SUPPRESSOR OF FRIGIDA3 Encodes a Nuclear ACTIN-RELATED PROTEIN6 Required for Floral Repression in Arabidopsis

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Flowering traits in winter annual Arabidopsis thaliana are conferred mainly by two genes, FRIGIDA (FRI) and FLOWERING LOCUS C (FLC). FLC acts as a flowering repressor and is regulated by multiple flowering pathways. We isolated an early-flowering mutant, suppressor of FRIGIDA3 (suf3), which also shows leaf serration, weak apical dominance, and infrequent conversion of the inflorescence shoot to a terminal flower. The suf3 mutation caused a decrease in the transcript level of FLC in both a FRI-containing line and autonomous pathway mutants. However, suf3 showed only a partial reduction of FLC transcript level, although it largely suppressed the late-flowering phenotype. In addition, the suf3 mutation caused acceleration of flowering in both 35S-FLC and a flc null mutant, indicating that SUF3 regulates additional factor(s) for the repression of flowering. SUF3 is highly expressed in the shoot apex, but the expression is not regulated by FRI, autonomous pathway genes, or vernalization. SUF3 encodes the nuclear ACTIN-RELATED PROTEIN6 (ARP6), the homolog of which in yeast is a component of an ATP-dependent chromatin-remodeling SWR1 complex. Our analyses showed that SUF3 regulates FLC expression independent of vernalization, FRI, and an autonomous pathway gene, all of which affect the histone modification of FLC chromatin. Subcellular localization using a green fluorescent protein fusion showed that Arabidopsis ARP6 is located at distinct regions of the nuclear periphery.

INTRODUCTION

Proper timing of flowering is pivotal for the reproductive success of plants; thus, they have evolved a sophisticated mechanism to determine flowering time in response to endogenous signals and environmental cues. Approximately two decades of genetic studies in Arabidopsis thaliana have revealed >80 flowering time genes that have been classified into four interdependent genetic pathways: long day, autonomous, vernalization, and gibberellin-dependent (reviewed in Mouradov et al., 1991, 1998; He et al., 2003). The long-day, vernalization, gibberellin, and autonomous pathways seem to respond to endogenous signals. Mutations in genes involved in the long-day pathway, such as constans (co), gigantea (gi), and ft, cause late flowering under long days but do not affect flowering time under short days. The expression of CO is regulated by circadian rhythm, and the coincidence of the peak expression of CO and light exposure during long days was shown to activate the expression of FT, which is sufficient to induce flowering (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004). By contrast, mutations in genes of the autonomous pathway, such as luminidependens (ld), fca, fve, fpa, flowering locus D (fld), and fy, cause late flowering under both long days and short days compared with the wild type, showing that the mutants have a normal response to environmental factors (Koornneef et al., 1991, 1998; He et al., 2003). The long-day, vernalization, gibberellin, and autonomous pathways converge on common downstream target genes, the so-called flowering pathway integrators, such as FT, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1/AGL20), and LEAFY, to promote flowering (Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000; Simpson and Dean, 2002; Moon et al., 2003, 2005).

In terms of flowering time traits, Arabidopsis accessions can be classified into winter annuals and summer annuals (Gazzani et al., 2003; Michaels et al., 2003). Winter annual accessions show a very late-flowering phenotype without prolonged cold exposure (vernalization), but flowering is dramatically accelerated by vernalization. By contrast, summer annual accessions flower early, and the effect of vernalization is minimal. This difference in flowering behavior is determined mainly by two genes, FRIGIDA (FRI) and FLOWERING LOCUS C (FLC), that act as floral repressors (Napp-Zinn, 1985; Burn et al., 1993; Lee et al.,
have a nonfunctional fri allele and/or a weak flc allele, whereas winter annual accessions have functional versions of both genes. FRI, encoding a coiled-coil protein, functions to increase RNA levels of FLC, and FLC, a MADS box transcription factor, represses the expression of the genes necessary for the transition to flowering (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999, 2000; Johanson et al., 2000). FLC expression is also negatively regulated by the autonomous pathway; thus, mutations in the autonomous pathway genes cause increased levels of FLC. It is noteworthy that the activity of FRI is dominant over the activity of autonomous pathway genes—that is, FRI increases the FLC expression level in the presence of all of the autonomous pathway genes (Michaels and Amasino, 1999, 2001). Vernalization promotes flowering in winter annuals and in the mutants of the autonomous pathway genes by epigenetic downregulation of FLC (Bastow et al., 2004; Sung and Amasino, 2004a, 2004b). Therefore, FLC is a convergence point for autonomous and vernalization pathways and the activity of FRI.

Vernalization is an epigenetic switch in that the vernalized state is maintained throughout vegetative growth by mitotic cell division and is completely reset at the next generation by passage through meiosis (Amasino, 2004; Sung and Amasino, 2004b). Epigenetic control that establishes and maintains a certain transcriptional pattern is usually mediated by the modification of chromatin structure, which is regulated by two types of chromatin-modifying complexes: ATP-dependent chromatin-remodeling complexes and histone-modifying complexes such as histone acetylase and histone deacetylase (Narlikar et al., 2002; reviewed in Turner, 2002). Recently, the epigenetic regulatory mechanism of vernalization was shown to involve the modification of FLC chromatin (Bastow et al., 2004; Sung and Amasino, 2004a, 2004b; He and Amasino, 2005). The establishment of the vernalized state, the transcriptional repression of FLC, is mediated by VERNALIZATION-INSENSITIVE3 (VIN3), which encodes a PHD domain protein and presumably a component of chromatin-modifying complexes (Sung and Amasino, 2004a). It was shown that the expression of VIN3 is induced not by short exposure to cold but only by vernalization (long exposure to cold), and VIN3 is necessary for the deacetylation of histone 3 (H3) in FLC chromatin during vernalization. Then, the maintenance of FLC repression is mediated by VERNALIZATION1 (VRN1) and VRN2 through the methylation of H3 at Lys-9 and Lys-27 (Bastow et al., 2004; Sung and Amasino, 2004a). VRN1 encodes a Myb-related DNA binding protein, whereas VRN2 encodes a polycomb group protein homologous with SUPPRESSOR OF ZESTE-12, a component of POLYCOMB REPRESSOR COMPLEX2, a complex with histone methyltransferase activity (Gendall et al., 2001; Kuzmichev et al., 2002; Levy et al., 2002; Chanvivattana et al., 2004).

A large number of genes involved in the modification of chromatin structure have been shown to regulate flowering, especially through the analyses of early-flowering mutants. For example, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1), encoding an Arabidopsis homolog of ISWI, a member of the ATP-dependent chromatin-remodeling protein SWI/SNF superfamily, controls multiple flowering pathways and is required for the upregulation of FLC in winter annuals and autonomous pathway mutants (Noh and Amasino, 2003). By contrast, Arabidopsis BRAHMA, encoding a homolog of SNF2, another member of the SWI/SNF superfamily, controls the phoptoperiod flowering pathway by negative regulation of CO, FT, and SOC1 but does not affect the expression of FLC (Farrona et al., 2004). EARLY BOLTING IN SHORT DAYS, encoding a nuclear protein that contains the bromoadjacent homology domain found in chromatin-remodeling factors in other organisms, regulates flowering by the repression of FT (Pifio et al., 2003). TERMINAL FLOWER2 (TFL2) encodes a homolog of HETEROCHROMATIN PROTEIN1 (HP1) that binds to methylated Lys-9 of histone H3 and maintains an inactive heterochromatin structure (Gaudin et al., 2001; Kotake et al., 2003). The mutations in TFL2 cause early flowering by the ectopic expression of FT as well as the conversion of the inflorescence shoot apex to a terminal flower (Gaudin et al., 2001; Kotake et al., 2003; Takada and Goto, 2003). It was also shown that the homologs of components of the PAF1 complex in yeast, VERNALIZATION INDEPENDENCE4 (VIP4), VIP5, VIP6/ELF8 (for EARLY FLOWERING8), and ELF7 are required for the upregulation of FLC in winter annuals and autonomous pathway mutants (Zhang and van Nocker, 2002; He et al., 2004; Oh et al., 2004). The PAF1 complex in yeast was shown to recruit SET1 methyltransferase, which catalyzes the trimethylation of histone H3 on Lys-4, a landmark of active transcription (Krogan et al., 2003; Ng et al., 2003). Indeed, the elf7 and elf8 mutants cause a decrease in the trimethylation of histone H3 on Lys-4 in FLC chromatin (He et al., 2004). Mutations in homologs of the PAF1 complex also cause early flowering independent of FLC, suggesting that they play roles in multiple flowering pathways.

In this study, we screened fast neutron–irradiated early-flowering mutants of FRI-containing Arabidopsis winter annuals and analyzed one of the mutants named suppressor of FRIGIDA3 (su3). Map-based gene cloning revealed that SUF3 encodes ACTIN-RELATED PROTEIN6 (ARP6), a putative component of a chromatin-remodeling complex. SUF3 is required for high expression of FLC in both FRI-containing lines and in autonomous pathway mutants. In addition to FLC, our results showed that SUF3 regulates additional flowering repressors. Arabidopsis ARP6 is located at specific regions of the nuclear periphery where gene activation may occur.

RESULTS

Isolation of suf Mutants by Fast Neutron Mutation Genes

To dissect the genetic mechanisms governing flowering behavior of Arabidopsis winter annuals, we performed fast neutron mutagenesis in the line Col;FRS2/FRS2 from the winter annual San Feliz-2 introgressed into Col by backcrossing eight times; thus, this line has a winter annual flowering trait; it was used as the wild type in our study (Michaels and Amasino, 1999; Lee et al., 2000). We screened early-flowering mutants that showed recessive single gene mutations. Genetic complementation
analysis disclosed a group of early-flowering mutants that were allelic to one another but not to either flic (FN231) or fri (FN235) (data not shown) (Michaels and Amasino, 1999). We named this mutant suf3. Seven suf3 alleles were obtained, and all of them showed the same phenotype; thus, we mainly discuss suf3-1 as the representative phenotype (Figure 1, Table 1). When grown under long days, suf3 showed much earlier flowering than wild-type Col:FRI(SF2) and similar flowering time to Col, which has a fri FLC genotype (Figures 1A and 1C, Table 1) (we refer to suf3 in the Col background as suf3 fri below). The suf3 mutant also showed delay in flowering under short days, similar to Col, suggesting that the suf3 mutation does not affect the photoperiod response (Table 1). However, suf3 showed much stronger acceleration of flowering by vernalization than Col, indicating that the vernalization response is not much affected by the suf3 mutation (Table 1). In addition to early flowering, all of the suf3 alleles showed additional phenotypes. suf3 consistently produced serrated leaves starting from the sixth leaf (Figures 1B and 1C). It also produced approximately twice as many coflorescence shoots as Col (6.44 ± 0.71 for suf3 and 5.06 ± 0.49 for suf3 fri versus 3.33 ± 0.49 for Col), which suggests the weakening of apical dominance in the suf3 mutants (Figure 1C). Although infrequent, the secondary shoot apices of suf3 occasionally converted to terminal flowers after producing racemic inflorescences (3 terminal flowers were observed among 62 secondary shoots from 13 suf3 mutant plants; Figure 1D). suf3 mutants occasionally produced flowers with extra petals; among 100 flowers, 63 had four petals and 27 had five or more petals (Figure 1E). The frequency of flowers with extra petals in suf3 was slightly less than that in pie1 mutants (47% for five or more petals) reported previously (Noh and Amasino, 2003). Otherwise, suf3 showed normal growth and development, similar to Col; for example, it exhibited similar size and the same leaf initiation rate as the wild type and Col (data not shown).

Positional Cloning of the SUF3 Gene

For positional cloning of SUF3, we crossed suf3-1 to Ler:FRI(SF2) FLC(SF2), a line obtained by backcrossing of San Feliu-2 to Ler six times (Lee and Amasino, 1995), and selected early-flowering progeny from the F2 population for mapping (Figure 2). The rough mapping showed linkage with two simple sequence length polymorphism markers, ciw11 and ciw4, on chromosome 3. Then, we generated more simple sequence length polymorphism markers for fine mapping and found that SUF3 is located between markers SH33 and SH34 (Figure 2A). From 504 chromatids analyzed, no recombinants were found at markers SH35, SH36, SH37, or SH38 loci. Interestingly, we could not amplify DNA by PCR at the marker SH39 locus from suf3 mutants (data not shown). Because fast neutron mutagenesis usually generates a deletion, we tested whether the region surrounding marker SH39 was deleted. All of the suf3 alleles showed a deletion in the region including At3g33530 (WD repeat protein), At3g33520 (ARP6), and At3g33448 (hypothetical protein); suf3-1 showed the smallest deletion, covering ~14 kb (Figure 2A; data not shown). A homozygous null mutant of gene At3g33530 encoding

Figure 1. Phenotypes of the suf3 Mutant.

(A) The wild type (Col:FRI(SF2)), Col, and suf3-1 grown under long days. Photographs were taken when flowering initiated.
(B) Comparison of leaf shape in the wild type, Col, and suf3-1. The leaves are shown in order of production from the first true leaf at left.
(C) Phenotypes of all seven suf3 alleles that show serrated leaves and increased numbers of coflorescence shoots.
(D) Conversion of the secondary shoot apex to a terminal flower in suf3. White arrows indicate terminal flowers.
(E) suf3-1 flower with five petals.
We could not obtain the T-DNA insertion mutant of At3g33520. Although the expression was slightly weaker in the leaf and shoot apex (Figure 4A). In situ hybridization showed that stronger expression was observed in the shoot apex (Figure 4A). The RNAi transformants also showed additional phenotypes in suf3, such as serrated leaves, increased coflorescence shoots, and terminal flowers (Figure 2C), although the range of flowering time was variable among the lines depending on the level of reduction in SuF3 (data not shown). Furthermore, RNA interference (RNAi) of SuF3 resulted in complementation, showing that it is not responsible for the suf3 mutant phenotype (data not shown).

We determined whether the early flowering of suf3 mutants was attributable to the decreased level of FLC by RNA gel blot analysis. All seven suf3 alleles showed ∼30 to 60% reduction in FLC transcript level compared with the Col;FRI<sup>SF2</sup> wild type (Figures 5A and 5B). The FLC transcript level in suf3 is fivefold higher than that in Col, although suf3 and Col exhibited a similar flowering time. In contrast with FLC, the SOC1 transcript level in suf3 was similar to that in Col (Figure 5A). Because FLC functions in the shoot apex and it was reported previously that a mutation in pie1 causes a reduction in FLC specifically in the shoot apex but not in the root (Noh and Amasino, 2003), we compared the level of FLC reduction attributable to the suf3 lesion among different tissues. As shown in Figure 5C, a similar reduction was observed in all of the tissues we tested, indicating that the suf3 mutation affects the expression of FLC in all tissues. It is noteworthy that the suf3 mutants we analyzed have complete deletion of the gene; thus, the residual expression of FLC is not the result of a weak mutation.

Because a relatively higher level of FLC remained in suf3, we determined whether the residual expression of FLC still delays flowering. When the suf3 mutants were vernalized for 5 weeks, a period of cold that is sufficient to suppress FLC expression, flowering was further accelerated in both long days and short days (Figure 5D, Table 1). Consistently, the genetic removal of either FRI or FLC from suf3 mutants caused similar acceleration of flowering as the vernalization treatment (Figure 5D). These results show that the residual FLC expression in suf3 represses flowering. However, the flowering time as well as the SOC1 transcript level of suf3 in the Col;FRI<sup>SF2</sup> background are similar to

Table 1. Flowering Time of suf3

<table>
<thead>
<tr>
<th>Growth Condition</th>
<th>Col;FRI&lt;sup&gt;SF2&lt;/sup&gt;</th>
<th>suf3-1</th>
<th>Col (fri FLC)</th>
<th>suf3-1 fri</th>
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<tr>
<td>Long days</td>
<td>64.27 ± 3.80</td>
<td>12.45 ± 0.52</td>
<td>10.64 ± 0.67</td>
<td>5.73 ± 0.47</td>
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<td></td>
<td>(10.09 ± 0.83)</td>
<td>(4.18 ± 0.60)</td>
<td>(3.64 ± 0.50)</td>
<td>(3.09 ± 0.54)</td>
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<tr>
<td>Long days + vernalization</td>
<td>12.7 ± 0.67</td>
<td>4.80 ± 0.63</td>
<td>7.00 ± 0.32</td>
<td>N.D.</td>
</tr>
<tr>
<td>Short days</td>
<td>&gt;100</td>
<td>63.00 ± 3.14</td>
<td>60.3 ± 3.71</td>
<td>17.1 ± 1.73</td>
</tr>
<tr>
<td>Short days + vernalization</td>
<td>35.5 ± 3.3</td>
<td>13.8 ± 1.81</td>
<td>35.3 ± 5.3</td>
<td>N.D.</td>
</tr>
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</table>

To measure flowering time, 10 plants were used to count the number of rosette leaves when flowering. Values shown are leaf number ± s.e. The numbers in parentheses are numbers of cauline leaves. For vernalization treatment, plants were germinated and grown at 4°C for 5 weeks in short days and transferred to normal growth conditions. N.D., not determined.

Expression of SUF3

The SUF3 transcript was detected in all of the tissues we tested, although the expression was slightly weaker in the leaf and stronger in the shoot apex (Figure 4A). In situ hybridization showed that SUF3 is highly expressed in the shoot apex at both the vegetative and reproductive phases (Figure 4D). During vegetative growth, the leaf primordia as well as the shoot apex showed strong expression, but the expression decreased as the leaves matured (Figure 4D, a). During flower development, SUF3 was expressed throughout the entire flower meristem until floral stage 3 (for floral stage description, see Smyth et al., 1990). Afterward, expression was reduced from the outermost floral organ primordia. Finally, strong expression was detected at the inner side of the carpel primordia as the flower matured (Figure 4D).

The transcript level of SUF3 in the wild type was not changed by 5 weeks of vernalization, suggesting that the expression of SUF3 is not affected by environmental factors (Figure 4B). To determine whether SUF3 expression is regulated by any of the flowering time genes, RNA gel blot analysis was performed using plants with different genetic backgrounds (Figure 4C). The SUF3 transcript level in the Col;FRI<sup>SF2</sup> wild type was similar to that in Col, showing that SUF3 expression is not affected by the presence of the FRI gene. Furthermore, the SUF3 transcript level was not affected by mutations in autonomous pathway genes such as id, fca, and fld or by mutations in long-day pathway genes such as gi, co, and ft. A mutation in SOC1, a flowering pathway integrator, also did not affect the level of the SUF3 transcript. Together, our results showed that SUF3 expression is not regulated by vernalization or other flowering time genes.

Effect of suf3 on the Expression of FLC and Flowering Time

We determined whether the early flowering of suf3 mutants is attributable to the decreased level of FLC. All seven suf3 alleles showed ∼30 to 60% reduction in FLC transcript level compared with the Col;FRI<sup>SF2</sup> wild type (Figures 5A and 5B). The FLC transcript level in suf3 is fivefold higher than that in Col, although suf3 and Col exhibited a similar flowering time.
those of Col. This strongly suggests that SUF3 regulates not only FLC but also additional factor(s) for the repression of flowering. Consistent with this idea, the suf3 mutation caused earlier flowering in line 35S-FLC, which ectopically overexpresses FLC (Figure 5D).

Mutations in autonomous pathway genes in Col cause late flowering as a result of the derepression of FLC (Figure 5E) (Michaels and Amasino, 1999). The double mutant analysis showed that the suf3 mutation largely suppresses the late-flowering phenotype in the autonomous pathway mutants (Figure 5F). Consistently, RNA gel blot analysis showed that the suf3 mutation caused a decrease in FLC and an increase in SOC1 in the autonomous pathway mutants (Figure 5E). Together, our results suggest that SUF3 is generally required for high levels of FLC expression independent of FRI and the autonomous pathway genes.
Effect of *suf3* on the Expression of Other Flowering Time Genes

We checked the effect of *suf3* on the expression of another flowering pathway integrator, *FT* (Figure 6A). Similar to *SOC1*, the *FT* transcript level was also increased by the *suf3* mutation. Interestingly, under short-day conditions, the *suf3 fri* (*suf3* in Col) plants flowered very early compared with Col or *suf3* (Table 1). RT-PCR analysis showed that both *FT* and *SOC1* were highly expressed in *suf3 fri*, whereas they were not detectable in Col or *suf3* when the plants were grown under short days (Figure 6A).

Because *SUF3* most likely regulates an additional flowering repressor as well as *FLC*, our results suggest that an additional repressor and *FLC* act partially redundantly to repress the expression of *FT* and *SOC1* in short days. Thus, the combination of *suf3* and weak expression of *FLC* causes a synergistic effect on flowering time in short days.

It was reported previously that *FLM/MAF1* (for *FLOWERING LOCUS M/MADS-AFFECTING FLOWERING1*) and *MAF2*, genes closely related to *FLC*, act as flowering repressors (Ratcliffe et al., 2001, 2003; Scortecci et al., 2003). In addition, similar to *FLC*, these *FLC* clade MADS box genes were shown to be regulated by homologs of the PAF1 complex, which mediates the trimethylation of histone H3 of Lys-4 (He et al., 2004). To address the possibility that the additional flowering repression caused by *SUF3* is attributable to these genes, we checked the effect of the *suf3* mutation on the expression of *FLM/MAF1* and *MAF2* (Figure 6B). The transcript levels of *FLM/MAF1* and *MAF2* in *suf3* mutants were similar to those in the wild type, suggesting that they are not the additional repressors regulated by *SUF3*.

Figure 3. Alignment of Deduced Amino Acids of ARP6.

The ARP6 homologs of *Arabidopsis* (At), rice (*Oryza sativa*; Os), human (Hs), *Drosophila* (Dm), and yeast (Sc) were aligned with *Arabidopsis* ACTIN2 (ACT2). ARP6s have a conserved core consisting of two α/β subdomains in the actin family. ARP6s have two peptide insertions (boxed regions) without disrupting the conserved actin fold structure. Dots indicate residues that have structurally equivalent roles in the nucleotide binding site. The amino acid sequences were aligned using ClustalW version 1.7 (Thompson et al., 1994).
SUPPRESSOR OF VEGETATIVE PHASE, a MADS box gene from another clade that acts as a flowering repressor (Hartmann et al., 2000; Scortecci et al., 2003), also did not show any difference in transcript level between the wild type and suf3 (Figure 6B). The expression of the other flowering time genes CO, LD, PIE1, and TFL2 also was not affected by the suf3 mutation.

Cellular Localization of SUF3

To understand the cellular function of SUF3, we determined the subcellular location of Arabidopsis ARP6. For this, a gene encoding the ARP6:green fluorescent protein (GFP) or yellow fluorescent protein (YFP):ARP6 fusion protein, with an N- or C-terminal fusion, respectively, was introduced transiently into Arabidopsis protoplasts. Genes encoding GFP alone, NLS:red fluorescent protein (RFP) (a nuclear localization signal from simian virus 40 large T antigen fused with red fluorescent protein) (Dingwall and Laskey, 1991; Lee et al., 2001), and TFL2:RFP were used as controls for subcellular localization (Figure 7). As expected, GFP alone was detected in both the cytoplasm and the nucleus, whereas NLS:RFP and TFL2:RFP were detected only in the nucleus (Figures 7A to 7C). As shown in Figure 7D, ARP6:GFP was also detected in the nucleus, as were the ARP6 homologs of yeast and human (Goodson and Hawse, 2002; Blessing et al., 2004). However, the subnuclear localization of ARP6:GFP was different from that of NLS:RFP or TFL2:RFP (Figures 7D to 7L). Although NLS:RFP and TFL2:RFP were detected throughout the nucleoplasm, ARP6:GFP was excluded from the central region of the nucleus but detected at several regions of the nuclear periphery in patches (Figure 7D). The C-terminally fused YFP:ARP6 also showed similar subnuclear localization (Figures 7E to 7H). Consistently, the colocalization experiment using ARP6:GFP and TFL2:RFP showed that ARP6 is localized at the nuclear periphery, whereas TFL2 is localized at the nucleoplasm. The nuclear periphery was thought to be a place where gene activation or gene silencing occurs (Casolari et al., 2004; Misteli, 2004); thus, subcellular localization studies may indicate that SUF3 regulates gene expression at the nuclear periphery. Our results also clearly showed that Arabidopsis ARP6 is not colocalized with TFL2 in the nucleus, in contrast with the colocalization of ARP6 and HP1, a TFL2 homolog in Drosophila cells (Frankel et al., 1997).

DISCUSSION

In this study, we screened for mutants that suppressed the late-flowering trait of a FRI-containing line and characterized a mutant named suf3 that flowers as early as Col. Map-based cloning revealed that SUF3 encodes Arabidopsis ARP6, homologs of which are components of ATP-dependent chromatin-remodeling complexes in other eukaryotes (Blessing et al., 2004). The suf3 mutation caused a reduction in FLC expression in both a FRI-containing line and several autonomous pathway mutants, suggesting that SUF3 is generally required for high levels of FLC expression.

ARP6 is a member of the ARP family, which is homologous with conventional actin and comprises divergent and evolutionarily conserved eukaryotic proteins (reviewed in Goodson and...
Hawse, 2002; Blessing et al., 2004; Kandasamy et al., 2004). Most eukaryotic cells contain at least eight ARPs with diverse functions and different subcellular localization. Among them, ARP1 to ARP3 and ARP10 are localized to the cytoplasm and function in cellular motility and actin polymerization, whereas ARP4 to ARP9 are localized to the nucleus and are found as components of chromatin-remodeling complexes. Arabidopsis contains eight ancient classes of ARPs that show differential expression (McKinney et al., 2002; Kandasamy et al., 2004). Recently, it was reported that silencing of Arabidopsis ARP4 causes multiple defects in plant development, including early flowering and delayed senescence (Kandasamy et al., 2005).

The ARP6 protein was originally thought to act with HP1 for heterochromatin organization, because colocalization of ARP6
and HP1 was maintained throughout development in Drosophila (Frankel et al., 1997). However, it was shown in fission yeast that the localization of ARP6 is independent of SWI6, a yeast HP1 homolog, and that ARP6 is required only for telomere silencing, whereas SWI6 is required for both centromere and telomere silencing (Ueno et al., 2004). In any case, ARP6 is generally implicated in gene silencing. By contrast, ARP6 was recently found as a component of an ATP-dependent chromatin-remodeling complex, SWR1-C in yeast and SRCAP (for SWI2/SNF2-related CBP activator protein) in human (Krokan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004; Cai et al., 2005). In addition, it was clearly demonstrated that the biochemical function of the SWR1 complex is to catalyze the ATP-driven exchange of the histone variant H2AZ with conventional H2A, thus remodeling chromatin structure (Mizuguchi et al., 2004). This result indicates that ARP6 may function in gene activation in addition to gene silencing. The Arabidopsis genome contains a homolog of both HP1 and SWR1; the HP1 homolog is TFL2 and the closest homolog of SWR1 in Arabidopsis is PIE1 (Gaudin et al., 2001; Kotake et al., 2003; Noh and Amasino, 2003). Interestingly, mutations in both TFL2 and PIE1 cause early flowering similar to the mutation in SUF3.

The tfl2 mutation causes early flowering, the frequent conversion of the inflorescence shoot to a terminal flower, small and curled leaf formation, and dwarfism (Larsson et al., 1998; Gaudin et al., 2001; Kotake et al., 2003). However, suf3 produced normal-sized serrated leaves instead of small curled leaves, did not show dwarfism, and produced terminal flowers very infrequently. More interestingly, the function of TFL2 in the regulation of flowering time is somewhat different from that of SUF3; the tfl2 mutation causes an increase in FT but does not affect the FLC transcript level, whereas the suf3 mutation causes both a decrease of FLC and an increase in the FT level (see Supplemental Figure 1 online) (Kotake et al., 2003; Takada and Goto, 2003). Moreover, ft is completely epistatic to tfl2, indicating that TFL2 regulates flowering through FT (Kotake et al., 2003). Therefore, it is likely that TFL2 acts only on the long-day pathway but SUF3 acts mainly on the autonomous pathway. The results of our subcellular localization analysis for Arabidopsis ARP6 and TFL2 are consistent with the idea that the two genes have different functions. Although the two proteins locate in the nucleus, the subnuclear localization is completely different, because TFL2 is located throughout the nucleoplasm but ARP6 is located at distinct regions of the nuclear periphery.

The pie1 mutation also caused pleiotropic phenotypes in addition to early flowering. The mutant produced serrated leaves and flowers with extra petals. Particularly in the Col background, it showed reduced fertility and the bushy phenotype, which indicates an extreme loss of apical dominance (Noh and Amasino, 2003). The phenotype of reduced fertility was not found in suf3 mutants in the same genetic background, suggesting that the two genes may have at least partially independent functions. However, the pie1 and suf3 mutants have interesting similarities: both of the mutants produce serrated leaves and flowers with extra petals, and both show loss of apical dominance, although the severity is different. In addition, the effect of the two mutations on flowering is very similar; both pie1 and suf3 cause reduced expression of FLC in the FRI-containing line and autonomous pathway mutants, and both mutants cause early flowering independent of FLC (Noh and Amasino, 2003). Thus, it is probable that the two genes act together to control flowering time. Further studies consisting of double mutant analysis and protein–protein interaction analysis will provide an answer to this question.

Although SUF3 is necessary for the high expression of FLC, the suf3 mutant shows only a partial reduction of FLC level in a FRI-containing line, and the remaining expression of FLC is functional in repressing flowering as well as in suppressing FT and SOC1 (Figures 5 and 6). However, the suf3 mutant showed similar flowering time to Col, which has fivefold less FLC expression. Thus, these results strongly suggest that SUF3 regulates additional factors for the repression of flowering in addition to FLC. Consistently, the suf3 mutation causes earlier flowering in both the flic null mutant and the 35S-FLC overexpression line. The function of additional factors for the repression of flowering is most prominent in short days. Although flc flowers just slightly earlier than Col (Michaels and Amasino, 2001) and suf3 flowers similarly to Col in short days, the suf3 flic double mutant flowers very early in short days. This finding suggests that the additional factors regulated by SUF3 have partially redundant function with FLC for repressing flowering in short days.

The presence of additional flowering factors that can act along with FLC to repress flowering was reported previously. Vernalization promotes flowering of the flc null mutant especially in short days, suggesting the presence of FLC-independent repression that is alleviated by vernalization (Michaels and Amasino, 2001). It was also shown that the transcript levels of

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**Figure 6.** Effect of the suf3 Mutation on the Expression of FT, SOC1, and Other Flowering Time Genes.

(A) FT and SOC1 expression in wild-type, suf3, Col, and suf3 flic plants grown under long days (LD) for 10 d or under short days (SD) for 20 d were determined by RT-PCR. High transcript levels of FT and SOC1 were detected in suf3 flic under short days. TUB, TUBULIN.

(B) Expression of other flowering time genes in suf3-1 and wild-type plants grown for 10 d under long days as determined by RT-PCR.
both FT and SOC1 were increased in the flc null mutant by vernalization, indicating that the additional factors regulate the same flowering pathway integrators (Moon et al., 2003). In addition, a mutation in any of the homologs of components of the PAF1 complex causes early flowering independent of FLC, suggesting that the additional factors may be regulated by the Arabidopsis PAF1 complex (Zhang and van Nocker, 2002; He et al., 2004; Oh et al., 2004). Currently, it is not known whether the same additional factors are regulated by vernalization, FRI, and the autonomous pathway. First, suf3 causes the suppression of FLC expression regardless of the presence of FRI—that is, suf3 further decreases the FLC transcript level in Col, a fri null (Figure 5D). Second, the suf3 lesion causes additive suppression of FLC with vernalization, thus resulting in earlier flowering (Table 1; see Supplemental Figure 2 online). Third, the suf3 mutation causes the suppression of FLC in fve, a mutation in the autonomous pathway gene that regulates the histone modification of FLC chromatin (Figure 5D) (He et al., 2003; Ausin et al., 2004). Therefore, SUF3 provides a distinct mechanism to regulate FLC gene expression. Future work to purify the Arabidopsis ARP6-containing protein complex and to analyze the components genetically and biochemically will help us understand the exact role of SUF3 in the regulation of flowering.

Figure 7. Localization of ARP6:GFP and YFP:ARP6 Fusion Proteins in Arabidopsis ProtoplastTransient Assay.
Chloroplasts appear red or blue (pseudocolor), GFP and YFP fluorescence is green, and RFP fluorescence is red. ARP6:GFP and YFP:ARP6 were localized in distinct regions of the nuclear periphery.
(A) Protoplast expressing GFP alone.
(B) Protoplast expressing NLS:RFP.
(C) Protoplast expressing TFL2:RFP.
(D) Protoplast expressing ARP6:GFP.
(E) to (H) Protoplasts expressing YFP:ARP6. (E) and (G) are transparent images.
(I) to (L) Protoplasts expressing both TFL2:RFP and ARP6:GFP.
(I) Section of a protoplast transient image.
(J) ARP6:GFP fluorescence.
(K) TFL2:RFP fluorescence.
(L) Merged image of TFL2:RFP and ARP6:GFP fluorescence. All images are projections except for (I).
METHODS

Plant Materials and Growth Conditions

The wild type used in this study was the Arabidopsis thaliana Col-FR152 strain, which is a Col near-isogenic line containing the FRI allele of San Felu-2 by eight backcrosses into Col (Lee et al., 1993; Michaels and Amasino, 1999). Plants were grown in long days (16 h of light/8 h of dark) or short days (8 h of light/16 h of dark) under cool white fluorescent lights (100 μmol·m⁻²·s⁻¹) at 22°C with 60% RH. For vernalization, seeds were soaked and allowed to germinate on Murashige and Skoog medium at 4°C in short days for 5 weeks. Flowering time was measured by counting the number of rosette leaves from at least 10 plants.

Mutagenesis and Cloning of SUF3

Fast neutron mutagenesis and mutagenized populations of the Col-FR152 strain have been described previously (Michaels and Amasino, 1999). Among early-flowering mutants that flower as early as Col, we obtained seven fast neutron alleles of suf3, suf3-1 to suf3-7 (FN6, FN7, FN24, FN108, FN115, FN202, and FN225) through complementation analysis. For the positional cloning of the SUF3 gene, we selected early-flowering F2 progeny from the crosses between suf3-1 and Ler-FR152 FLOC52, which was obtained by six backcrosses of San Felu-2 to Ler (Lee and Amasino, 1995). Bulked segregation analysis was performed with the pool of 30 F2 individuals using molecular markers described by Lukowitz et al. (2000). For fine mapping, molecular markers based on small insertion–deletion polymorphisms on chromosome 3 were made using an alignment program, EditPlus 2, provided at http://www.ch.embnet.org/software/LALIGN_form.html, after extracting Col and Ler sequences (http://www.arabidopsis.org/Cereon/index.jsp). The sequences of primers for the markers made are shown in Supplemental Table 1 online.

Analysis of Gene Expression

Total RNA was extracted from Arabidopsis seedlings using TRIZOL reagent (Sigma-Aldrich). For RNA gel blot analyses, 20 μg of total RNA was separated by 1.2% denaturing formaldehyde–agarose gel and transferred to nylon membranes (Hybond N; Amer sham). The digoxigenin (DIG)-labeled mRNA probes were prepared from plasmid vectors containing the cDNA fragments lacking the MADS box domains for FLC and SOCI and the full cDNA for SUF3 using the DIG application manual (Roche). For constructing an RNAi construct, the SUF3 cDNA fragment was obtained by RT-PCR with forward primer 5’-ATGCCAGATCCGTAAGAATCGTCTACGGAC-3’ and reverse primer 5’-ATGCCAGATCCGTAAGAATCGTCTACGGAC-3’ , which remove the stop codon at the C terminus and bear a BarnHI restriction site. The fragment was inserted at the BarnHI restriction site of the p326-GFP vector between the cauliflower mosaic virus 35S promoter and the N terminus of GFP (Lee et al., 2001). For the YFP:AtARP6 fusion construct, the SUF3 cDNA fragment was obtained by RT-PCR with forward primer 5’-ATGCCAGATCCGTAAGAATCGTCTACGGAC-3’ and the full cDNA for SUF3 using the BarnHI and Stul restriction sites. The fragment was amplified by PCR with forward primer 5’-ATGCCAGATCCGTAAGAATCGTCTACGGAC-3’ and reverse primer 5’-ATTAGGCGCTCGAAGAATCGTCTACGGAC-3’ , which was cloned in a plant expression vector containing the cassava vein mosaic virus promoter (Verdaguer et al., 1998) and the nopaline synthase terminator using BarnHI and Stul restriction sites. For the TFL2:RFP fusion construct, TFL2 cDNA was amplified by PCR with forward primer 5’-ATGCCAGATCCGTAAGAATCGTCTACGGAC-3’ and the product was inserted at the N terminus of RFP in p326-RFP vector. For a positive control of nuclear localization, the fusion construct NLS-RFP was used (Dingwall and Laskey, 1991; Lee et al., 2001).

Protoplast Transient Expression Assay

Rosette leaves of plants grown for 4 to 6 weeks were used for the isolation and transformation of protoplasts essentially as described at http://genetics.mgh.harvard.edu/sheenweb/. Protoplasts were electroporated with 20 μg of plasmid DNA prepared with the Qiagen Plasmid Maxi Kit and cultured at 22°C in the dark. After 12 h of electroporation, protoplasts were observed with a confocal laser scanning microscope equipped with an argon/krypton laser (Bio-Rad). The GFP fusion and YFP proteins were excited at 488 nm, whereas the RFP fusion protein and chlorophylls were excited at 588 and 568 nm. GFP/YFP, RFP, and chlorophyll autofluorescence were analyzed with the HQ515/30, HQ660/50, and E600LP emission filters, respectively. The resulting green and red images were overlaid and processed using Confocal Assistant 4.02 (Todd Clark Brelje) and Adobe Photoshop 6.0.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AT3G33520.

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SUPPRESSOR OF FRIGIDA3 Encodes a Nuclear ACTIN-RELATED PROTEIN6 Required for Floral Repression in Arabidopsis
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