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

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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., 2002; Simpson and Dean, 2002; Amasino, 2004; Boss et al., 2004). The long-day and vernalization pathways respond to the environmental factors light and temperature, respectively, whereas the autonomous and gibberellin 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.,

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1993, 1994; Clarke and Dean, 1994; Koornneef et al., 1994; Gazzani et al., 2003; Michaels et al., 2003). Summer annual accessions such as Landsberg *erecta* (L*er*) and Columbia (Col) 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 photoperiod 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* (Piñeiro 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* (*suf3*). 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 Radiation Mutagenesis

To dissect the genetic mechanisms governing flowering behavior of *Arabidopsis* winter annuals, we performed fast neutron mutagenesis in the line Col:*FRI*^{SF2} (*FRI*^{SF2} from the winter annual San Feliu-2 was 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 *flc* (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 $\pm$ 0.71 for *suf3* and 5.06 $\pm$ 0.49 for *suf3 fri* versus 3.33 $\pm$ 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 L*er*:*FRI*^{SF2} *FLC*^{SF2}, a line obtained by backcrossing of San Feliu-2 to L*er* 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

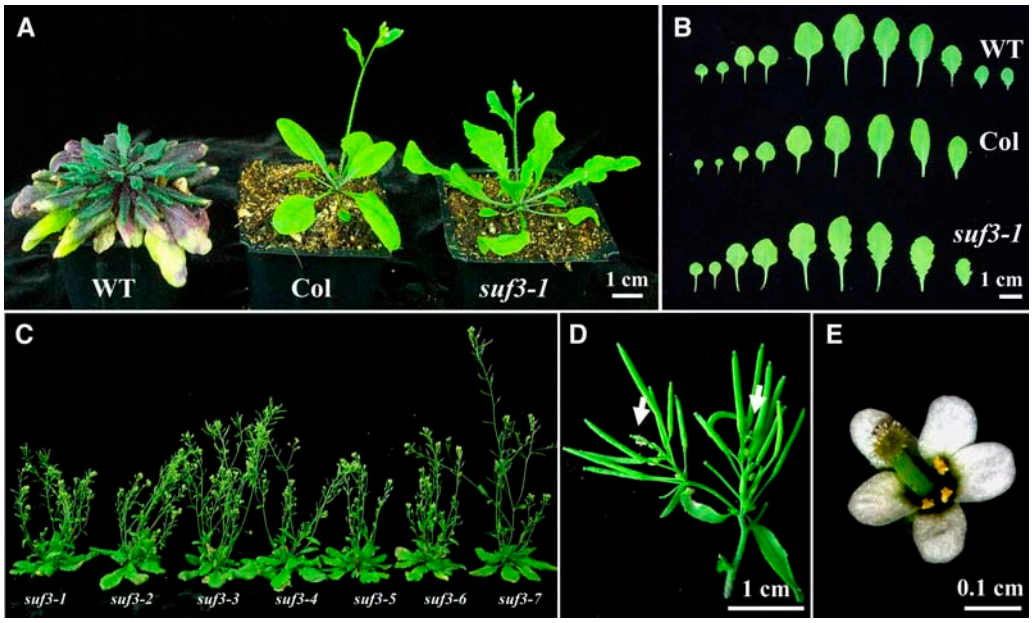

Figure 1. Phenotypes of the *suf3* Mutant.

(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.

Table 1. Flowering Time of *suf3*

Growth Condition	Col:*FRI*^{SF2}	*suf3-1*	Col (*fri FLC*)	*suf3-1 fri*
Long days	64.27 ± 3.80	12.45 ± 0.52	10.64 ± 0.67	5.73 ± 0.47
	(10.09 ± 0.83)	(4.18 ± 0.60)	(3.64 ± 0.50)	(3.09 ± 0.54)
Long days + vernalization	12.7 ± 0.67	4.80 ± 0.63	7.00 ± 0.32	N.D.
Short days	>100	63.00 ± 3.14	60.3 ± 3.71	17.1 ± 1.73
Short days + vernalization	35.5 ± 3.3	13.8 ± 1.81	35.3 ± 5.3	N.D.

To measure flowering time, 10 plants were used to count the number of rosette leaves when flowering. Values shown are leaf number ± SD. 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.

the WD repeat protein was selected from the SALK line (SALK_003098; T-DNA was inserted in the first exon), and the cross with *suf3* resulted in complementation, showing that it is not responsible for the *suf3* mutant phenotype (data not shown). We could not obtain the T-DNA insertion mutant of At3g33520 encoding ARP6; thus, we introduced the *35S-ARP6* transgene into the *suf3* mutant. All 14 transformants showed a very late-flowering phenotype similar to the wild type (Figure 2C). In addition, none of the transformants showed serrated leaves, terminal flowers, or extra petals, the phenotypes observed in *suf3* (data not shown). Furthermore, RNA interference (RNAi) of *ARP6* in the wild type consistently caused early flowering, although the range of flowering time was variable among the lines depending on the level of reduction in *ARP6* (Figures 2B and 2C). The RNAi transformants also showed additional phenotypes observed in *suf3*, such as serrated leaves, increased coflorescence shoots, and terminal flowers (Figure 2C, d; data not shown). Therefore, we concluded that *SUF3* encodes the ARP6 protein. As reported previously, *Arabidopsis* ARP6 consists of six exons and encodes proteins of 422 amino acids (McKinney et al., 2002). ARP6 is highly conserved among eukaryotes and has two peptide insertions that seemingly provide divergent surface features from conventional actin (Figure 3).

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*^{SF2} 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 *ld*, *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* by RNA gel blot analysis. All seven *suf3* alleles showed ~30 to 60% reduction in *FLC* transcript level compared with the Col:*FRI*^{SF2} 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*^{SF2} background are similar to

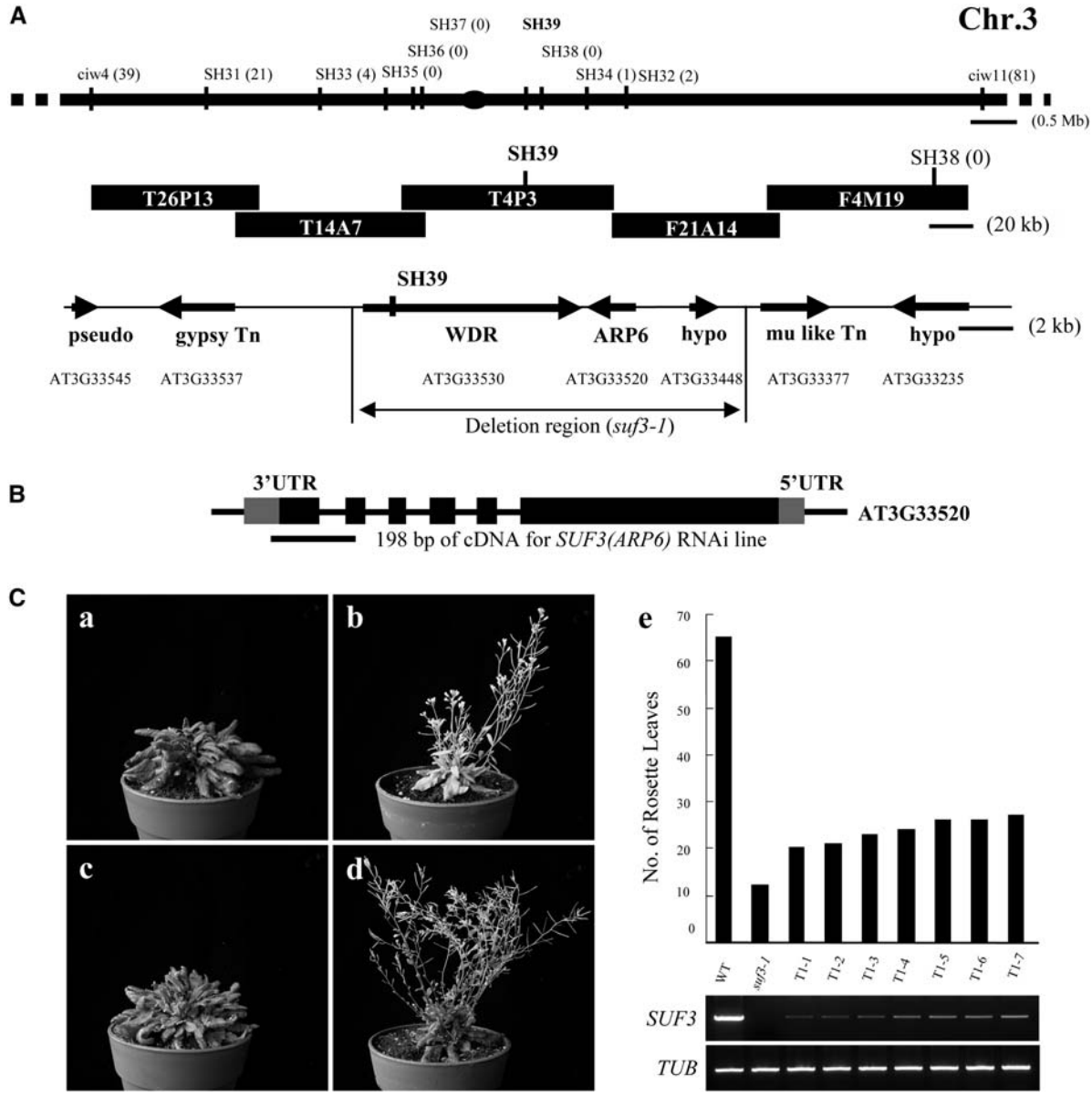

Figure 2. Positional Cloning of the *SUF3* Gene.

(A) The genetic interval, molecular markers, BAC clones, and deletion region are shown. Genomic DNA with marker SH39 could not be amplified by PCR, indicating that the region is deleted in *suf3-1*. Marker SH39 is located in the first intron of At3g33530. The deletion region in *suf3-1* is indicated.
(B) *SUF3* gene structure showing exons (thick bars) and introns (thin bars). The gray bars represent untranslated regions (UTRs). The underlined region was used to generate *AtARP6* RNAi transgenic plants.
(C) Phenotype of transgenic plants. (a) Wild type. (b) *suf3-1*. (c) *35S-ARP6* in *suf3-1*. (d) *ARP6* RNAi in the Col:*FRI*^{SF2} wild type. (e) Flowering time and *ARP6* expression level of individual *ARP6* RNAi transgenic T1 plants. *TUB*, *TUBULIN*.

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.

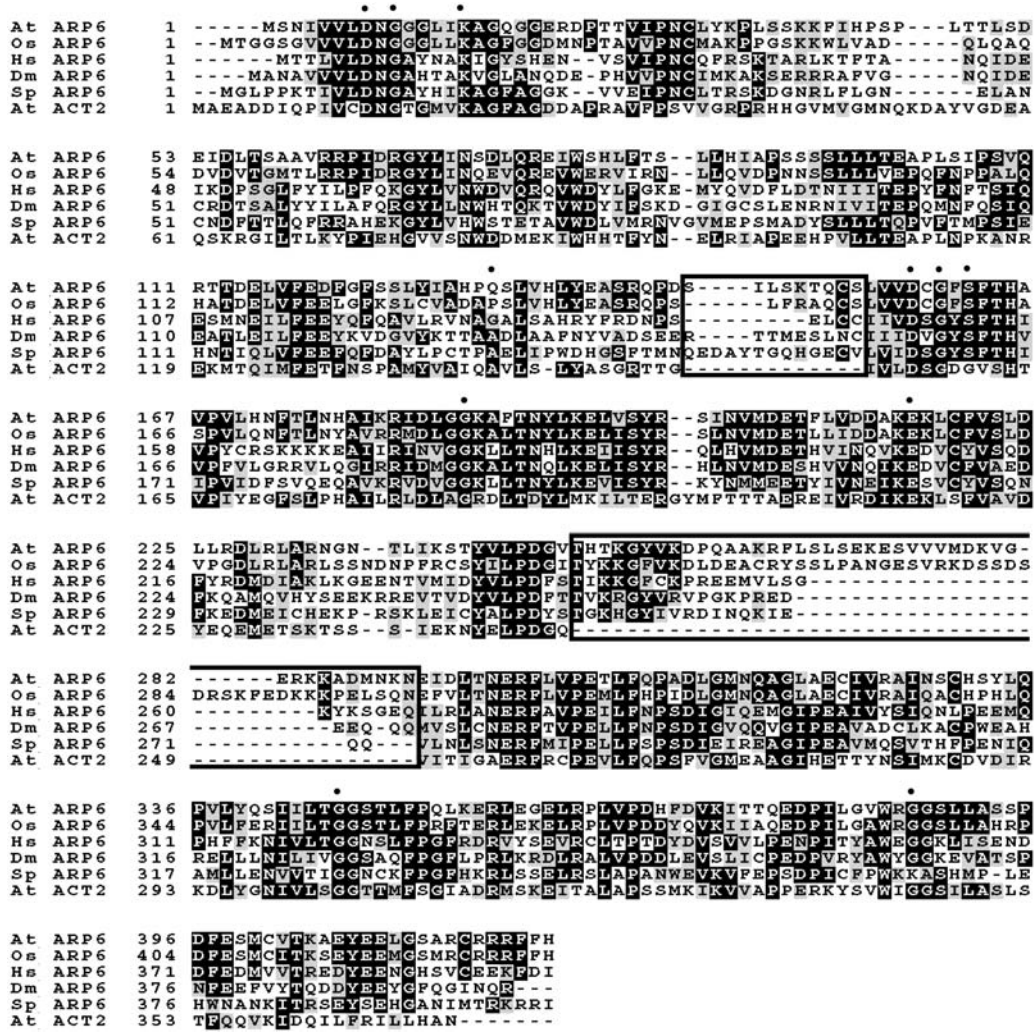

Figure 3. Alignment of Deduced Amino Acids of ARP6.

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).

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.*

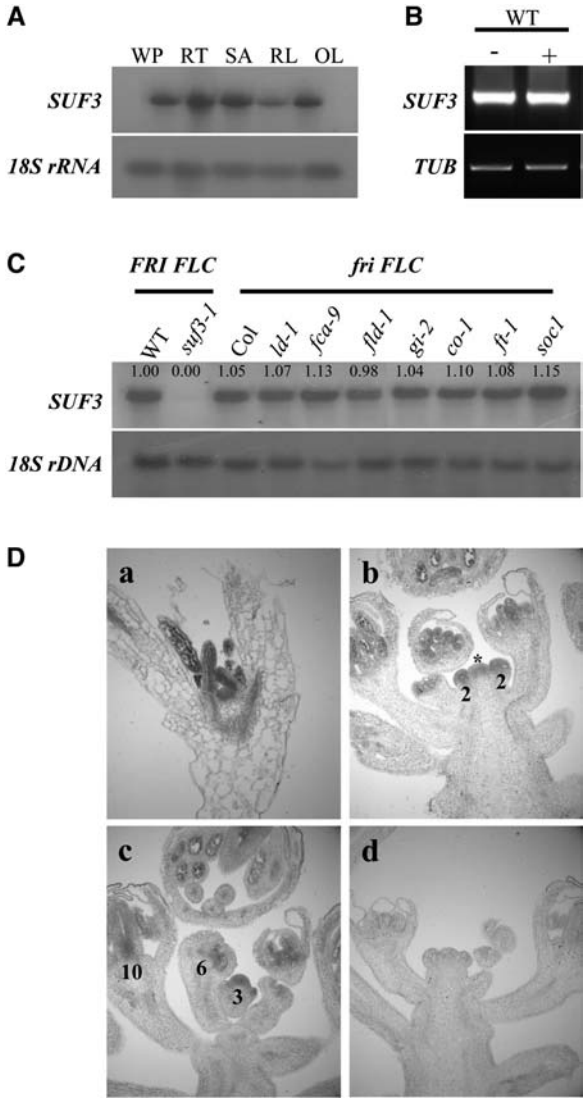

Figure 4. Expression Pattern of *SUF3*.

(A) *SUF3* expression in different tissues. Tissues were harvested from 20-d-old Col:*FRI*^{SF2} wild-type plants grown in long days for whole plant (WP), root (RT), shoot apex (SA), and rosette leaves (RL). The tissues for old leaves (OL) were harvested from 60-d-old wild-type plants. The expression level was determined by RNA gel blot analysis.

(B) *SUF3* expression in the wild type treated with (+) and without (−) vernalization. Total RNAs for RT-PCR analysis were extracted from plants grown at 4°C for 40 d under short days. *TUB*, *TUBULIN*.

(C) *SUF3* expression in *FRI* or the flowering time mutants *ld-1*, *fca-9*, *fld-1*, *gi-2*, *co-1*, *ft-1*, and *soc1-2*. All plants were grown for 10 d under long days.

(D) In situ hybridization analysis. (a) *SUF3* expression in 12-d-old Col plants grown in long days. (b) *SUF3* expression in the inflorescence of a Col plant. The asterisk indicates the shoot apical meristem, and the numbers below the flower meristems indicate the floral stages. (c) *SUF3* expression in the flower meristem. The numbers inside the flower meristems indicate the floral stages. (d) In situ hybridization with a sense control. No signal was detected.

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

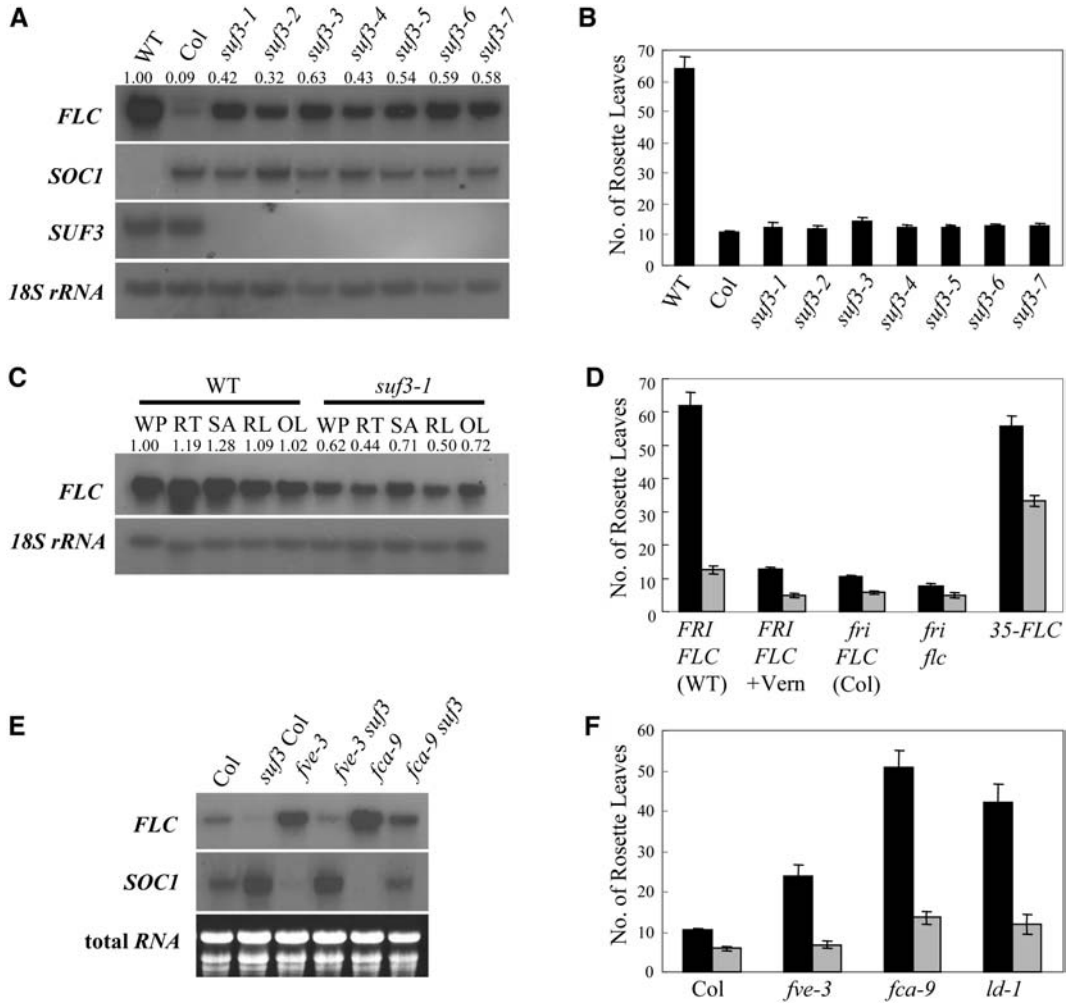

Figure 5. *FLC* Expression in *suf3* Mutants and the Effect on Flowering Time.

(A) RNA gel blot analysis in all *suf3* alleles. Numbers above the lanes are relative transcript levels of *FLC* compared with the wild type. *FLC*, *SOC1*, and *SUF3* transcript levels in *suf3* alleles were compared with those in the wild type and Col. Plants were grown for 10 d in long days. *SUF3* expression was not detected in all *suf3* alleles.

(B) Flowering time of all *suf3* alleles. Error bars indicate SD based on means obtained for 15 plants for each line.

(C) *FLC* expression in different tissues of *suf3-1*. Numbers above the lanes are relative transcript levels of *FLC* compared with whole plants from the wild type. WP, whole plant; RT, root; SA, shoot apex; RL, rosette leaves; OL, old leaves.

(D) Effect of the *suf3* mutation on flowering time of plants with different genetic backgrounds. Flowering times of *FRI FLC* (wild type), *fri FLC* (Col), *fri flc*, and *35S-FLC* were compared in the presence or absence of the *suf3* mutation. The effect of 5 weeks of vernalization on the flowering time of wild type and *suf3* plants was also determined. Black bars represent plants with *SUF3*, and gray bars represent plants with the *suf3* mutation. Error bars are as in **(B)**.

(E) *FLC* and *SOC1* transcript levels in double mutants of *suf3* and autonomous pathway mutants were determined by RNA gel blot analysis. All plants were grown for 10 d under long days.

(F) Flowering time of double mutants of *suf3* and autonomous pathway mutants. The *suf3* mutation suppressed late flowering of autonomous pathway mutants. Error bars are as in **(B)**.

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

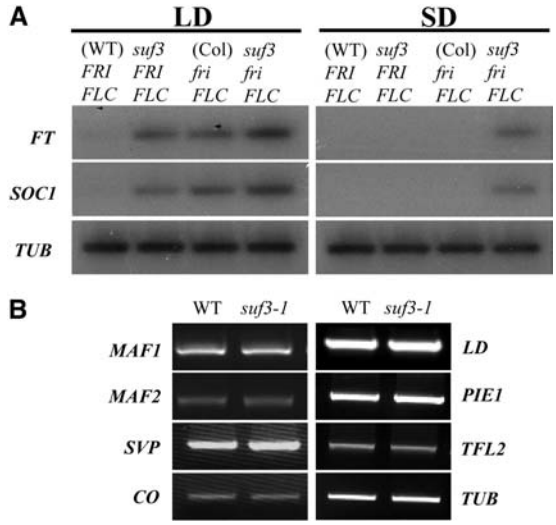

Figure 6. Effect of the *suf3* Mutation on the Expression of *FT*, *SOC1*, and Other Flowering Time Genes.

(A) *FT* and *SOC1* expression in wild-type, *suf3*, Col, and *suf3 fri* 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 fri* 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.

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 (Krogan 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 *flc* 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 fri* 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

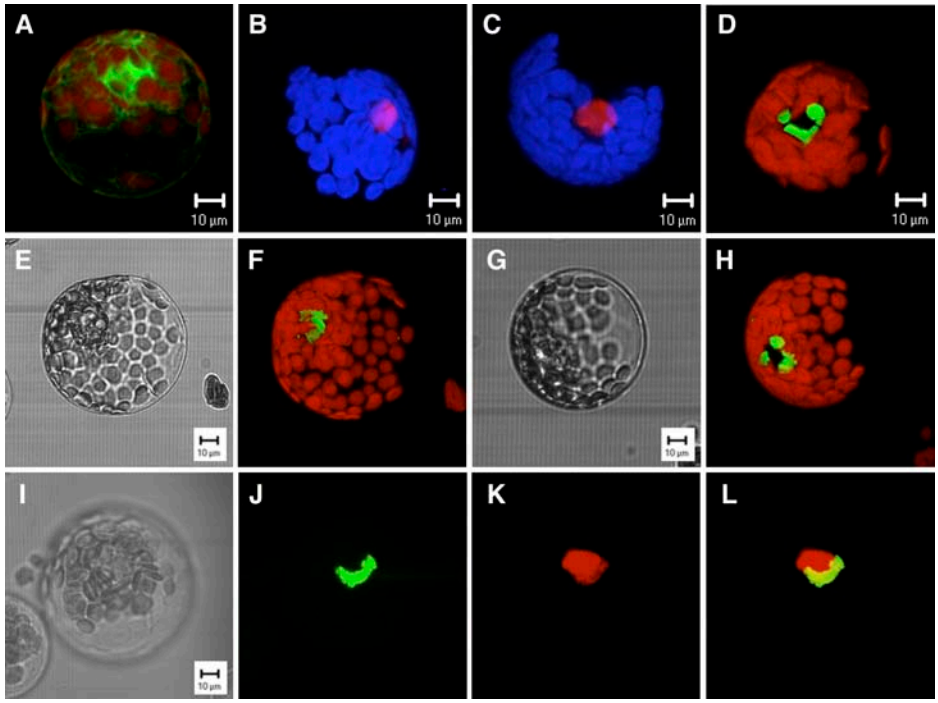

Figure 7. Localization of ARP6:GFP and YFP:ARP6 Fusion Proteins in *Arabidopsis* Protoplast Transient Assay.

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 transparent 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)**.

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, the PAF1 complex, and *SUF3*. At least the PAF1 complex and *SUF3* seem to have different targets, because the expression of *FLM* and *MAF2*, two *FLC* clade flowering repressors, was regulated not by *SUF3* but by the PAF1 complex (He et al., 2004).

Because *SUF3* encodes *Arabidopsis* ARP6, a putative component of an ATP-dependent chromatin-remodeling complex, it is tempting to speculate that *SUF3* regulates *FLC* through chromatin remodeling. However, the putative function of SUF3 for *FLC* chromatin remodeling seems to be independent of the histone modification of *FLC* chromatin 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.

METHODS

Plant Materials and Growth Conditions

The wild type used in this study was the *Arabidopsis thaliana* Col:*FRI*^{SF2} strain, which is a Col near-isogenic line containing the *FRI* allele of San Feliu-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:-*FRI*^{SF2} 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 L*er*:*FRI*^{SF2} *FLC*^{SF2}, which was obtained by six backcrosses of San Feliu-2 to L*er* (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 L*er* 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 electrophoresis and transferred to nylon membranes (Hybond N$^+$; Amersham). The digoxigenin (DIG)-labeled mRNA probes were prepared from plasmid vectors containing the cDNA fragments lacking the MADS domain for *FLC* and *SOC1* and the full cDNA for *SUF3* using the DIG RNA labeling kit (Roche). Prehybridization, hybridization, and washes were performed as described in the DIG application manual (Roche). As a quantitative RNA loading control, membranes were stripped and probed with 18S rDNA labeled with [α-^{32}P]dCTP. The RT-PCR procedure and primers used for *SOC1*, *FLC*, *FT*, and *TUB2* were described previously (Lee et al., 2000; Moon et al., 2005). For *SUF3*, primers SUF3-F (5′-ATCACGCCATTAAGAGGATTG-3′) and SUF3-R (5′-CTTGGTGACACACATGGACTC-3′) were used.

In Situ Hybridization

Tissues from 12-d-old Col seedlings and 25-d-old Col inflorescence shoots grown under long days were collected, fixed, and treated according to the Irish laboratory protocol provided at http://pantheon.yale.edu/%7Evi5/In%20situ%20protocol.pdf. The sections were made in 8 μm. As a template for the *SUF3* probe, we used pYB31 containing full-length cDNA amplified by PCR. For the antisense probe, pYB31 plasmid DNA was digested with *Hin*dIII, which resulted in a probe of 660 nucleotides at the C terminus. For the sense probe, the DNA was digested with *Eco*RI, which resulted in a full-length probe with 1300 nucleotides. The entire procedure of in situ hybridization followed the Irish laboratory protocol.

Plasmid Construction

To generation the *35S-SUF3* construct, the cDNA of *SUF3* was amplified by RT-PCR with forward primer 5′-ATGCAGGATCCGTATGTCAAACATCGTTGTTCTA-3′ and reverse primer 5′-AACCCGGATCCTCAATGAAAGAATCGTCTACGAC-3′. The *Bam*HI fragment of the PCR product was cloned into pCGN18 binary vector containing the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator (Jack et al., 1994). To produce the *SUF3* RNAi construct, an inverted-repeat construction including 198 bp of the 3′ untranslated region (25 bp) and the C-terminal region (173 bp) of *AtARP6* was made using pKANNIBAL vector in which a spliceable intron separates the two repeats (Helliwell and Waterhouse, 2003). Primers designed for PCR amplification of two identical 198-bp fragments for the *AtARP6* RNAi construct were RNAi *Xho*I (5′-ATGCCCTCGAGCCACTTGTCCCAGATCACTTT-3′), RNAi *Kpn*I (5′-ATGCCGGTACCCTCATGTGATATGTTTTGGT-3′), RNAi *Bam*HI (5′-ATGCCGGATCCCCACTTGTCCCAGATCACTTT-3′), and RNAi *Cla*I (5′-ATGCCATCGATCTCATGTGATATGTTTTGGT-3′). The product was subcloned into a binary vector, pART27, for transformation of the wild type (Gleave, 1992).

For construction of a gene encoding a GFP fusion, a PCR fragment containing the *AtARP6* open reading frame was amplified with forward primer 5′-ATGCAGGATCCGTATGTCAAACATCGTTGTTCTA-3′ and reverse primer 5′-AACCCGGATCCAATGAAAGAATCGTCTACGACAC-3′, which remove the stop codon at the C terminus and bear a *Bam*HI restriction site. The fragment was inserted at the *Bam*HI 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′-ATGCGGATCCATGTCAAACATCGTTGTTCTA-3′ and reverse primer 5′-AATTAGGCCTATGAAAGAATCGTCTACGACA-3′ and was cloned in a plant expression vector containing the cassava vein mosaic virus promoter (Verdaguer et al., 1998) and the nopaline synthase terminator using *Bam*HI and *Stu*I restriction sites. For the *TFL2:RFP* fusion construct, *TFL2* cDNA was amplified by PCR with forward primer 5′-ATGCAAGATCTATGAAAGGGGCAAGTGGTGCT-3′ and reverse primer 5′-ATGCAAGATCTAAGGCGTTCGATTGTACTT-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 568 nm. GFP/YFP, RFP, and chlorophyll autofluorescence were analyzed with the HQ515/30, HQ600/50, 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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