patterns of ARP6

(A) Immunoblot of protein extracts from whole plants (7 day, 7-day-old seedling; 15 day, 15-day-old plant) or various plant organs probed for the 47 kD ARP6 with the monoclonal antibody mAbARP6a (upper panel) and for the 115 kD PEP-carboxylase with anti-PEP carboxylase (PEPC, lower panel) polyclonal antibody as a loading control. Each lane was loaded with approximately 25 μg of total protein and the two panels shown were from different segments of

the same blot. The flower protein sample was prepared from the entire inflorescence tip and thus contains flowers of all stages. **(B)** Diagram of the *β-glucuronidase* (*GUS*) coding sequence under control of the *ARP6* regulatory sequences. The *ARP6* promoter / 5′ UTR is defined as the 2 kb region upstream of the *ARP6* start codon and the *ARP6* 3′ UTR consists of the 400 bp region downstream of the *ARP6* stop codon. **(C-G)** Transgenic plants carrying the reporter transgene shown in (B). **(C)** Seven-day-old seedling. **(D)** Twenty-day-old plant. **(E)** Inflorescence tip showing flowers, stems, and cauline leaves. **(F)** A single flower. **(G)** Mature green silique opened to expose seeds.

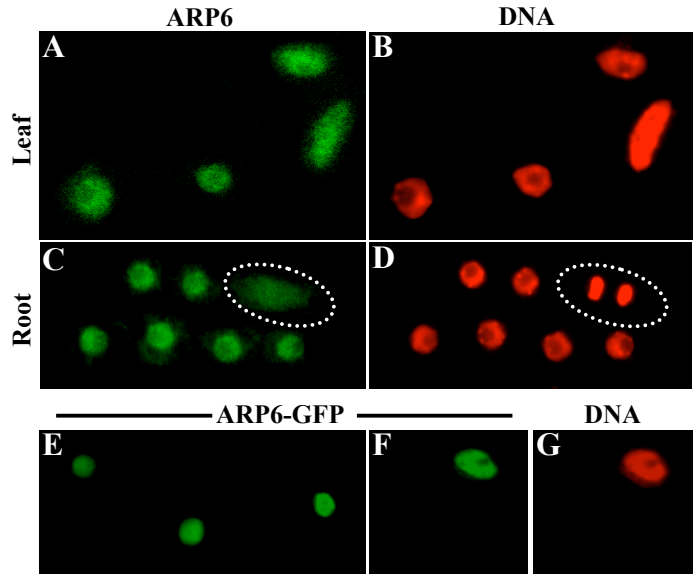

Figure 2.4 Nuclear Localization of ARP6

(A) Isolated leaf cells probed with the mAbARP6a monoclonal antibody. **(B)** DAPI channel image of cells shown in (A). **(C)** Root cells probed with the mAbARP6a monoclonal antibody. Dotted oval indicates an anaphase cell. **(D)** DAPI channel image of the cells shown in (C). **(E)** Maize mesophyll protoplasts expressing the ARP6-GFP fusion protein. **(F)** Close-up photo of a single maize mesophyll protoplast expressing the ARP-GFP fusion protein. **(G)** DAPI channel image of the cell shown in (F).

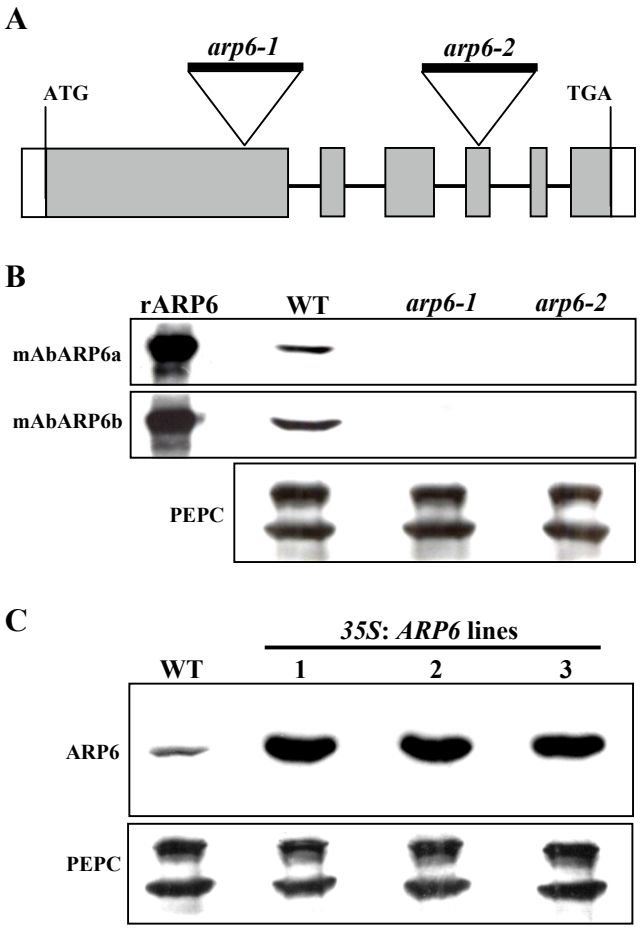

Figure 2.5 *ARP6* Gene Structure, T-DNA Insertion Alleles, and Overexpression Lines

(A) Structure of the *ARP6* gene. Exons are depicted as gray boxes, introns as black lines, and the 5′ and 3′ UTRs as white boxes. The locations of T-DNA insertions in *arp6-1* and *arp6-2* alleles are shown as triangles above the gene diagram. **(B)** Immunoblots of SDS gels loaded with 5 ng of recombinant ARP6 (rARP6) and 25 μg of total protein extract from 15-day-old wild type (WT), *arp6-1*, and *arp6-2* plants. Blots were probed with monoclonal antibody mAbARP6a (upper panel), mAbARP6b (middle panel) and anti-PEP carboxylase polyclonal (PEPC) as a loading control (lower panel). **(C)** Immunoblot of and SDS gel loaded with 25 μg of total protein from 15 day old wild type (WT) plants and three independent transgenic lines expressing *ARP6*

from the *CaMV 35S* promoter (*35S*:*ARP6* lines). Blot was probed with monoclonal antibody mAbARP6a (top panel) and anti-PEPC polyclonal antibody (lower panel).

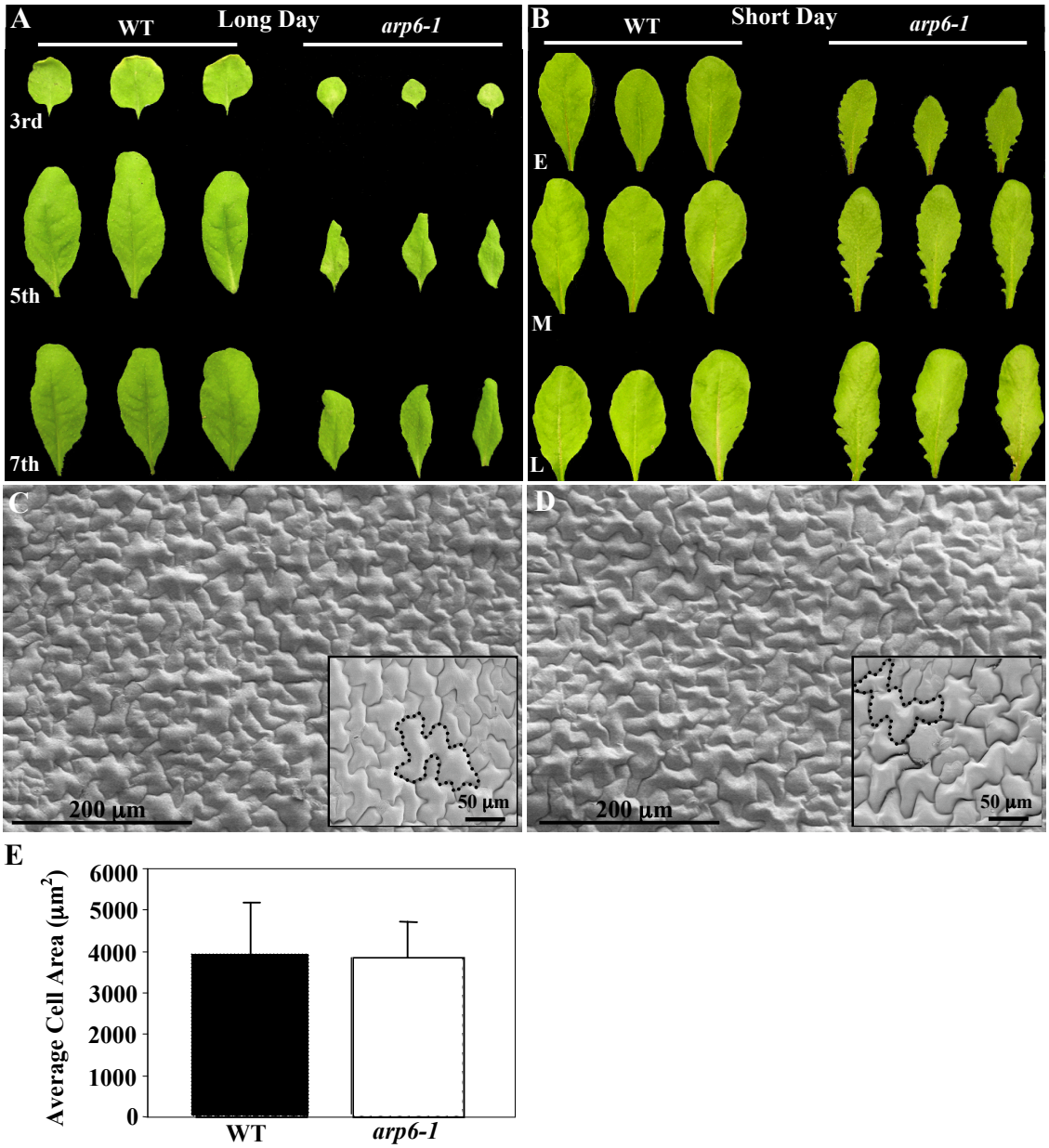

Figure 2.6 Leaf Development in Long- and Short-Day Grown *arp6-1* Plants

(A) Third, 5th, and 7th rosette leaves of 27-day-old wild type (WT) 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-day-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 3rd rosette leaves from 27-day-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 (μm^2) of adaxial epidermal cells from 3rd rosette leaves of 27-day-old plants grown in long days.

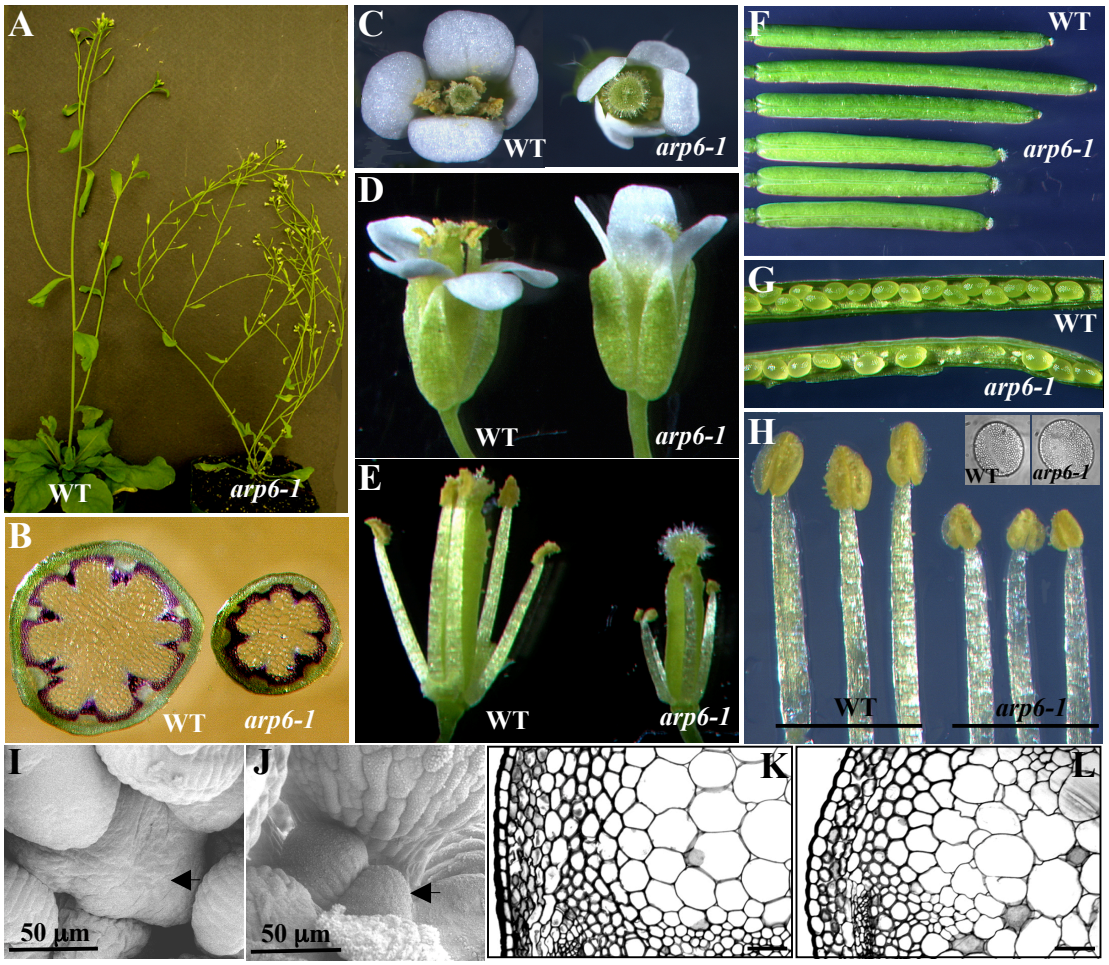

Figure 2.7 Inflorescence and Flower Development in Long Day Grown *arp6-1* Plants

(A) Inflorescences of wild type (WT) and *arp6-1* plants. **(B)** Cross sections of wild type and *arp6-1* inflorescence stems taken just below the first internode. Sections were stained for lignin with phloroglucinol. **(C)** Top view of wild-type and *arp6-1* flowers. **(D)** Side view of wild type and *arp6-1* flowers. **(D)** Side view of wild type and *arp6-1* flowers without sepals and petals. **(F)** Fully expanded wild type and *arp6-1* siliques. **(G)** Representative siliques of wild type and *arp6-1* plants opened to reveal developing seeds and unfertilized ovules in the *arp6-1* fruit. **(H)** Wild-type and *arp6-1* stamens. Inset pictures show pollen grains of each genotype. **(I)** Toluidine blue stained sections of wild type (WT) and *arp6-1* inflorescence stems shown at 40X magnification.

Figure 2.8 Flowering of *arp6* Mutants and Overexpression Lines

(A) Twenty three-day-old wild type (WT), *arp6-1*, *arp6-2*, and *arp6-1*/*arp6-2* plants grown under long-day conditions. **(B)** 50-day-old wild-type, *arp6-1*, *arp6-2* plants grown under short-day conditions. **(C)** 30 day old wild type (WT) and *35S*:*ARP6* transgenic lines grown in long days. Representatives of three independent transgenic lines are shown.

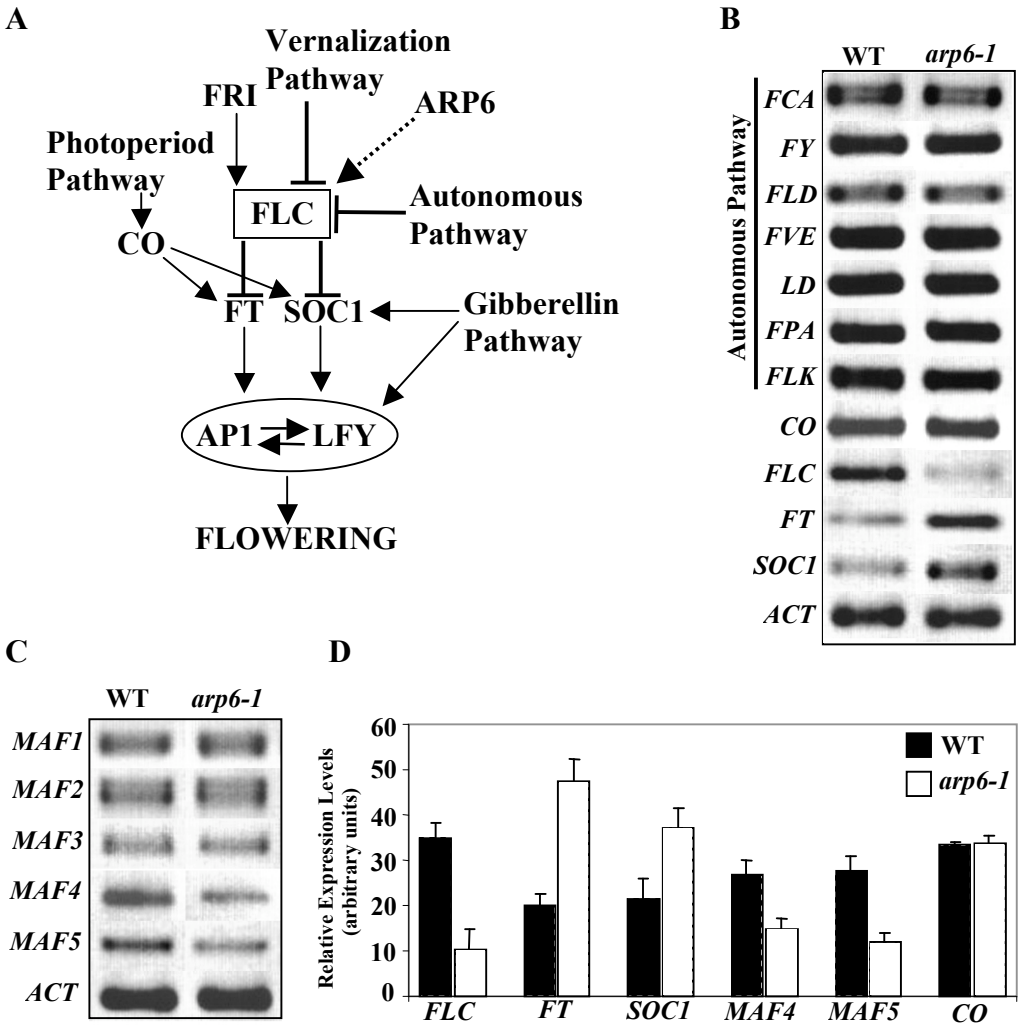

Figure 2.9 Expression of Flowering Time Control Genes in *arp6-1* Plants

(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 (rectangular 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 *AP1* and *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. 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)** Semi-quantitative RT-PCR analysis of multiple flowering time control genes was performed using total RNA from 10-day-old wild type (WT) and *arp6-1* shoots. Total actin (*ACT*) transcript levels were also assayed as a control for the amount of input cDNA. **(C)** Semi-quantitative 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 between the three independent experiments.

Table 2.1 Average Number of Seeds Per Silique From Wild Type, *arp6-1* and Reciprocal Crosses

Natural Pollination		Manual Pollination		
WT	*arp6-1*	WT	WT female x *arp6-1* male	*arp6-1* female x WT male
52.7 +/- 2.5	21.8 +/- 4.9	39.6 +/- 8.1	38.8 +/- 4.2	20.5 +/- 2.1

Values shown are the mean number +/- SD of seeds per silique. These data represent the seed set of at least 6 siliques for each category. Each set of crosses was repeated at least twice.

Table 2.2 Rosette Leaf Number at the Time of Flowering in Wild Type and Mutant Plants

Light Condition	WT	*arp6-1*	*arp6-2*	*flc-3*	*FRI*-Col	*FRI*-Col/*arp6-1*
Long Days	12.0 +/- 1.1	6.0 +/- 0.4	5.22 +/- 0.4	8.2 +/- 0.4	65.0 +/- 2.2	12.0 +/- 1.4
Short Days	43.1 +/- 5.0	19.9 +/- 1.9	20.0 +/- 1.8	36.4 +/- 5.0	ND	ND

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.

Table 2.3 Primer Sequences used for RT-PCR

Gene name (AGI #)	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
FCA (At4g16280)	GTTTCATCTTCTGCCACATT	TAAATTTTGGTTTGGTTGCT
FY (At5g13480)	GCAGCAAGGGTATCAGCAACA	ATCTGTGGACGGTTGAAACCA
FLD (At3g10390)	GAGCACAGATCTCGATACCTT	TCGTAATCATCGCCTGAAGCT
FVE (At2g19520)	GTTGTTTGATCGTAGGAAGC	AACATGCGACTTGAACTTCT
LD (At4g02560)	GTTTATGCACTAACTTCGGG	GTTCCCTAACTGCATACGAG
FPA (At2g43410)	TAGCGAGCCTCTCAGAATAC	AACAGGATGATTTGACGAAC
FLK (At3g04610)	GCAGCAAATGCAGATTCCACT	TGAGCTGTAATCCGTAGCGTA
CO (At5g15840)	TCGAGTATGGACTACAAATTCACA	TGTTGTACATTAGCATCGTGTTGA
FLC (At5g10140)	ACAAAAGTAGCCGACAAGTCACCT	GGAAGATTGTCGGAGATTTGTCCA
FT (At1g65480)	AGACGTTCTTGATCCGTTTA	GTAGATCTCAGCAAACTCGC
SOC1 (At2g45660)	TGTTGAAGAAAGCCTTTGAGCTCT	ATCACTTTCTTGAAGAACAAGGTA
MAF1 (At1g77080)	GTGAGCTAGGAAGGCAGAACTGA	CCGAAGGAGGTACAACACTGATCC
MAF2 (At5g65050)	TGTGGGTCTCCGGTGATTAGGATC	AATCAGGCTGTAAGTTTAAGGTGAAAGC
MAF3 (At5g65060)	AAAAAAGCAAACACATTTTGGGTCC	AACTCTGATATTTGTCTACTAAGGTAC
MAF4 (At5g65070)	GTCAGAAGAATTAGTCGGAGAAAAC	GATGACTTTTCCGTAGCAGGGGGAAG
MAF5 (At5g65080)	ATTAGATGTGTCGGAAGAGTGAAG	CCTGTCTTCCAAGGTAACACAAAGG
ACT*	GARAARATGACNCARATNATGTTYGARACNTT	TCYTTNCTNATRTCNACRTCRCAYTTCATDAT

* This primer set amplifies all eight actin transcripts

CHAPTER III:

REPRESSION OF FLOWERING IN *ARABIDOPSIS* REQUIRES ACTIVATION OF *FLOWERING LOCUS C* (*FLC*) EXPRESSION BY THE HISTONE VARIANT H2A.Z[2]

[2] Deal, R.B., Topp, C.N., McKinney, E.C., and Meagher, R.B. 2006. Submitted to *Plant Cell*, 10/23/06

ABSTRACT

The histone variant H2A.Z has been implicated in numerous chromatin-mediated processes including transcriptional activation, euchromatin maintenance, and heterochromatin formation. In yeast and humans, H2A.Z is deposited into chromatin by a conserved protein complex known as SWR1 or SRCAP, respectively. Here we show that mutations in the *Arabidopsis* homologs of two components of this complex, ACTIN-RELATED PROTEIN 6 (ARP6) and PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), produce similar developmental phenotypes and result in the misregulation of a common set of genes. Using H2A.Z-specific antibodies we demonstrate that ARP6 and PIE1 are required for the deposition of H2A.Z at multiple loci including the *FLOWERING LOCUS C* (*FLC*) gene, a central repressor of the transition to flowering. Loss of H2A.Z from chromatin in *arp6* and *pie1* mutants results in reduced *FLC* expression and premature flowering, indicating that this histone variant is required for high-level expression of *FLC*. In addition to defining a novel mechanism for the regulation of *FLC* expression, these results support the existence of a SWR1-like complex in *Arabidopsis* and show that H2A.Z can serve to potentiate transcriptional activation in plants. The finding that H2A.Z remains associated with chromatin throughout mitosis suggests that it may serve an epigenetic memory function by marking active genes and poising silenced genes for reactivation.

INTRODUCTION

In order to ensure reproductive success, plants must align their transition to flowering with favorable environmental conditions. This is achieved through the integration of signals from multiple pathways that sense and respond to various environmental and endogenous cues such as day length, temperature, and developmental state (Simpson and Dean 2002). In *Arabidopsis*, several of these signaling pathways converge on the central floral repressor gene *FLOWERING LOCUS C* (*FLC*) and either activate or repress its expression to suppress or promote the transition to flowering, respectively (Henderson and Dean 2004; Simpson 2004; Sung and Amasino 2004). The terminal effectors of these pathways controlling *FLC* expression are now known to represent many types of chromatin modifying factors including histone acetyltransferases, histone deacetylases, histone methyltransferases, polycomb-type proteins, and a putative histone demethylase (He and Amasino 2005). Thus, *FLC* serves as a model for how chromatin remodeling and modification can regulate a critical developmental switch. In contrast to histone modifications, there has been no evidence to date for the role of histone variants in the control of *FLC* expression.

The fundamental repeating unit of chromatin, known as the nucleosome, consists of roughly 150 bp of DNA wrapped around a protein particle composed of two copies of each of the four core histones: H2A, H2B, H3, and H4. These histone proteins are encoded by multiple gene copies and are produced in large quantities to accommodate the nascent genome during DNA replication. In addition to these bulk histones, eukaryotic genomes also encode variant histones that are deposited independently of DNA replication and serve to functionally specialize or differentiate specific chromatin regions. In *Saccharomyces*, *Drosophila*, and humans, the histone variant H2A.Z is deposited into chromatin by a conserved protein complex known as SWR1,

Tip60, or SRCAP, respectively (Krogan et al. 2003; Kobor et al. 2004; Kusch et al. 2004; Mizuguchi et al. 2004; Ruhl et al. 2006). The yeast H2A.Z variant plays important roles in both activating transcription and antagonizing the spread of heterochromatin into euchromatic regions (Santisteban et al. 2000; Adam et al. 2001; Larochelle and Gaudreau 2003; Meneghini et al. 2003). Several genome-wide studies of yeast H2A.Z occupancy have shown that this variant localizes to the promoters of thousands of euchromatic genes and its presence is required for the optimal transcriptional activation of many of these genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005). In contrast, cytological and genetic studies suggest that in metazoans H2A.Z may also play a role in heterochromatin formation and/or maintenance (Rangasamy et al. 2003; Swaminathan et al. 2005), although this variant has been observed at the 5′ ends of several active genes in human and chicken cell lines (Bruce et al. 2005; Farris et al. 2005). However, null mutations in mouse and *Drosophila* H2A.Z result in embryonic lethality (van Daal and Elgin 1992; Faast et al. 2001), making it difficult to study the developmental functions of H2A.Z in these organisms.

Previous work in *Arabidopsis* showed that loss-of-function mutations in two genes encoding putative homologs of components of the SWR1/SRCAP complex, ACTIN-RELATED PROTEIN 6 (ARP6) and the Snf2 protein PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), resulted in premature flowering due to reduced *FLC* expression (Noh and Amasino 2003; Choi et al. 2005; Deal et al. 2005; Martin-Trillo et al. 2006). However, the mechanism by which these proteins regulate *FLC* expression has not been addressed. Here we provide evidence for the existence of a SWR1-like complex in plants and show that ARP6 and PIE1 are both required for the deposition of H2A.Z into chromatin at *FLC* and the *FLC* paralogous genes *MADS AFFECTING FLOWERING 4* (*MAF4*) AND *MAF5*. Loss of H2A.Z

from chromatin in *arp6* and *pie1* mutants results in reduced expression of *FLC*, *MAF4*, and *MAF5*, indicating that H2A.Z acts to potentiate transcriptional activation of these genes. Thus, in addition to the host of chromatin modifying factors identified previously, the histone variant H2A.Z and its deposition machinery define a novel mechanism for promoting *FLC* expression and thereby ensuring the proper timing of the transition from vegetative growth to flowering.

RESULTS

arp6 and *pie1* mutants have similar developmental and molecular phenotypes

In order to investigate the similarities in phenotype caused by disruption of the *ARP6* and *PIE1* genes, we compared the null mutants *arp6-1* (Deal et al. 2005) and *pie1-5* (Figure 3.1). Our observations indicated that *arp6* and *pie1* mutants showed a strikingly similar array of developmental phenotypes including reduced leaf size and early flowering (Figure 3.2A). In addition to these phenotypes, real-time RT-PCR analysis showed that a common set of seven genes was downregulated in both *arp6-1* and *pie1-5* mutants (Figure 3.2B). Among these genes was the MADS-box floral repressor *FLC* (Michaels and Amasino 1999), which was reduced approximately ten-fold in each mutant as compared to wild type (WT). Expression of the *FLC* paralogs *MAF4* and *MAF5* (Ratcliffe et al. 2003) was also decreased in *arp6-1* and *pie1-5*, but the reduction of both transcripts was more severe in the *pie1-5* mutant, suggesting that PIE1 has additional effects on these genes that are independent of ARP6. Preliminary microarray experiments comparing WT and *arp6-1* plants (R.B. Deal and R.B. Meagher, unpublished data) revealed a large number of genes whose expression was disrupted by the loss of *ARP6*. As such, we chose to examine the expression of several of these candidate genes in both *arp6-1* and *pie1-5* mutants using real-time RT-PCR. We found that both mutants were defective in the expression

of a *CONSTANS- LIKE* (*COL*) transcription factor, the *WRK70* transcription factor, and the putative disease resistance genes *PRB1* and *TIR* (Figure 3.2B). We also found several transcripts that were unchanged in the mutants and one whose expression was increased in both *arp6-1* and *pie1-5* (data not shown). The common developmental phenotypes and gene expression defects observed in these mutants suggested that ARP6 and PIE1 acted in a common pathway and perhaps the same protein complex.

We next sought to determine whether the ARP6 and PIE1 proteins were actually part of the same protein complex, as predicted based on their homology to yeast SWR1 complex components. Using gel-filtration chromatography and an ARP6 monoclonal antibody (Deal et al. 2005) we found that the major native form of ARP6 migrated with a molecular weight of approximately 600 kDa. In these experiments the 50 kDa monomeric ARP6 was not detectable as a discrete peak, suggesting that ARP6 exists mainly as part of a protein complex *in vivo* (Figure 3.2C). Based on a previous genomic survey, the observed mass of 600 kDa was consistent with the expected size of an *Arabidopsis* SWR1 complex (Meagher et al. 2005). When the same experiment was performed with *pie1-5* mutant extracts, the peak of native ARP6 shifted down to approximately 250 kDa (Figure 3.2C), as would be expected if the 230 kDa PIE1 protein, and perhaps other subunits, were lost from the complex. These results strongly suggested that PIE1 and ARP6 were normally part of the same protein complex, and that at least some of the components of the complex remained associated with ARP6 in the absence of PIE1. This result was consistent with the previous finding that yeast ARP6 forms a subcomplex with the Swc2, Swc3, and Swc6 components of the SWR1 complex (Wu et al. 2005).

ARP6 interacts with H2A.Z

The *Arabidopsis* genome encodes 13 H2A histones that fall into four distinct phylogenetic classes, each of which is also conserved in the monocot *Oryza sativa*. Of these 13 H2As, four are clearly H2A.Z variants, being more closely related to yeast and metazoan H2A.Z proteins than to other plant H2As (Figure 3.3A). Using synthetic peptides as antigens, we raised polyclonal antibodies that reacted with the relatively conserved N-termini of the *Arabidopsis* H2A.Z subclass proteins HTA9 and HTA11, but not with representatives of any of the other three H2A subclasses (Figure 3.3B and C). Immunoprecipitations performed on plant protein extracts with the H2A.Z-specific antibodies efficiently precipitated ARP6, while parallel experiments using pre-immune (PI) rabbit serum as primary antibody failed to precipitate ARP6 (Figure 3.3D). These results confirmed that ARP6 interacts with H2A.Z, either directly or indirectly, and further supported the notion that ARP6 was involved in the deposition of H2A.Z into chromatin.

H2A.Z is excluded from chromocenters and retained in mitotic chromosomes

In order to examine the distribution of H2A.Z in chromatin we performed immunolocalization on isolated leaf and root cells with the H2A.Z antibody. We found that H2A.Z was dispersed widely throughout the nucleus but was conspicuously absent from the chromocenters (Figure 3.4A-C), which are composed of highly condensed heterochromatin containing centromeric and pericentromeric repeats (Fransz et al. 2002). This indicated that H2A.Z was found mainly in euchromatic regions and was likely responsible for regulating the expression of many genes. The incorporation pattern observed was in contrast to mammalian and *Drosophila* H2A.Zs, which accumulate in both heterochromatic and euchromatic chromatin (Leach et al. 2000; Rangasamy et al. 2003). In dividing cells we observed H2A.Z throughout the condensed chromosomes

(Figure 3.4D-F), indicating that this variant persisted in the chromatin through mitosis and therefore may serve as a stable epigenetic mark.

ARP6 and PIE1 are required for H2A.Z deposition

We next sought to examine the possibility that ARP6 and PIE1 were involved in the deposition of H2A.Z at the *FLC* gene, because *FLC* expression was greatly reduced in both *arp6-1* and *pie1-5* mutants (Figure 3.2B). Chromatin immunoprecipitation (ChIP) using the *Arabidopsis* H2A.Z antibody was employed to examine H2A.Z abundance across the *FLC* gene in WT and mutant plants. In WT plants H2A.Z was predominantly enriched in two discrete domains, one at each end of the transcribed region, with relatively low H2A.Z levels between these two domains (Figure 5A and E). In contrast, the *arp6-1* and *pie1-5* mutants showed little or no H2A.Z enrichment across the entire *FLC* gene, and the pattern of enrichment was nearly indistinguishable between the two mutants (Figure 3.5A).

ChIP experiments examining the *MAF4* and *MAF5* genes in WT plants revealed a pattern of H2A.Z distribution similar to that found at *FLC*, with the highest levels near the beginning and end of each transcribed region (Figure 3.5B and F). In the case of *MAF4* we observed only a small decrease in H2A.Z over the middle of the transcribed region, whereas H2A.Z levels were quite low in the middle of the *MAF5* gene (Figure 3.5B and F). Again, in contrast to WT, there was little or no H2A.Z accumulation across the *MAF4* and *MAF5* genes in *arp6-1* and *pie1-5* mutants. Control ChIP experiments using a histone H2B antibody, which should react with all nucleosomes, showed that there was not a significant difference in overall H2B distribution between WT and *arp6-1* mutants on *FLC*, *MAF4*, or *MAF5* (Figure 3.5C and D). Thus, the observed differences in H2A.Z enrichment on these genes were not simply due to gross

differences in nucleosome occupancy among genotypes. These results indicated that ARP6 and PIE1 were each required for the deposition of H2A.Z at the three genes examined, supporting the existence of a SWR1/SRCAP-like complex in *Arabidopsis*. In addition, H2A.Z was normally distributed across the *FLC* and *MAF* genes with highest levels at the beginning and end of the transcribed regions, and loss of H2A.Z from chromatin in the mutants was correlated with decreased gene expression and early flowering. Thus, *Arabidopsis* H2A.Z is required for high-level expression of the *FLC*, *MAF4*, and *MAF5* genes, suggesting that it plays a role similar to yeast H2A.Z in promoting transcription.

H2A.Z occupancy is inversely correlated with *FLC* transcript levels

In order to gain insight into the role of H2A.Z in promoting high-level expression of *FLC*, we examined H2A.Z distribution in plant samples with widely different *FLC* transcript levels. H2A.Z accumulation on *FLC* was measured in cauline leaves, 10-day-old WT shoots, and 10-day-old shoots carrying the strong *FLC* activator *FRIGIDA* (*FRI*) (Lee and Amasino 1995). Among these samples the *FRI*-expressing line showed the highest levels of *FLC* expression, WT was intermediate, and cauline leaves had a 10-fold lower level of *FLC* than did the *FRI* line (Figure 3.6A). Interestingly, the spatial distribution of H2A.Z on *FLC* was the same in each sample and the overall levels of H2A.Z in each were also surprisingly similar, showing only minor differences except at the 5´ and 3´ ends of the gene, where an inverse correlation between transcript level and H2A.Z occupancy was observed (Figure 3.6B and D). This correlation was most clear at the 3´ end of the gene.

Because nucleosome occupancy on *FLC* might have differed among these samples, we performed parallel ChIP experiments with an H2B antibody to quantify total nucleosome

distribution, allowing us to measure H2A.Z enrichment relative to that of H2B. We found that the H2A.Z/H2B ratio across the *FLC* gene was also nearly indistinguishable among the three samples at most sites tested, but the H2A.Z/H2B ratio at the 5´ and 3´ ends of the gene still showed an inverse correlation with *FLC* transcript levels (Figure 3.6C and D). Thus, the higher the transcript level, the less H2A.Z was present at the 5´ and 3´ ends of the gene, similar to the trend previously observed for yeast H2A.Z at the 5´ ends of genes (Guillemette et al. 2005; Zhang et al. 2005). Collectively, our results indicate that H2A.Z is required for high-level expression of *FLC*, but its presence does not directly induce transcriptional activation, suggesting that this variant poises the gene in a state competent for activation by other factors.

DISCUSSION

Previous studies of *Arabidopsis* ARP6 and PIE1 revealed that these proteins were both involved in regulating multiple developmental processes including leaf development, inflorescence and flower development, and repression of the transition to flowering. In the case of flowering time control, both ARP6 and PIE1 were shown to be required for high-level expression of the floral repressor gene *FLC* (Noh and Amasino 2003; Choi et al. 2005; Deal et al. 2005; Martin-Trillo et al. 2006), indicating that these proteins were likely to be involved in transcriptional regulation. Concurrent with the elucidation of ARP6 and PIE1 function in *Arabidopsis*, several groups discovered that in *Saccharomyces cerevisiae*, ARP6 was a component of the SWR1 chromatin remodeling complex (Krogan et al. 2003; Kobor et al. 2004; Kusch et al. 2004; Mizuguchi et al. 2004; Ruhl et al. 2006). This complex was shown to have the novel activity of replacing histone H2A with the variant H2A.Z in particular nucleosomes, thus functionally specializing the surrounding chromatin and in many cases potentiating the

expression of nearby genes. Given this information we considered the possibility that such a complex might also exist in *Arabidopsis*. A comparison of the yeast SWR1 ATPase subunit to all *Arabidopsis* proteins indicated that of the 42 Swi2/Snf2 family proteins encoded by the *Arabidopsis* genome, PIE1 was the most closely related to SWR1. In addition to ARP6 and PIE1, the *Arabidopsis* genome also encodes clear orthologs of most other SWR1 complex components (Meagher et al. 2005), as well as multiple H2A.Z isovariants (Figure 3.3). Could the developmental functions of *Arabidopsis* ARP6 and PIE1 be attributable to their activity within a plant SWR1-like complex? In yeast the SWR1 complex deposits the histone H2A.Z variant into euchromatic regions near telomeres (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004) and in the promoters of many euchromatic genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005), thereby preventing the spread of silent heterochromatin into euchromatic regions (Meneghini et al. 2003) and promoting transcriptional activation, respectively. Perhaps a plant SWR1-like complex could have an analogous function of depositing H2A.Z into *FLC* chromatin, ensuring competence of the gene for high-level expression and thus allowing the flowering program to be repressed and vegetative growth to continue. In this investigation we explored the hypothesis that the contribution of ARP6 and PIE1 to the developmental program in *Arabidopsis* was a manifestation of their role in depositing H2A.Z into chromatin and thus regulating the expression of multiple developmentally important genes.

A direct comparison of *arp6* and *pie1* mutants indicated that these mutants shared many developmental and molecular phenotypes. Both mutations caused aberrations in leaf development and early flowering, and resulted in the misregulation of a common set of genes including the flowering regulators *FLC*, *MAF4* and *MAF5*. In addition, we found that ARP6 was

a component of a high-molecular weight protein complex and that the size of this complex was dramatically reduced in the absence of PIE1, suggesting that ARP6 and PIE1 were indeed part of the same protein complex (Figure 3.2). Furthermore, polyclonal antibodies that reacted with at least two of the four *Arabidopsis* H2A.Z proteins efficiently immunoprecipitated ARP6 from plant extracts, confirming an interaction between ARP6 and H2A.Z (Figure 3.3). Collectively these results suggested that ARP6 and PIE1 were part of a plant SWR1-like complex.

Chromatin Immunoprecipitation (ChIP) was employed to determine whether ARP6 and PIE1 were involved in the deposition of H2A.Z into chromatin at the *FLC* and *MAF* genes, since the expression of these genes was reduced in both *arp6* and *pie1* mutants. We found that H2A.Z was normally enriched in two discrete domains on each of these genes, one near the transcription start site and another near the end of the gene, and that little or no H2A.Z accumulated in the chromatin of *arp6* or *pie1* mutants (Figure 3.5). These results indicated that ARP6 and PIE1 were indeed necessary for deposition of H2A.Z into chromatin at the three loci examined. Loss of H2A.Z from chromatin in *arp6-1* and *pie1-5* mutants was correlated with reduced expression of *FLC*, *MAF4*, and *MAF5* indicating that H2A.Z is normally required to promote the expression of each of these genes. This observation suggested that, like yeast H2A.Z, the *Arabidopsis* variant can also potentiate transcriptional activation. ChIP analysis of the *PRB1*, *COL*, *WRKY70*, and *TIR* genes, whose transcript levels were also reduced in the mutants (Figure 3.2B), indicated that even in WT plants these loci did not accumulate significant amounts of H2A.Z (data not shown), and thus these genes were likely to be indirectly regulated by ARP6 and PIE1. This finding indicated that not all *Arabidopsis* genes require H2A.Z for high-level expression and that many of the transcriptional defects in *arp6* and *pie1* mutants were likely secondary effects

resulting from the misregulation of a smaller number of primary target genes that do require H2A.Z for proper expression.

In contrast to the situation in yeast where H2A.Z is normally found mainly in nucleosomes around the transcription start site (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005), *Arabidopsis* H2A.Z occupies regions near both the transcription start and termination sites at the three genes examined. Previous studies have shown a role for H2A.Z in recruiting RNA polymerase (Adam et al. 2001) and acting in concert with nucleosome remodeling complexes (Santisteban et al. 2000) to initiate transcription. However, the data presented here suggest that in *Arabidopsis* this histone variant also has a function beyond the 5´ end of genes, perhaps in facilitating transcript elongation and/or termination. The tendency of H2A.Z to destabilize nucleosomes that contain it (Suto et al. 2000; Abbott et al. 2001; Zhang et al. 2005) may facilitate nucleosome remodeling and thus polymerase passage and transcript elongation. In addition, multiple lines of evidence now indicate that chromatin remodeling enzymes regulate all phases of the transcription cycle, including termination (Alen et al. 2002; Morillon et al. 2003). Thus, *Arabidopsis* H2A.Z may facilitate remodeling at both ends of the gene in order to effect proper initiation and termination of transcription. The occupancy of H2A.Z at sites beyond the transcription initiation region has also been observed for several other *Arabidopsis* genes (A.P. Smith, R.B. Deal, and R.B. Meagher, unpublished data), suggesting that this phenomenon is not specific to the MADS-box transcription factor genes.

As a means of gaining insight into the role of H2A.Z in promoting transcriptional activation, we examined the relationship between *FLC* transcript levels and H2A.Z abundance on the *FLC* gene. H2A.Z occupancy on *FLC* was measured in three different tissues with *FLC* expression levels spanning a 10-fold range, from very high to very low. In each sample we observed

essentially the same spatial distribution of H2A.Z across the gene, with a peak at the beginning and end of the transcribed region. However, there was an inverse correlation between *FLC* expression levels and H2A.Z abundance on the gene such that the higher the transcript level, the less H2A.Z was present on the gene (Figure 3.6). This inverse relationship between H2A.Z occupancy and gene expression may result from a shift in the balance between ARP6/PIE1-mediated deposition of H2A.Z and loss of H2A.Z due to nucleosome disruption by RNA polymerase. During high-level transcription, the rate of transcription-induced depletion of H2A.Z might exceed the rate of replacement by ARP6/PIE1, resulting in an inverse correlation between transcript level and H2A.Z occupancy. In any case, these results clearly indicated that there was not a positive correlation between *FLC* expression level and H2A.Z abundance on the gene, even over a 10-fold range of *FLC* transcript levels. This suggests that the H2A.Z variant serves to poise the gene in a state competent for activation by other factors, rather than activating transcription directly. This may reflect the ability of H2A.Z to facilitate nucleosome remodeling (Santisteban et al. 2000) and/or to recruit the transcription machinery (Adam et al. 2001) or other activators in order to allow high-level transcription under the appropriate conditions. Thus, in the absence of H2A.Z in *arp6* and *pie1* mutants, *FLC* levels remain low even in the presence of strong activators such as *FRI* (Noh and Amasino 2003; Choi et al. 2005; Deal et al. 2005; Martin-Trillo et al. 2006), resulting in early flowering.

In addition to ChIP studies on the distribution of H2A.Z across single genes, we also used our H2A.Z antibodies to examine the nuclear localization of this histone variant during interphase and mitosis. In contrast to mammalian and *Drosophila* H2A.Zs, which accumulate in both heterochromatic and euchromatic chromatin (Leach et al. 2000; Rangasamy et al. 2003), we found that *Arabidopsis* H2A.Z was widely distributed throughout euchromatin, but was excluded

from the heterochromatic chromocenters (Figure 3.4). This indicated that H2A.Z is likely responsible for regulating the expression of many genes in euchromatin, but is not likely to be involved in heterochomatin formation or maintenance in *Arabidopsis*. The observation that H2A.Z is incorporated into euchromatin during interphase and remains in chromatin through mitosis suggests that it may serve an epigenetic memory function by marking actively transcribed genes and providing competence for the reactivation of silenced genes. Such a function, coupled with the ability of H2A.Z to promote transcriptional activation, is likely to be important in the establishment and maintenance of cell fate during development.

In conclusion, it appears that ARP6 and PIE1 act together to control multiple developmental processes, most likely by regulating the expression of a large number of genes through the incorporation of H2A.Z into chromatin. Our results support the notion that the H2A.Z deposition machinery is conserved in plants as it is in yeast and metazoans, requiring both ARP6 and a Snf2 protein of the SWR1/SRCAP class. Furthermore, the loss of H2A.Z from chromatin in *arp6-1* and *pie1-5* mutants correlates with decreased expression of *FLC*, *MAF4*, and *MAF5* indicating that H2A.Z deposition is essential for the full transcriptional activation of these genes. Thus, H2A.Z can act to potentiate the transcriptional activation of *Arabidopsis* genes, similar to its role in yeast. In terms of flowering time control, H2A.Z and its deposition machinery now define a novel mechanism for promoting *FLC* expression, and thus repressing the transition from vegetative to reproductive development in *Arabidopsis*. During vegetative growth H2A.Z allows high-level *FLC* expression and floral repression, yet it remains associated with the inactive gene post-flowering, perhaps to poise the gene for reactivation and reestablishment of the vegetative growth program in the next generation.

Future work in this area will be focused on large-scale approaches to identifying all of the genes that require H2A.Z for proper expression and elucidating the DNA sequence determinants and other factors that promote the deposition of H2A.Z into chromatin at particular loci. Insight into the mechanism by which H2A.Z promotes transcriptional activation could be gleaned from protein-protein interaction studies and the identification of second-site mutations that suppress the early flowering of *arp6* and *pie1* mutants. Because ARP6 and PIE1 act as a hub through which the expression of many genes is controlled, identification of the full set of genes misregulated in *arp6* and *pie1* mutants should allow the assignment of many currently anonymous genes to particular developmental pathways.

METHODS

Plants and growth conditions

All plants were of the Columbia (Col-0) ecotype and were germinated by sowing on wet soil and storing at 4° C for 2 days prior to moving to the growth chamber. Plants were grown at 22° C under 16 hours of light per day. The *pie1-5* mutation was a T-DNA insertion allele from the Salk Institute (SALK_011204). The T-DNA insertion is in exon 15 and is a null mutation based on RNA levels (Figure 3.1). The *arp6-1* allele was described previously (Deal et al. 2005).

Gel filtration chromatography

Gel filtration was performed on a Sephacryl S-300HR column (Amersham Biosciences) in a buffer consisting of 20 mM Tris (pH 7.5), 200 mM NaCl, 10 mM $MgCl_2$, and 10% v/v glycerol. The column was calibrated with a mixture of standard proteins ranging in size from 670 kDa to 60 kDa. Plant extracts were prepared by grinding a mixture of leaf and flower tissue in two

volumes of the gel filtration buffer supplemented with 1 mM β-mercaptoethanol and Roche Complete protease inhibitors (Roche). After grinding, samples were cleared by centrifugation and filtration. The column was run at room temperature with a flow rate of 0.25 mL/min and 0.5 mL fractions were collected. Two independent biological replicates of the gel filtration experiment were done and both gave very similar results.

Phylogenetic analysis

The following *Arabidopsis* histone H2A protein sequences were used for phylogenetic analysis: HTA1 (At5g54640), HTA2 (At4g27230), HTA3 (At1g54690), HTA4 (At4g13570), HTA5 (At1g08880), HTA6 (At5g59870), HTA7 (At5g27670), HTA8 (At2g38810), HTA9 (At1g52740), HTA10 (At1g51060), HTA11 (At3g54560), HTA12 (At5g02560), HTA13 (At3g20670). Other H2A proteins used for the analysis were from *Oryza sativa*: HTA701 (Os01g31800), HTA702 (Os08g33100), HTA703 (Os12g25120), HTA704 (Os03g51200), HTA705 (Os10g28230), HTA706 (Os05g38640), HTA707 (Os05g02300), HTA708 (Os07g36140), HTA709 (Os07g36130), HTA710 (Os03g17100), HTA711 (Os12g34510), HTA712 (Os03g06670), HTA713 (Os03g53190); Human: Hs H2A.X (NP_002096), Hs H2A.Z (NP_002097); *Drosophila*: Dm H2Av (NP_524519); and *Saccharomyces*: Sc Htz1 (NP_014631). Sequences were aligned with Clustal W using default settings, and trees were constructed with PAUP using the neighbor-joining method.

Antibody preparation

Polyclonal antibodies specific to *Arabidopsis* H2A.Zs were produced in rabbits by injecting peptides representing the N-termini of HTA9 and HTA11. The peptide sequences N-

SGKGAKGLIMGKPSGSDKDKDKKKPIT-C (HTA9) and N-AGKGGKGLVAAKTMAANKDKDKDKKKPIS-C (HTA11) were synthesized as four-fold multiple antigenic peptides. The primary injection and three subsequent boosts were done with 250 μg of each peptide. Antibodies were affinity purified on resin to which the H2A.Z peptides had been coupled.

Immunoprecipitation

Plant extracts were prepared by grinding a mixture of leaf and flower tissue in 2 volumes of IP buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 0.1% NP-40, 1mM β-mercaptoethanol, and Roche Complete protease inhibitors) followed by centrifugation and filtration to clear the extracts. Extracts were then aliquoted into multiple tubes (900 μL each) and 40 μL of Protein A-agarose beads (Roche) were added to each tube. Tubes were rocked at 4° C for 1 hour to pre-clear the extracts, and then beads were removed by centrifugation. Antibodies or pre-immune serum were then added and tubes were kept on ice for 1 hour to allow antibody binding. H2A.Z antibodies were used at a 1:100 dilution and an equivalent amount of pre-immune serum was used based on antibody concentration. Antibody-antigen complexes were captured by adding 50 μL of Protein A-agarose beads and rocking for 1 hour at 4° C. Beads were collected by centrifugation and washed 3 times for 20 minutes in IP buffer at 4° C.

Western blotting and Immunofluorescence microscopy

Both of these techniques were performed as described previously (Deal et al. 2005).

Reverse transcriptase-mediated PCR (RT-PCR)

RNA was isolated from 10-day-old seedlings (minus roots) using the Qiagen RNeasy Plant Mini Kit (Qiagen). Prior to reverse transcription, RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. Three micrograms of each RNA was reverse transcribed with the Superscript III first strand synthesis kit (Invitrogen). Real-time PCR was used to analyze the cDNA populations using 18S RNA as an endogenous control. The genes assayed by this method were: *FLC* (At5g10140), *MAF4* (At5g65070), *MAF5* (At5g65080), *PRB1* (At2g14580), *COL* (At5g24930), *WRKY70* (At3g56400), and *TIR* (At1g72930).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described previously (Gendrel et al. 2005). For each experiment 1-2 g of 10-day-old seedlings (minus roots) were used. For experiments with cauline leaves, 1-2 g of leaves from 30-day-old plants were used. The H2A.Z antibody was used at a dilution of 1:100, and the H2B antibody (Abcam, ab1790) was used at a dilution of 1:150. DNA was analyzed by real-time PCR with the *ACT2* 3´ UTR sequence as the endogenous control for all ChIP experiments. The relative quantity value calculated by the 2^{-ddCt} method in ChIP experiments is reported as "fold enrichment".

Real-time PCR

Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR system using SYBR Green detection chemistry (Applied Biosystems). The 2^{-ddCt} method (Livak and Schmittgen 2001) of relative quantification was used in all experiments. The data presented for

both RT-PCR and ChIP experiments are the average relative quantity from at least two biological replicates +/- standard error. All primer sequences are available upon request.

Accession numbers

The accession numbers for the *ARP6* and *PIE1* genes are At3g33520 and At3g12810, respectively.

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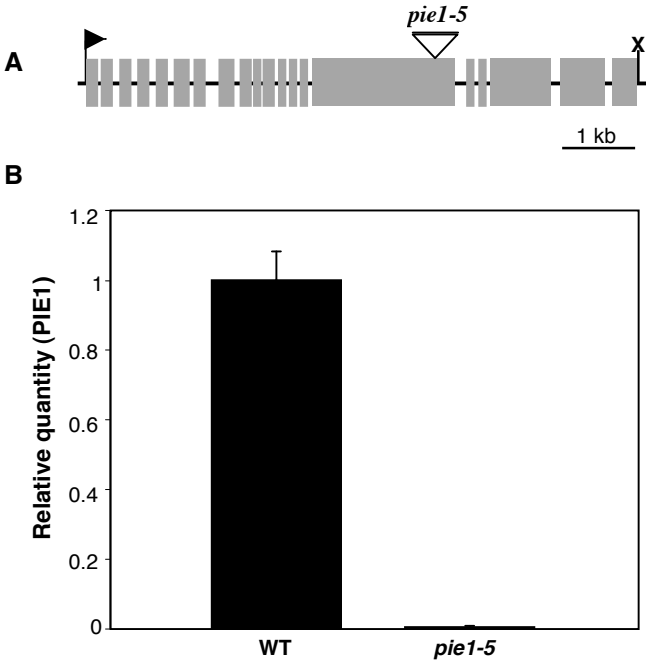

Figure 3.1 The *pie1-5* Mutant Allele is Null

(**A**) Diagram of the *PIE1* gene. The transcription start site is shown as a right-facing arrow and termination site is indicated by an "X". Exons are depicted as gray boxes. The location of the T-DNA insertion in *pie1-5* is shown as a triangle above exon 15. (**B**) Relative quantity of the *PIE1* transcript in wild-type (WT) and *pie1-5* plants as detected by real-time RT-PCR. Bars represent standard error. The transcript is essentially undetectable in the *pie1-5* mutant indicating that the allele is null.

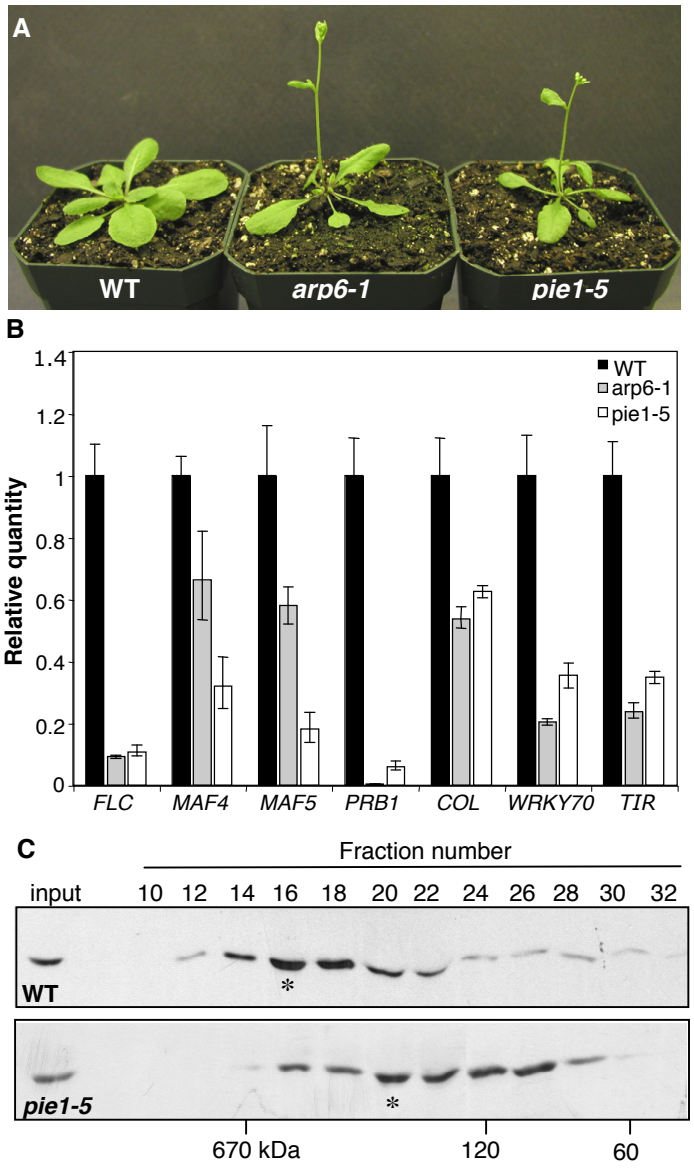

Figure 3.2 *arp6* and *pie1* Mutants Have Similar Developmental and Molecular Phenotypes

(**A**) Twenty-day-old wild type (WT), *arp6-1*, and *pie1-5* plants grown under long day conditions. (**B**) Real-time RT-PCR data showing relative expression of the indicated genes in WT, *arp6-1* and *pie1-5* plants. Average relative quantities (RQ) +/- standard errors (SE) are shown for each sample. (**C**) Western blots of even-numbered gel filtration fractions from a Sephacryl S-300 column run with extract from WT (upper panel) or *pie1-5* plants (lower panel). Blots were

probed with an ARP6 monoclonal antibody. Asterisks indicate the ARP6 peak fractions, and calibrated molecular weights are given below the blots.

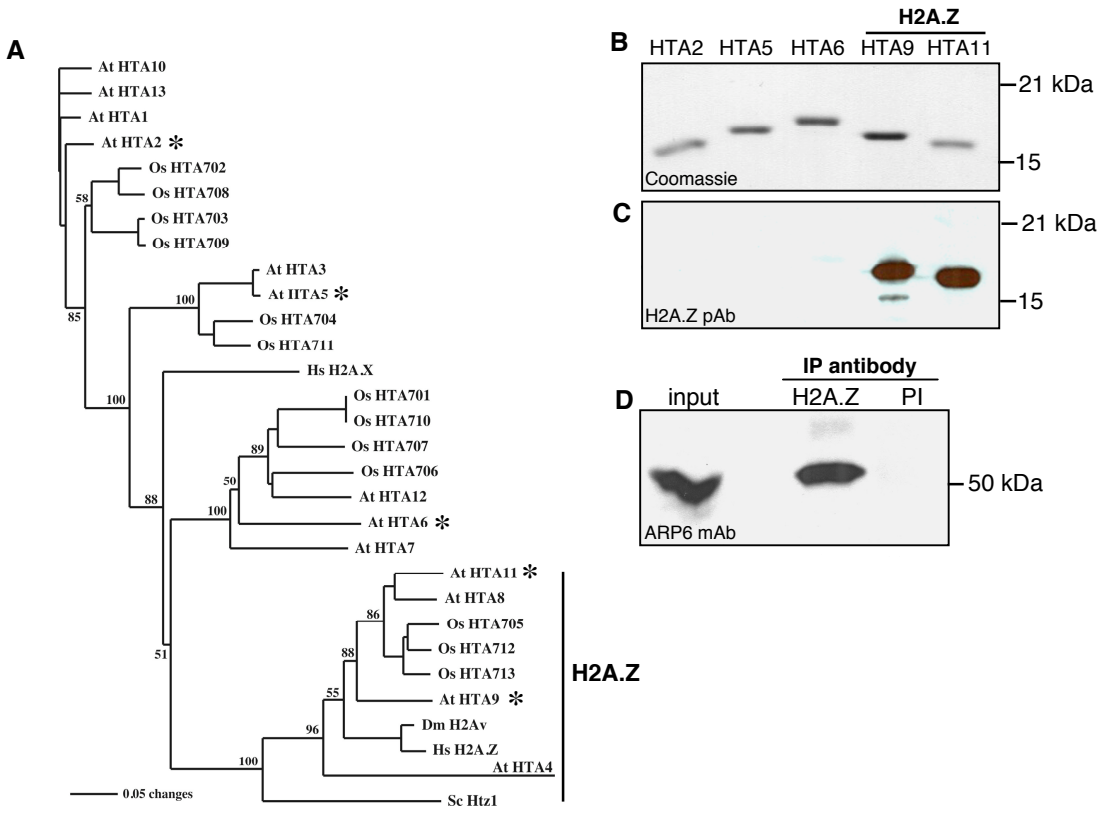

Figure 3.3 H2A Phylogeny and H2A.Z-specific Antibodies

(**A**) Neighbor-joining protein sequence phylogeny showing H2A proteins from *Arabidopsis* (At), Rice (Os), *Drosophila* (Dm), Human (Hs), and *Saccharomyces* (Sc). Bootstrap values are shown on the branch points of the tree. The H2A.Z clade is indicated by a vertical bar to the right of the tree and asterisks indicate proteins used in (B) and (C). (**B**) Coomassie blue-stained SDS-PAGE gel showing purified recombinant *Arabidopsis* H2A histones. The H2A.Z variants are indicated by a horizontal bar above the protein names. Molecular weights are shown to the right of the gel. (**C**) Western blot of a gel loaded as in (B), probed with the H2A.Z-specific antibody. Molecular weights are shown to the right of the gel. The antibody recognized HTA9 and HTA11, and was predicted to react with HTA8 because it is nearly identical to HTA11 in the N-terminal region. The HTA4 protein is highly divergent from the other H2A.Zs at the N-terminus and is not

expected to be recognized by the antibody. (**D**) Western blot of immunoprecipitates probed with an ARP6 monoclonal antibody. Input sample is 5% of the total protein used in each immunoprecipitation. The antibody used for immunoprecipitation is indicated above the blot: either the H2A.Z antibody or pre-immune serum (PI) from the same rabbit.

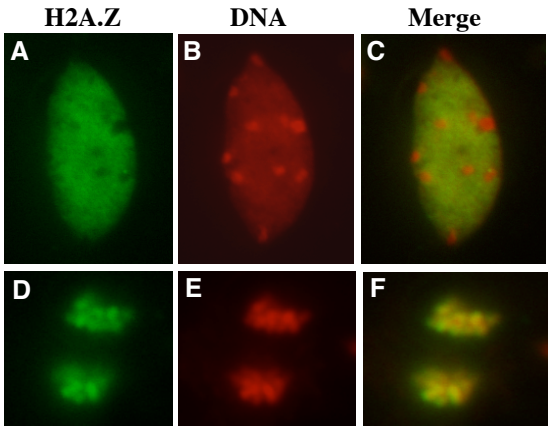

Figure 3.4 H2A.Z Localizes to Euchromatic Regions but not Heterochromatic Chromocenters

(**A**) Isolated leaf cell nucleus probed with the H2A.Z antibody. (**B**) The 4´, 6-diamidino-2-phenylindole (DAPI) channel image of the nucleus shown in (A). Chromocenters appear as densely stained spots throughout the nucleus. (**C**) Merge of images in (A) and (B). (**D**) Anaphase stage root cell probed with the H2A.Z antibody. (**E**) DAPI channel image of the cell shown in (D). (**F**) Merge of images shown in (D) and (E).

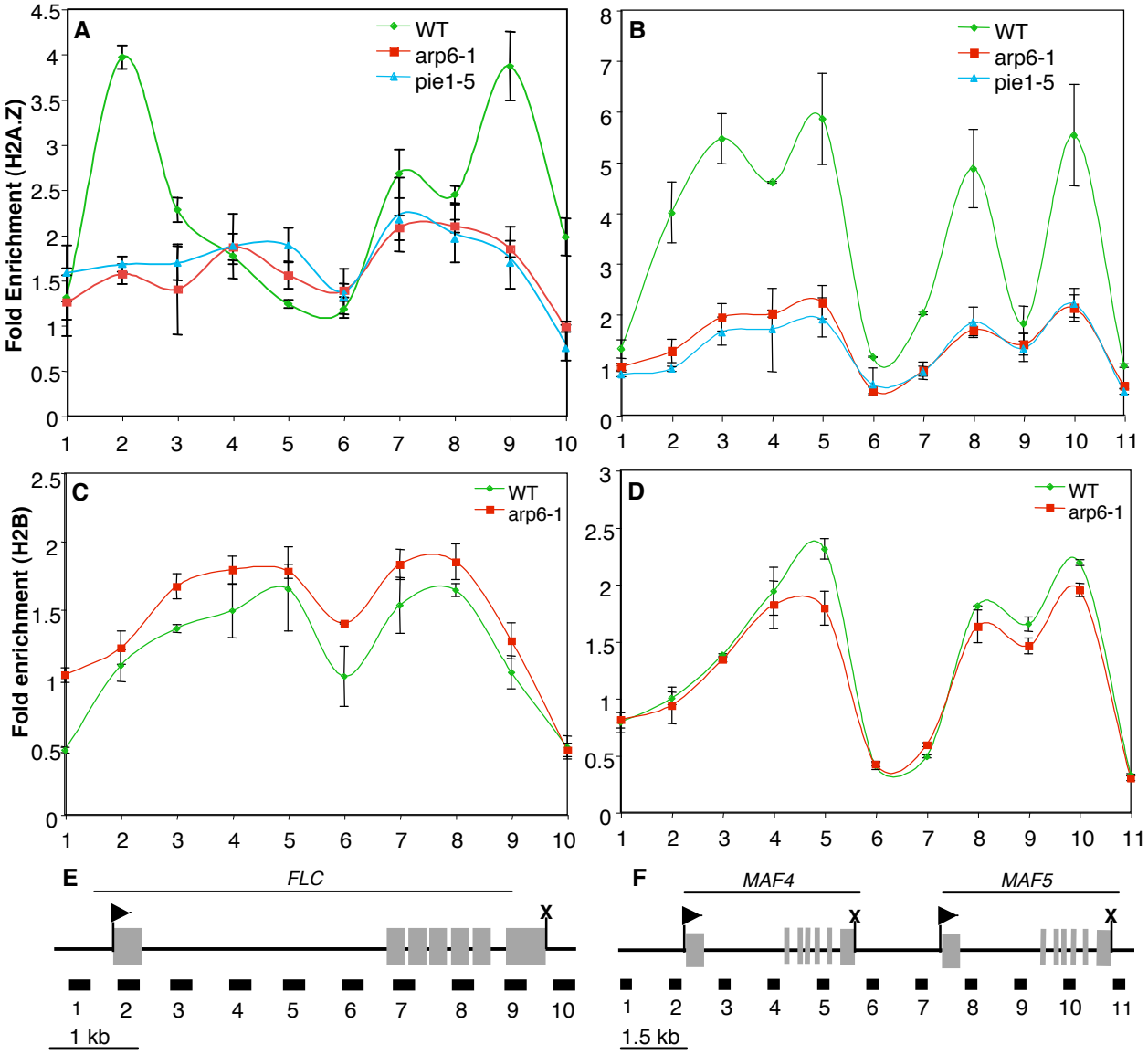

Figure 3.5 ARP6 and PIE1 are Required for Deposition of H2A.Z at *FLC*, *MAF4*, and *MAF5*

(**A**) Enrichment of H2A.Z on the *FLC* gene in WT and mutants as measured by chromatin immunoprecipitation (ChIP) with the H2A.Z antibody. Graph shows average fold enrichment +/- SE as measured by real-time PCR. (**B**) Enrichment of H2A.Z on the *MAF4* and *MAF5* genes in WT and mutants as measured by ChIP and real-time PCR. (**C**) Enrichment of H2B on the *FLC*

gene in WT and *arp6-1*. (**D**) Enrichment of H2B on the *MAF4* and *MAF5* genes in WT and *arp6-1*. (**E**) Diagram of the *FLC* gene with exons indicated as gray boxes. Transcription start is shown as an arrow and termination site as an "X". PCR primer sets are shown as black boxes below the diagram. Primer set numbers correspond to the numbers on the X-axis of the graphs in (A) and (C). (**F**) Diagram of the *MAF4* and *MAF5* genes and locations of PCR primer sets, depicted as in (E). Primer set numbers correspond to the numbers on the X-axis of the graphs in (B) and (D).

Figure 3.6 Relationship Between *FLC* Expression Levels and H2A.Z Abundance on the Gene

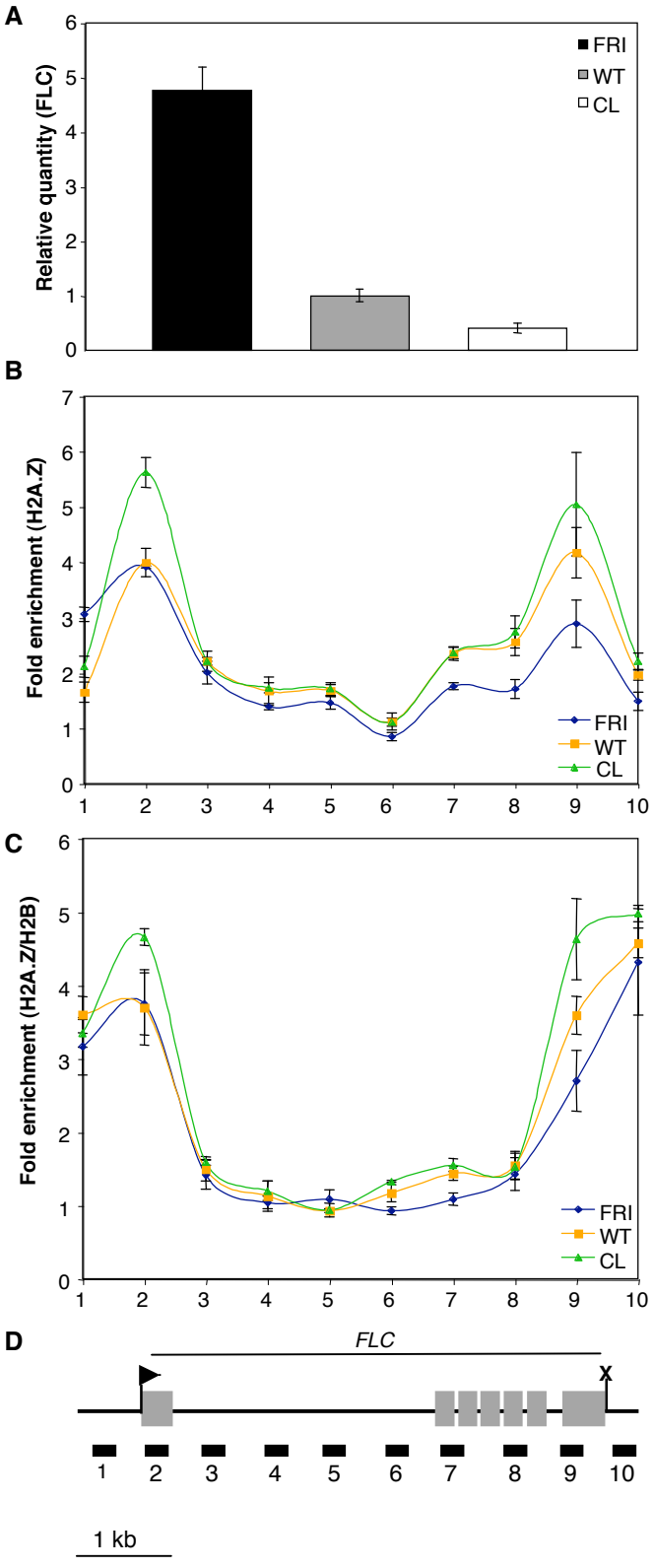

Figure 3.6 Relationship Between *FLC E*xpression Levels and H2A.Z Abundance on the Gene

(**A**) Real-time RT-PCR results showing the relative *FLC* level in each genotype: FRI is a Col-0 line carrying the strong *FRI* allele from the Sf-2 ecotype, WT is Col-0, and CL is cauline leaf. Graph shows average RQ +/- SE. (**B**) Enrichment of H2A.Z on the *FLC* gene in the indicated genotypes as measured by chromatin immunoprecipitation (ChIP) and real-time PCR. Graph shows average fold enrichment +/- SE. (**C**) ChIP was performed on the indicated genotypes using either an H2A.Z antibody or an H2B antibody, and data are reported as H2A.Z enrichment/H2B enrichment to correct the H2A.Z levels for total nucleosome occupancy. (**D**) Diagram of the *FLC* gene with exons indicated as gray boxes. Transcription start is shown as an arrow and termination site as an "X". Locations of PCR primer sets are shown as black boxes below the diagram. Primer set numbers correspond to the numbers on the X-axis of the graphs in (B) and (C).

CHAPTER IV:

SUMMARY AND CONCLUSIONS

At the outset of this study, ARP6 was known only as a ubiquitous eukaryotic protein, and our knowledge of the nature of its function was limited to a few qualitative observations made in yeast and *Drosophila*. In both of these organisms the protein was shown to be localized to the nucleus, and in *Drosophila* it co-localized with heterochromatin protein 1 (HP1) in pericentric heterochromatin, suggesting a possible role in heterochromatin function (Frankel et al. 1997; Harata et al. 2000). These observations, in conjunction with the evolutionary conservation of ARP6, indicated that this protein was part of the core set of eukaryotic proteins and suggested that it was likely to play an essential role in the nucleus. As such, we sought to investigate the role of ARP6 in the growth and development of *Arabidopsis* and to explore the underlying molecular functions of this protein. Our approach to this problem was to employ a reverse genetic strategy to elucidate the developmental roles of ARP6, and to use this knowledge as a means to explore the molecular functions of ARP6.

We first sought to examine the spatial and temporal patterns of *ARP6* expression and to determine the location of the protein at the subcellular level. Expression of *ARP6* was observed in nearly all tissues throughout vegetative and reproductive development (Figure 2.3) and the protein was found to be localized to the nucleus in all cell types examined (Figure 2.4). The broad expression patterns and nuclear localization observed for ARP6 suggested that this protein was likely to play a fundamental role in the growth and development of the plant. Based on our

analysis of two null alleles, we indeed found that ARP6 had a function in the growth and development of nearly every plant organ. Most notably, we found that ARP6 was required for proper development of the leaf, inflorescence, and flower, and was essential for the proper timing of the transition from vegetative to reproductive development (Figures 2.6-2.8).

A major role for ARP6 that emerged from this study was the promotion of cell proliferation and the control of organ size, particularly during rapid growth such as in long-day conditions. This was evidenced by the reduction in size of all aboveground organs in *arp6* mutants, and the fact that these organs were not composed of smaller cells, but rather fewer cells of normal size (Figures 2.6 and 2.7). Although the organ size defect of *arp6* mutants was quite severe during growth under long-day conditions, this defect was not as prevalent under short-day conditions, indicating that photoperiod and/or growth rate had a substantial impact on ARP6 function with regard to cell proliferation. However, *arp6* mutants grown under short-day conditions exhibited narrow leaves with deeply serrated margins (Figure 2.6), which may have reflected local changes in the degree or rate of cell proliferation along the margins of leaf primordia. Thus, ARP6 regulates cell proliferation and patterning in order to achieve the proper size and shape of organs such as leaves, stems, and flowers.

In addition to its role in the control of cell proliferation and patterning, ARP6 also regulates one of the most important developmental phase changes in the life of the plant, namely the transition from vegetative to reproductive development. Our analysis of *arp6* mutants indicated that ARP6 represses the switch from vegetative growth to flowering in both long- and short-day conditions. However, while ARP6 was necessary to repress flowering, overexpression studies indicated that it was not sufficient to do so alone (Figure 2.8). Because the molecular genetic control of flowering time has been extensively studied and the major pathways involved have

been elucidated (Mouradov et al. 2002; Simpson and Dean 2002; Putterill et al. 2004), this system represented a tractable entry point for understanding the molecular basis of a major phenotype resulting from the loss of ARP6 function. As such, we used semi-quantitative RT-PCR to investigate changes in gene expression in *arp6-1* mutants that might have been responsible for the observed early flowering phenotype. We discovered that expression of the main flowering repressor gene, the MADS-box transcription factor *FLC*, was greatly reduced in *arp6* mutants, as were the *FLC* paralogs *MAF4* and *MAF5* (Figure 2.9). Furthermore, *arp6* mutations were able to suppress the *FLC*-mediated late flowering phenotype of plants carrying a strong *FRI* allele, indicating that ARP6 is normally required to render *FLC* competent for high-level expression (Table 2.2 and Figure S1).

Concurrent with our elucidation of the developmental functions of *Arabidopsis* ARP6, several groups discovered that *Saccharomyces cerevisiae* ARP6 was a component of the SWR1 chromatin remodeling complex (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). This complex was shown to have the novel activity of replacing histone H2A with the H2A.Z variant in particular nucleosomes, thus functionally specializing the surrounding chromatin and in many cases promoting the expression of nearby genes. Given this information we considered the possibility that such a complex might also exist in *Arabidopsis*. A BLAST (Altschul et al. 1997) comparison of the SWR1 ATPase subunit to all *Arabidopsis* proteins indicated that of the 42 Swi2/Snf2 family proteins encoded by the *Arabidopsis* genome, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1) was the most closely related to SWR1. Interestingly, the reported phenotypes of *pie1* mutants were 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 (Noh and Amasino 2003). In addition to ARP6 and PIE1, the *Arabidopsis*

genome also encoded clear orthologs of most other SWR1 complex components (Meagher et al. 2005), as well as multiple H2A.Z isovariants (Figure 3.2). Could the developmental functions of *Arabidopsis* ARP6 be attributable to its activity within a plant SWR1-like complex? In yeast the SWR1 complex deposits the histone H2A.Z variant into euchromatic regions near telomeres (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004) and in the promoters of many euchromatic genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005), thereby preventing the spread of silent heterochromatin into euchromatic regions (Meneghini et al. 2003) and promoting transcriptional activation, respectively. Perhaps a plant SWR1-like complex could have an analogous function of depositing an H2A.Z variant(s) into *FLC* chromatin, ensuring competence of the gene for high-level expression and thus allowing the flowering program to be repressed and vegetative growth to continue. In the absence of such an activity, *FLC* levels would remain low even in the presence of strong activators like FRI, resulting in early flowering. Such a scenario would be consistent with our data and the known epigenetic regulation of *FLC* by multiple chromatin modifying factors (He and Amasino 2005).

In the next phase of our investigation we explored the hypothesis that the contribution of ARP6 to the developmental program in *Arabidopsis* was a manifestation of its role in depositing H2A.Z into chromatin and thus regulating the expression of multiple developmentally important genes. We therefore compared *arp6* and *pie1* mutants and found that these mutants shared many developmental and molecular phenotypes. Both mutations caused aberrations in leaf development and early flowering, and resulted in the misregulation of a common set of genes including the flowering regulators *FLC*, *MAF4*, and *MAF5*. In addition, we found that ARP6 was a component of a high-molecular weight protein complex and that the size of this complex was

dramatically reduced in the absence of PIE1, suggesting that ARP6 and PIE1 were indeed part of the same protein complex (Figure 3.1).

We next developed polyclonal antibodies that recognized at least two of the four H2A.Z variants in *Arabidopsis*, which allowed us to further investigate the role of ARP6 in the deposition of H2A.Z into chromatin. These antibodies efficiently immunoprecipitated ARP6 from plant extracts, confirming an interaction between these two proteins (Figure 3.2) and further supporting the notion that ARP6 was involved in H2A.Z deposition in *Arabidopsis*. Chromatin Immunoprecipitation (ChIP) was then employed to determine whether ARP6 and PIE1 were involved in the deposition of H2A.Z into chromatin at the *FLC* and *MAF* genes, since the expression of these genes was reduced in both *arp6* and *pie1* mutants. We found that in wild-type plants H2A.Z was normally enriched in two discrete domains on each of these genes, one near the transcription start site and another near the end of the gene. In contrast, little or no H2A.Z accumulated in the chromatin of *arp6* or *pie1* mutants (Figure 3.4). Collectively, these results indicated that ARP6 and PIE1 were both required for H2A.Z deposition and that *Arabidopsis* H2A.Z was required for high-level expression of the *FLC*, *MAF4*, and *MAF5* genes, suggesting that it plays a role similar to yeast H2A.Z in promoting transcription. In contrast to the situation in yeast where H2A.Z is found mainly in nucleosomes around the transcription start site (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005), *Arabidopsis* H2A.Z occupies regions near both the transcription start and termination sites at the three genes examined. Previous studies have shown a role for H2A.Z in recruiting RNA polymerase (Adam et al. 2001) and acting in concert with nucleosome remodeling complexes (Santisteban et al. 2000) to initiate transcription. However, our data suggested that in *Arabidopsis* this histone

variant also has a function beyond the 5′ ends of genes, perhaps in facilitating transcript elongation and/or termination.

In order to gain insight into the role of H2A.Z in promoting high-level expression of *FLC*, we examined the relationship between *FLC* expression level and H2A.Z abundance on the gene. H2A.Z accumulation on *FLC* was measured in cauline leaves, 10-day-old WT shoots, and 10-day-old shoots carrying the strong *FLC* activator *FRIGIDA* (*FRI*) (Lee and Amasino 1995). Among these samples the *FRI*-expressing line showed the highest levels of *FLC* expression, WT was intermediate, and cauline leaves had a 10-fold lower level of *FLC* than did the *FRI* line. We found that the spatial distribution of H2A.Z across the gene was essentially the same in each sample, but there was an inverse correlation between *FLC* expression levels and H2A.Z abundance at the 5′ and 3′ ends of the gene (Figure 3.5). Thus, the higher the level of *FLC* expression, the less H2A.Z was present on the gene. This is consistent with the observed depletion of yeast H2A.Z from chromatin upon transcriptional induction (Guillemette et al. 2005; Li et al. 2005; Zhang et al. 2005), and points to a model in which H2A.Z is continually deposited at target genes, but the level of H2A.Z is shifted towards depletion from chromatin upon transcriptional activation. This phenomenon is most likely due to increased nucleosome disruption associated with polymerase passage and may reflect the decreased stability of H2A.Z-containing nucleosomes, a feature that likely aids in transcriptional activation (Suto et al. 2000; Abbott et al. 2001; Zhang et al. 2005). In any case, our data clearly showed that there was not a positive correlation between *FLC* expression levels and H2A.Z abundance on the gene. This is in contrast to the histone H3.3 variant (Mito et al. 2005) and histone post-translational modifications such as H3 lysine 4 methylation and H3 lysine 9 acetylation (Liu et al. 2005), whose presence is directly correlated with gene expression levels. Collectively our results

indicated that H2A.Z was necessary for full transcriptional activation of *FLC*, but its presence was not sufficient to cause activation, suggesting that the H2A.Z variant serves to poise the gene in a state competent for activation by other factors, rather than activating transcription directly (Figure 4.1).

In addition to examining H2A.Z distribution at the level of single genes, we also used our H2A.Z antibodies to observe the behavior of this variant in interphase and mitotic chromosomes. We found that during interphase H2A.Z was dispersed widely throughout the nucleus but was conspicuously absent from the chromocenters, which are composed of highly condensed heterochromatin containing centromeric and pericentromeric repeats (Fransz et al. 2002). This indicated that H2A.Z is found mainly in euchromatic regions and is likely responsible for regulating the expression of many genes. This incorporation pattern is in contrast to mammalian and *Drosophila* H2A.Zs, which accumulate in heterochromatic and euchromatic chromatin (Leach et al. 2000; Rangasamy et al. 2003), and suggests that in plants H2A.Z is not likely to be involved in heterochromatin formation and/or maintenance. In dividing root cells we observed H2A.Z throughout the condensed chromosomes, indicating that it persists in the chromatin through mitosis (Figure 3.3). The observation that H2A.Z is incorporated into euchromatin during interphase and remains in chromatin through mitosis suggests that it may serve an epigenetic memory function by marking actively transcribed genes and providing competence for the reactivation of silenced genes. Such a function would aid in the establishment and perpetuation of cell lineage-specific gene expression programs during development. In addition, H2A.Z might add robustness to the epigenetic control of gene expression in that it serves as an additional requirement for the transcriptional activation of particular genes.

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, male and female reproductive development, and repression of the transition from vegetative growth to flowering. Our data support the concept that the multiple developmental functions of *Arabidopsis* ARP6 reflect its critical role in depositing H2A.Z into chromatin as part of a SWR1-like complex. Furthermore, the loss of H2A.Z from chromatin in the *arp6-1* and *pie1-5* mutants correlates with decreased expression of *FLC*, *MAF4*, and *MAF5* indicating that H2A.Z deposition is essential for the full transcriptional activation of these genes. Thus, H2A.Z can act to potentiate the transcriptional activation of *Arabidopsis* genes, similar to its role in yeast. Collectively, these findings suggest a model in which the ARP6-containing SWR1-like complex deposits H2A.Z into chromatin at many high-level developmental regulatory genes, thereby regulating their expression, and indirectly that of their downstream targets, thus ensuring proper elaboration of the developmental program in *Arabidopsis* (Figure 4.2).

With the complete genome sequence of the model plant *Arabidopsis thaliana* in hand, the goal of the research community is now to define the function of each gene and the networks of interactions between genes, with a view toward truly understanding the growth and development of the plant in molecular terms. The research described herein contributes to this goal by identifying the developmental functions of *Arabidopsis* ARP6 and illuminating the basic mechanism by which these functions are carried out. Future work in this area should be focused on large-scale approaches to identify all of the genes that require H2A.Z for proper expression and elucidating the mechanism by which H2A.Z potentiates transcriptional activation. Because ARP6 acts as a hub through which the expression of many genes is controlled, identification the

full set of genes misregulated in *arp6* mutants will allow the assignment of many currently anonymous genes to particular developmental pathways.

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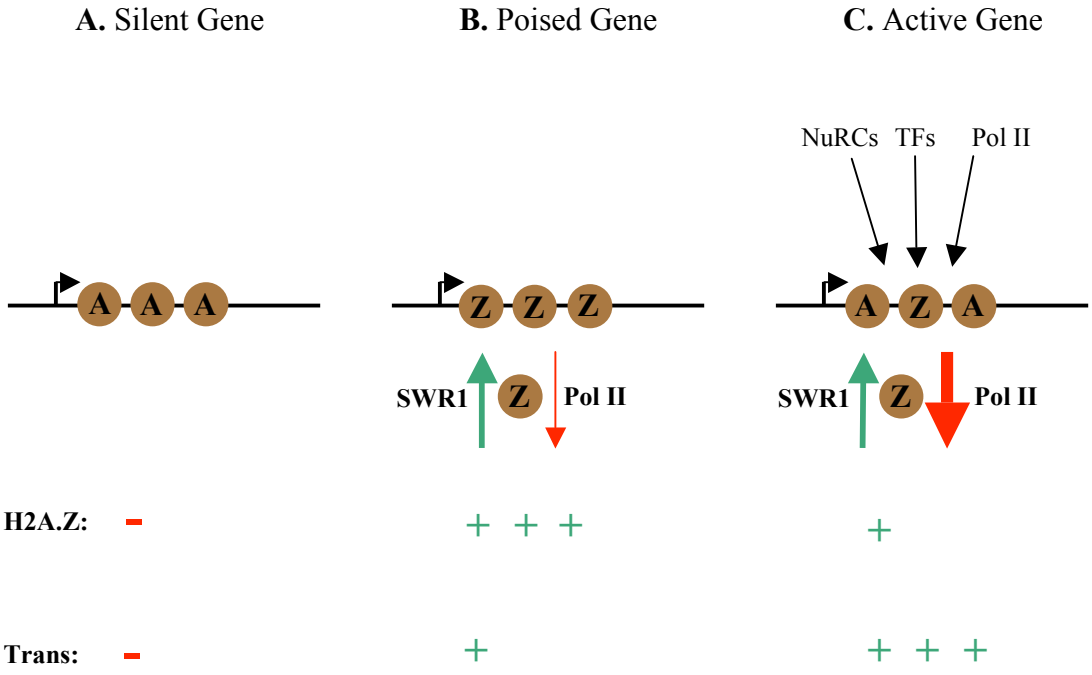

Figure 4.1 A Model For the Role of H2A.Z in Transcriptional Activation

A hypothetical gene in various states of activity is shown as a black line with the transcription start site depicted as a right-facing arrow. Brown circles represent nucleosomes containing either H2A (A) or H2A.Z (Z). Shown below the gene diagrams are the levels of H2A.Z and the rate of transcription (Trans). (-) indicates low level/rate and (+) indicates higher level/rate. (**A**) A gene in the transcriptionally silent state. It is not transcribed and has no H2A.Z nucleosomes. (**B**) A gene poised for activation. The SWR1 complex deposits H2A.Z into chromatin (upward-facing green arrow) resulting in high abundance of H2A.Z nucleosomes, and low-level transcription removes H2A.Z from chromatin by RNA polymerase (Pol II) passage (downward-facing red arrow). (**C**) A poised gene becomes activated. This can occur when H2A.Z facilitates chromatin remodeling by nucleosome remodeling complexes (NuRCs) targeted to the gene and/or by H2A.Z-mediated recruitment of transcriptions factors (TFs) and RNA polymerase (Pol II). High-

level transcription results in nucleosome disruption and loss of H2A.Z from chromatin. In this state the rate of transcription-induced depletion of H2A.Z exceeds the rate of replacement by SWR1. This model incorporates data from this study and references cited in the text.

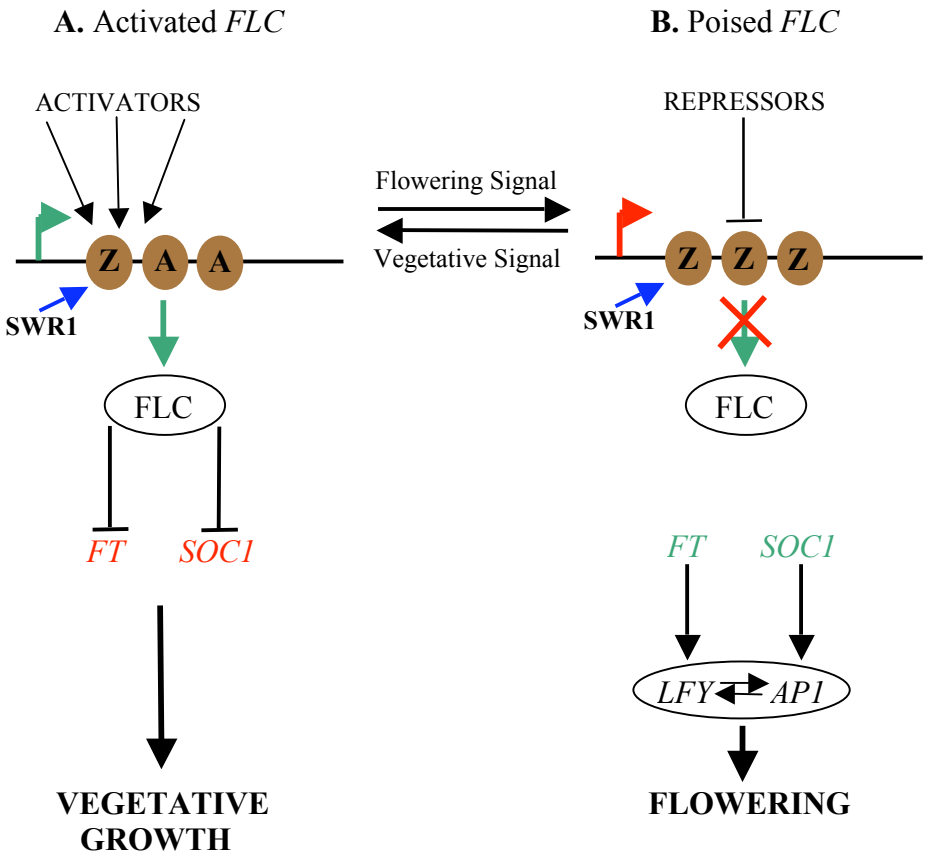

Figure 4.2 The Role of H2A.Z Deposition in the Regulation of Flowering Time

The *FLC* gene is shown in either the transcriptionally activated or poised state within meristematic cells. The gene is shown as a black line with the transcription start site depicted as a right-facing arrow. Green arrow indicates high-level transcription and red arrow indicates low or no transcription. Nucleosomes are shown as brown circles containing either H2A (A) or H2A.Z (Z). Gene activation is indicated by arrows and repression is indicated by lines with bars. (**A**) *FLC* in the transcriptionally active state where H2A.Z facilitates the effects various activator proteins. SWR1 replaces H2A with H2A.Z in nucleosomes (indicated as a blue arrow) to promote activation, but H2A.Z levels are relatively low due to transcription-induced loss as described in the text and Figure 4.1. In the active state the FLC protein is produced in abundance

and it represses expression of the flowering-inducing genes *FT* and *SOC1*, thus maintaining the vegetative growth state. (**B**) When flowering signals are received by the meristem, *FLC* expression is silenced by a variety of factors. Under these conditions the FLC protein is not produced at high levels and its target genes *FT* and *SOC1* become active. The FT and SOC1 proteins activate the floral-meristem identity genes *AP1* and *LFY*, which induce the switch to flowering. The SWR1 complex continues to deposit H2A.Z into *FLC* chromatin such that the gene is now poised for reactivation and reestablishment of the vegetative program when the proper signals are received and activating factors are once again present, presumably during embryogenesis. In the absence of SWR1 activity in *arp6* and *pie1* mutants, H2A.Z is not deposited into *FLC* chromatin and the gene remains permanently in a state incompetent for activation, resulting in early flowering in each generation.

APPENDIX A:

SUPPLEMENTAL FIGURES

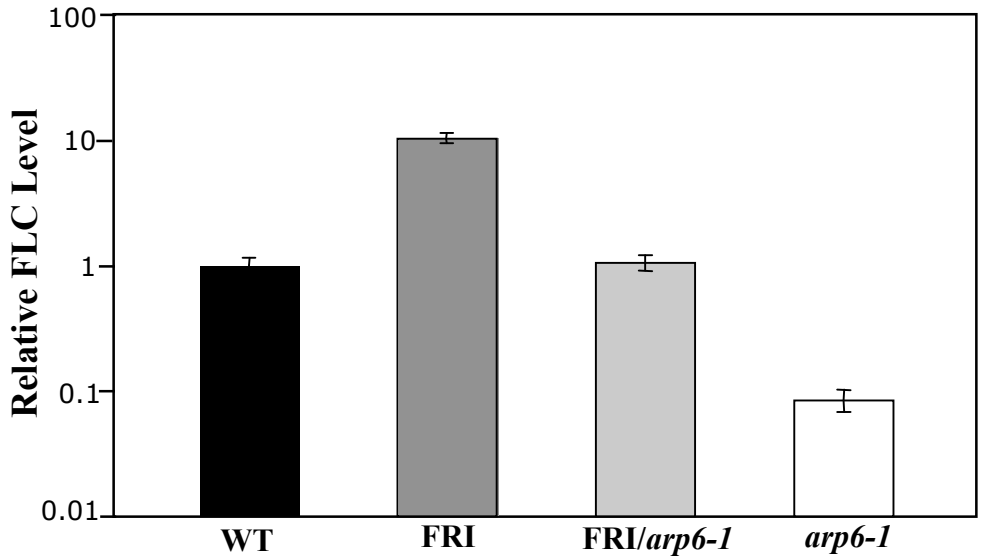

Figure S1 ARP6 is Required for Activation of *FLC* by FRI

RT-PCR analysis of *FLC* transcript levels in the indicated genotypes. WT is wild-type Columbia ecotype, *FRI* is a Columbia line into which the strong *FRI* allele from the Sf-2 ecotype had been introgressed, *FRI*/*arp6-1* is the *FRI* line homozygous for *arp6-1*, and *arp6-1* is Columbia line homozygous for *arp6-1*. For this experiment, RNA was isolated from young rosette leaves of 26-day-old plants grown under long-day conditions.