

# ***SUPPRESSOR OF FRIGIDA4*, Encoding a C2H2-Type Zinc Finger Protein, Represses Flowering by Transcriptional Activation of *Arabidopsis FLOWERING LOCUS C*** <sup>Ⓜ</sup>

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***FLOWERING LOCUS C (FLC)*, a strong floral repressor, is one of the central regulators of flowering in *Arabidopsis thaliana*. The expression of *FLC* is increased by *FRIGIDA (FRI)* but decreased by vernalization, a long period of cold exposure that accelerates flowering. Although many aspects of *FLC* regulation have been reported, it is not known how *FLC* is transcriptionally activated by *FRI* at the molecular level. We isolated *suppressor of FRIGIDA4 (suf4)*, a mutant that flowers early as a result of low *FLC* expression. *SUF4* encodes a nuclear-localized protein with two C2H2-type zinc finger motifs and a Pro-rich domain. *SUF4* protein interacts with *FRI* and *FRIGIDA-LIKE1 (FRL1)*, two genes for which single mutations have the same phenotype as *suf4*. *SUF4* also bound to the promoter of *FLC* in a chromatin immunoprecipitation assay, suggesting that *SUF4* acts as a transcriptional activator of *FLC* after forming a complex with *FRI* and *FRL1*. In addition, *suf4* suppresses *luminidependens (ld)*, a late-flowering mutation that causes an increase of *FLC*, and *SUF4* protein directly interacts with *LD*. Thus, we propose that *LD* binds to *SUF4* to suppress its activity in the absence of *FRI*.**

## **INTRODUCTION**

Since proper timing of flowering is critical for the survival of plant species, plants have evolved a complex genetic network that fine-tunes flowering time in response to endogenous signals and environmental cues. *Arabidopsis thaliana* accessions can be classified into summer annuals and winter annuals based upon their flowering behavior (Gazzani et al., 2003; Michaels et al., 2003). Summer annuals flower rapidly and thus complete their life cycle in a single growing season. By contrast, winter annuals begin vegetative growth in the fall and through winter as rosettes and then flower in the following spring. Thus, winter annuals require a mechanism to prevent flowering in the fall and to permit rapid flowering in the spring after a long period of winter cold. This mechanism is the vernalization response. The difference in the flowering behavior of winter-annual and summer-annual accessions is mainly determined by two genes, *FRIGIDA (FRI)* and *FLOWERING LOCUS C (FLC)* (Napp-Zinn, 1985; Koornneef et al., 1994; Lee et al., 1994). While winter annuals have functional versions of *FRI* and *FLC*, summer annuals such as *Landsberg erecta (Ler)* and *Columbia (Col)* have a null allele of *FRI* and/or a weak allele of *FLC* (Gazzani et al., 2003; Michaels et al., 2003). *FRI* encodes a coiled-coil protein that increases the transcript

level of *FLC*; in turn, *FLC*, a MADS box transcription factor, represses the expression of the so-called flowering pathway integrators *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1/AGL20)*, and *LEAFY* (Blázquez and Weigel, 2000; Johanson, et al., 2000; Lee et al., 2000; Samach et al., 2000; Simpson and Dean, 2002). Thus, high levels of *FLC* expression cause very late flowering in winter annuals. By contrast, vernalization represses *FLC* expression, thus causing rapid flowering (Michaels and Amasino, 1999; Sheldon et al., 1999).

In summer annuals, *FLC* expression is low due to the absence of *FRI*. However, a group of mutants in summer-annual backgrounds shows high levels of *FLC*, late flowering, and a vernalization requirement for rapid flowering similar to *FRI*-containing winter annuals (Sheldon et al., 1999, 2000; Michaels and Amasino, 2001). They are called autonomous pathway mutants because they normally respond to environmental factors such as photoperiod and vernalization. Genetic analysis showed that the *flc* null mutant is completely epistatic to all of the autonomous pathway mutations (Michaels and Amasino, 2001). It indicates that the function of autonomous pathway is the repression of *FLC*, while that of *FRI* is to overcome such repression.

Recently, aspects of the molecular mechanism of vernalization have been elucidated. Vernalization induces expression of *VERNALIZATION INSENSITIVE3 (VIN3)*, which encodes a PHD domain protein that may function as a component of the histone deacetylase complex. The *VIN3*-dependent deacetylation of H3 (histone 3) in *FLC* chromatin initiates the establishment of the vernalized state (Sung and Amasino, 2004). Afterwards, *VERNALIZATION1 (VRN1)* and *VRN2*, which encode a myb-related DNA binding protein and a polycomb group protein, respectively (Gendall et al., 2001; Kuzmichev et al., 2002; Levy

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<sup>Ⓜ</sup> Online version contains Web-only data.  
www.plantcell.org/cgi/doi/10.1105/tpc.106.045179

et al., 2002; Chanvivattana et al., 2004), maintain the repressed state by inducing the methylation of H3K9 and H3K27 (Lys-9 and Lys-27) in *FLC* chromatin (Bastow et al., 2004; Sung and Amasino, 2004).

The transcriptional regulation of *FLC* through histone modification is also observed in the *Arabidopsis* homolog of the PAF1 complex (Zhang and van Nocker, 2002; He et al., 2004; Oh et al., 2004). This complex is required for the trimethylation of H3K4 in *FLC* chromatin, a hallmark of the active chromatin state (He et al., 2004). Mutations in components of the PAF1 complex cause suppression of *FLC* in both *FRI*-containing winter annuals and autonomous pathway mutants. In addition, these mutants show a decrease in the transcript level of other floral repressors, *FLOWERING LOCUS M (FLM)* and *MADS AFFECTING FLOWERING2 (MAF2)*; thus, PAF1 complex mutants flower earlier than *fri* or *flc* (Zhang and van Nocker, 2002; He et al., 2004; Oh et al., 2004). A mutation in *EARLY FLOWERING IN SHORT DAYS (EFS)*, a homolog of SET domain methyltransferase, results in the same phenotype as mutants in the *Arabidopsis* homolog of the PAF1 complex (Soppe et al., 1999; Kim et al., 2005). It has been reported that the *efs* mutation causes reduced trimethylation of H3K4 or dimethylation of H3K36 in chromatin associated with the *FLC* promoter (Kim et al., 2005; Zhao et al., 2005). *FLC* expression is also regulated by putative components of an ATP-dependent chromatin remodeling complex, *PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1)*; a homolog of ISWI) and *ACTIN-RELATED PROTEIN6 (ARP6)* (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006).

At least three different classes of genes are involved in the autonomous pathway. The first class, including *FVE* and *FLOWERING LOCUS D*, represses *FLC* through histone deacetylation of *FLC* chromatin (He et al., 2003; Ausin et al., 2004). The second class, including *FCA*, *FY*, *FPA*, and *FLOWERING LOCUS K (FLK)*, encodes RNA binding or processing protein (Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003; Lim et al., 2004). The molecular mechanism of how *FLC* is regulated by the second class is unknown. The last component of the autonomous pathway, *LUMINIDEPENDENS (LD)*, encodes a homeodomain protein that is localized to the nucleus (Lee et al., 1994; Aukerman et al., 1999). How *LD* represses *FLC* expression is not known either.

By screening early-flowering mutants in *FRI*-containing winter-annual backgrounds, *FRIGIDA-LIKE1 (FRL1)* and *FRIGIDA ESSENTIAL1 (FES1)* have been identified as genes that are required for the upregulation of *FLC* by *FRI* (Michaels et al., 2004; Schmitz et al., 2005). *FRL1*, a relative of *FRI*, encodes a protein with a coiled-coil domain, whereas *FES1* encodes a CCCH-type zinc finger protein. Both *fri1* and *fes1* mutants are unable to suppress the late-flowering phenotype of autonomous pathway mutants; thus, the function of these two genes is dependent on *FRI*. Because all three single mutants, *fri*, *fri1*, and *fes1*, show the same phenotype, it is likely that the three genes act cooperatively to promote *FLC* expression. However, the molecular function of these genes is not disclosed yet.

Although many of the components that influence the chromatin state of *FLC*, whether active or inactive, have been reported, it is unknown what drives the transcription of *FLC*. In this study, we isolated an early-flowering mutant, *suppressor of FRIGIDA4 (suf4)*, in a *FRI*-containing winter-annual strain. The *suf4* mutant

showed a similar flowering phenotype as *fri* without any other morphological defects. The map-based gene cloning found that *SUF4* encodes a C2H2-type zinc finger protein. The *SUF4* protein is localized to the nucleus and binds to the promoter of *FLC* in vivo. Interaction analysis showed that *SUF4* binds to *FRI* and *FRL1*, thus suggesting the formation of a protein complex that acts as a transcriptional activator of *FLC*. Our results also showed that when *FRI* is absent, *LD* binds to *SUF4* and suppresses *SUF4* activity.

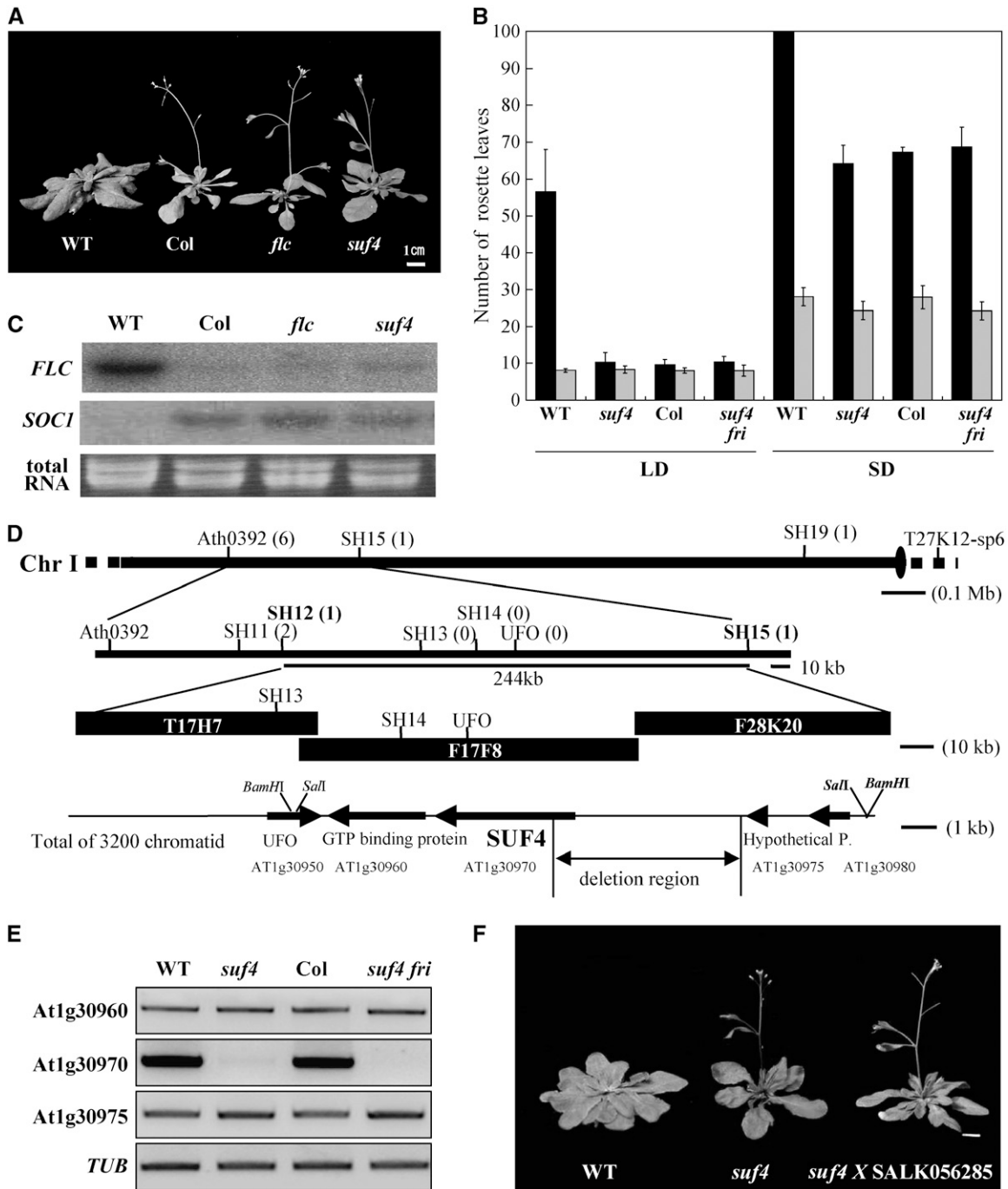
## RESULTS

### Isolation of the *suf4* Mutant

To elucidate the *FRI*-mediated *FLC* regulatory mechanism, we screened early-flowering mutants from *FRI*-containing Col (Col: *FRI*<sup>SF2</sup>) after fast neutron mutagenesis as reported previously (Michaels and Amasino, 1999; Choi et al., 2005). From this screen, we isolated a recessive mutant designated as *suf4* that showed early flowering almost identical to Col, which is a *fri* null, and did not display any other morphological alterations (Figure 1A). An F2 population derived from the cross with the wild type after three generations of backcrossing showed an ~3:1 segregation ratio (37 late versus 13 early,  $\chi^2 = 0.027$ ), indicating that a single recessive locus was the cause of the phenotype. The flowering responses of *suf4* to photoperiod and vernalization were similar to those of Col (i.e., *suf4* showed similar delays in flowering in short days and similar acceleration of flowering by vernalization as Col) (Figure 1B). In addition, the *suf4 fri* double mutant showed the same phenotype as *suf4 (FRI suf4)* or Col (*fri SUF4*) (Figure 1B, Table 1). To determine if the early-flowering phenotype caused by the *suf4* mutation is due to a defect in *FLC* activation by *FRI*, *FLC* expression was checked by RNA gel blot analysis using 10-d-old seedlings (Figure 1C). Similar to Col, the *FLC* transcript was barely detectable in *suf4*, while *SOC1* transcript was increased. This demonstrates that *SUF4* is necessary for *FRI*-mediated *FLC* activation.

### Positional Cloning of the *SUF4* Gene

For positional cloning of the *SUF4* gene, *suf4* in Col: *FRI*<sup>SF2</sup> was crossed to *Ler: FRI*<sup>SF2</sup>*FLC*<sup>SF2</sup>, which was obtained by six backcrosses of San Feliu-2 to *Ler* (Lee and Amasino, 1995; Choi et al., 2005). A total of 1600 early-flowering F2 plants were selected for mapping analysis. *suf4* was located near the centromere of chromosome 1 between the two simple sequence length polymorphic (SSLP) markers, SH12 and SH15, in the 244-kb interval that is covered by three BAC clones (Figure 1D). Because fast-neutron-induced mutagenesis often creates genomic deletions of various lengths, we searched restriction fragment length polymorphisms between the wild type and *suf4* using the three BAC clones as probes. The different restriction patterns were detected when BAC F17F8 and At1g30960, one of the genes located within F17F8, were used as probes (see Supplemental Figure 1 online). Further analysis of this region by PCR revealed that the genomic DNA of *suf4* contains a deletion of ~6 kb, which includes two zinc finger domains in the N-terminal region of At1g30970 (Figure 1D). The sequences of the two neighboring



**Figure 1.** Mutant Characteristics and Positional Cloning of *SUF4*.

**(A)** Morphology of the wild type, Col, *flc*, and *suf4* grown for 20 d under long days.

**(B)** Comparison of flowering time in the wild type, *suf4*, Col, and *suf4 fri* grown under long days (LD) and short days (SD) after 0 or 9 weeks of vernalization treatment. Black bars, plants vernalized for 0 week; gray bars, plants vernalized with 9 weeks. Bars represent mean values  $\pm$  SD of rosette leaf number. For each line, 20 plants were scored.

**(C)** The expressions of *FLC* and *SOC1* in the wild type, Col, *flc*, and *suf4* grown under long days. Total RNA was extracted from 10-d-old seedlings.

**(D)** Genetic map of *SUF4* on chromosome 1. The genetic interval, molecular markers, BAC clones, and deletion region are shown. The numbers in parenthesis are recombinants among 3200 chromatids analyzed.

**(E)** RT-PCR analysis shows that At1g30970 expression is not detected in *suf4*.

**(F)** Complementation analysis of *suf4* with SALK\_056285 that has a T-DNA insertion in the third exon of At1g30970. F1 plants from the cross of *suf4* with SALK\_056285 flower as early as *suf4*.

**Table 1.** Flowering Time of *suf4* and Double Mutants with Other Flowering Time Mutants

Genotype	No. of Rosette Leaves
Col: <i>FRI</i> <sup>SF2</sup> background in LD	
Wild type	55.6 ± 4.03
<i>fri</i>	10.9 ± 0.72
<i>suf4</i>	10.5 ± 1.12
<i>35S-FLC</i>	61.5 ± 6.56
<i>35S-FLC suf4</i>	58.7 ± 7.45
<i>vip4</i>	7.4 ± 0.78
<i>suf4 vip4</i>	7.8 ± 0.62
Col background in LD	
Col	10.9 ± 0.72
<i>suf4 fri</i>	10.3 ± 0.83
<i>flc-3</i>	9.3 ± 1.06
<i>suf4 flc-3</i>	9.1 ± 1.10
<i>fve-3</i>	25.4 ± 1.71
<i>suf4 fve-3</i>	18.9 ± 2.08
<i>fca-9</i>	40.2 ± 2.48
<i>suf4 fca-9</i>	32.4 ± 3.08
<i>ld-1</i>	42.7 ± 3.22
<i>suf4 ld-1</i>	25.8 ± 2.45
<i>soc1-2</i>	15.0 ± 0.92
<i>suf4 soc1-2</i>	15.8 ± 1.49
<i>ft-1</i>	25.2 ± 4.02
<i>suf4 ft-1</i>	29.5 ± 3.40
<i>co-1</i>	17.5 ± 2.00
<i>suf4 co-1</i>	19.6 ± 2.61
Col background in SD	
Col	67.2 ± 1.52
<i>suf4 fri</i>	68.7 ± 3.72
<i>flc-3</i>	45.4 ± 2.75
<i>suf4 flc-3</i>	45.1 ± 2.71

For measuring flowering time, at least 20 plants were used for counting the number of rosette leaves when flowering. Values are ± SD. LD, long days; SD, short days.

genes, At1g30960 and At1g30975, were intact, and the expression levels of them were not altered in *suf4* as expected (Figure 1E). By contrast, the expression of At1g30970 was not detected in *suf4*. To confirm that the loss of At1g30970 leads to the *suf4* phenotype, *suf4* was crossed to *SALK\_056285*, which has a T-DNA insertion in the third exon of At1g30970. The *SALK\_056285* line was most likely a null allele because it did not show any expression of At1g30970 when checked by RT-PCR, and similar to the *suf4*, it did not show any flowering phenotype in the Col background (data not shown). All the resulting F1 progenies exhibited an early-flowering phenotype, and 56 F2 progenies derived from the selfing of the F1 flowered in the same manner as *suf4*, confirming that At1g30970 is indeed *SUF4* (Figure 1F).

The *SUF4* gene contains seven exons and encodes a protein with two C2H2-type zinc finger domains at its N-terminal region and a Pro-rich domain in the central region (see Supplemental Figure 1 online). The deduced amino acid sequence of *SUF4* was distinct from those of other zinc finger proteins in the *Arabidopsis* database (data not shown). The comparison of amino acid sequence of *SUF4* with those of other plant proteins with two zinc fingers showed no significant similarity except one homol-

ogous protein in the rice (*Oryza sativa*) genome (see Supplemental Figure 1 online). However, the *SUF4* amino acid sequence showed similar characteristics with ZP207 class zinc finger proteins reported in animals (Pahl et al., 1998; Taguchi et al., 1998; Bergqvist et al., 2006); it has a potential nuclear localization signal at its N terminus, the two zinc fingers are separated by two amino acids, and it has a Pro-rich domain that is usually found in transcription factors (see Supplemental Figure 1 online).

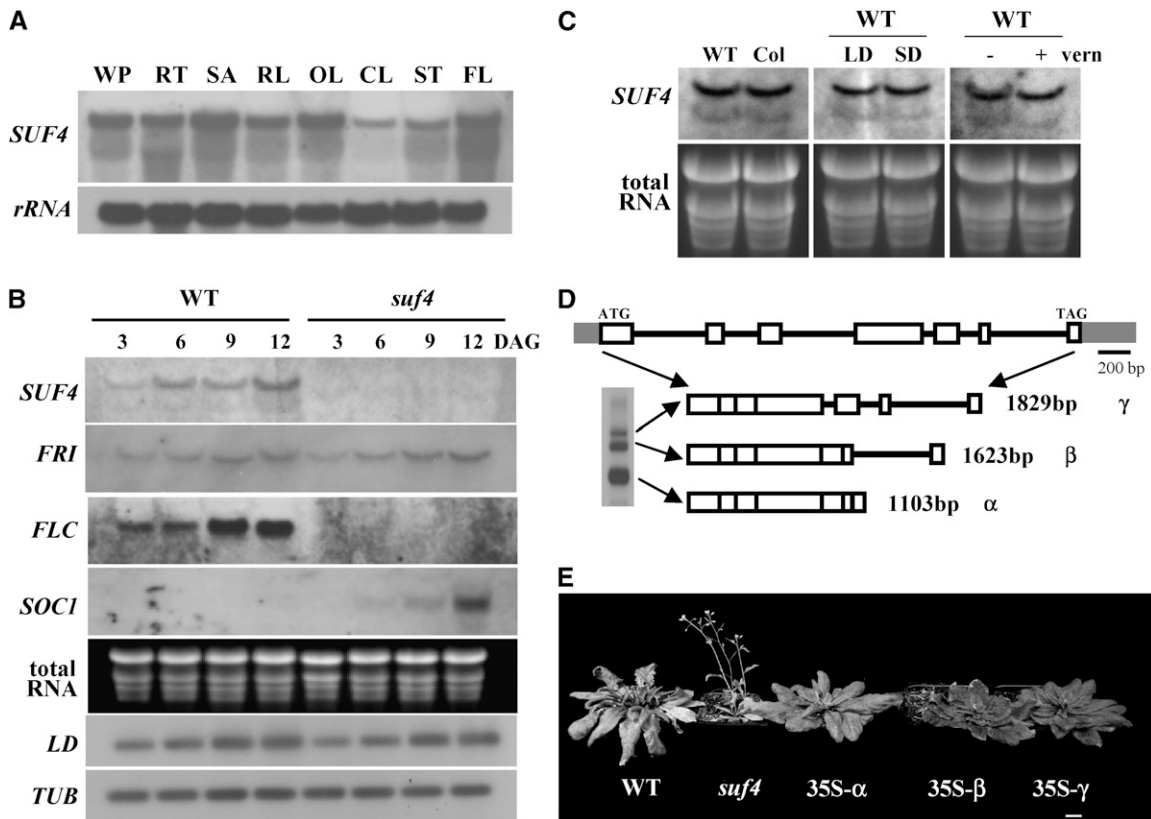
### Expression of *SUF4*

The *SUF4* transcript was detected in all of the tissues tested, although the expression was weaker in the cauline leaves and stems (Figure 2A). The time-course experiment showed that *SUF4* expression is gradually increased during development similar to *FRI* expression (Figure 2B). By contrast, *SUF4* transcript was not detectable in *suf4*, showing that this mutant is a null allele. The *LD* gene involved in the autonomous pathway also showed gradual increase during development, although the increase was less pronounced. When checked if the expression of *SUF4* is affected by environmental conditions, *SUF4* expression level was not influenced by photoperiod or vernalization (Figure 2C). It is noteworthy that *FRI* expression was not affected by *suf4* nor was *SUF4* expression affected by *fri* (Figures 2B and 2C), which suggests that *SUF4* and *FRI* do not regulate each other at transcriptional level.

We always detected two bands for *SUF4* transcript in RNA gel blot analysis: a major larger form and a minor smaller form. To confirm that *SUF4* produces differently sized transcripts, RT-PCR was performed using the primer sets designed to amplify the whole open reading frame (Figure 2D). The result showed three transcripts of 1103, 1623, and 1829 bases designated as  $\alpha$ ,  $\beta$ , and  $\gamma$ , respectively. The sequencing of individual RT-PCR products showed that  $\beta$  and  $\gamma$  forms were derived from incomplete RNA processing; the last three introns remained in the  $\gamma$  form, whereas the last intron remained in the  $\beta$  form (Figure 2D). The amount of transcripts from RT-PCR did not reflect the real transcript level detected in RNA gel blot analysis (for example, the  $\gamma$  form was higher than the  $\alpha$  form in the RNA gel blot analysis). This difference may be due to the preferential amplification of small size by PCR.

Because each of the three transcripts produces a different amino acid sequence at the C terminus, we wondered which transcript is functional. For this, we generated transgenic lines overexpressing the three transcripts from the constitutive 35S promoter in *suf4* and Col (*fri* null). The transgenic lines overexpressing either  $\alpha$ ,  $\beta$ , or  $\gamma$  in *suf4* showed similar late flowering as the wild type (Figure 2E). When the transgenic lines overexpressing the  $\gamma$  form and the  $\beta$  form were analyzed by RT-PCR, only the smallest transcript was overexpressed (see Supplemental Figure 2 online). Thus, the smallest  $\alpha$  form is most likely functional, and the  $\beta$  and  $\gamma$  transcripts are intermediate forms that have not completed the splicing process. When  $\alpha$ ,  $\beta$ , or  $\gamma$  forms were overexpressed in Col, none of the transgenic lines showed apparent alteration in flowering time compared with Col (data not shown), indicating that *FRI* activity is necessary for *SUF4* function in delaying flowering.

The mRNA processing of *SUF4* was not changed by any of the mutations in *FLK*, *FCA*, *FY*, *FPA*, and *ABH1*, genes encoding



**Figure 2.** Expression Pattern of *SUF4*.

**(A)** *SUF4* expression in different tissues. Tissues were harvested from 20-d-old wild type grown in long days for the whole plant (WP), root (RT), shoot apex (SA), and rosette leaves (RL). The tissues for old leaves (OL), stem (ST), cauline leaves (CL), and flowers (FL) were harvested from 50-d-old wild type. Expression level was determined by RNA gel blot analysis.

**(B)** Temporal expression of *SUF4*, *FRI*, *FLC*, *SOC1*, and *LD*. Total RNAs were extracted from plants grown for 3, 6, 9, 12 d after germination (DAG). *LD* expression level was detected by RT-PCR and all others were by RNA gel blot analysis. Total RNA was presented as loading control.

**(C)** Comparison of *SUF4* expression between the wild type and *Col* (left), between long days (LD) and short days (SD) (center), and between with and without vernalization (right). Total RNAs for RNA gel blot analysis were extracted from plants grown for 10 d under long days or short days. Vernalization was treated for 4 weeks.

**(D)** Three differently sized transcripts were detected for *SUF4* from RT-PCR analysis. White boxes indicate exons, gray boxes indicate untranslated regions, and lines indicate introns.

**(E)** The phenotype of transgenic plants. Early flowering of *suf4* was rescued by overexpression of all three transcripts.

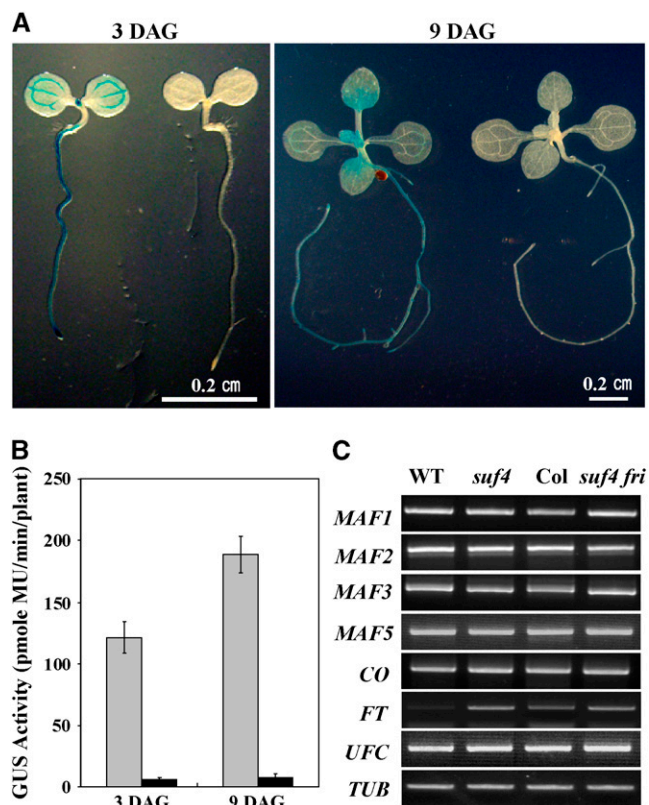
RNA binding or processing proteins that are involved in the regulation of *FLC* (see Supplemental Figure 3 online). In addition, *SUF4* expression was not affected by any of the flowering time mutants, such as *ld*, *fve*, and *fld* (autonomous pathway mutants), *co*, *gi*, and *ft* (photoperiod pathway mutants), and *soc1* (a flowering pathway integrator) (see Supplemental Figure 4 online).

***suf4* Causes Early Flowering through the Suppression of *FLC***

In the *suf4* mutant, *FLC* expression was suppressed, although *FRI* expression was not changed (Figure 2B). In the wild type, *FLC* is expressed at the highest levels in shoot and root apices (Michaels and Amasino, 1999). To evaluate whether the *suf4* mutation leads to a reduction of *FLC* expression in these regions, we introduced *FLC*- $\beta$ -glucuronidase (*GUS*) into *suf4* (Figure 3A). Consistent with

the previous report, *FLC-GUS* in the wild type was easily detected in germinating seedlings, and the expression remained strong in the shoot and root apices afterwards. By contrast, *FLC-GUS* in *suf4* was greatly reduced in both shoots and root tips, which is different from *pie1*, which shows *FLC* reduction only in shoots (Noh and Amasino, 2003). *FLC-GUS* expression was reduced to 5.3% in 3-d-old seedlings and 4.0% in 6-d-old seedlings by *suf4* mutation (Figure 3B). This is noteworthy because the *SUF4* transcript was barely detectable in 3-d-old seedlings (Figure 2B). This result suggests that the low expression of *SUF4* is even necessary for the activation of *FLC* in young seedlings.

To address if *SUF4* regulates other floral repressors as well as *FLC*, we examined the expression of *FLM/MAF1*, *MAF2*, *MAF3*, and *MAF5*, which were previously reported as *FLC* clade genes (Ratcliffe et al., 2001, 2003; Scortecci et al., 2003). The *suf4* mutation did not affect the transcript levels of these genes (Figure 3C).



**Figure 3.** Effect of *suf4* on the Expression of *FLC* and Other Flowering Time Genes.

(A) *FLC* expression is suppressed in *suf4* mutants. In each panel, left is *FLC-GUS* in *flic-3* and right is *FLC-GUS* in *suf4*. Plants were grown 3 and 9 d after germination (DAG) under long days before GUS staining.

(B) GUS activity was measured by 4-methyl umbelliferyl glucuronide (MUG) assay using plants 3 and 9 d after germination. GUS activities are reduced in *suf4 FLC-GUS* (black bars) compared with *FLC-GUS flic-3* (gray bars). Bars indicate means  $\pm$  SD of GUS activity in three MUG assay replicates. In each MUG assay, 10 plants per genotype were used.

(C) RT-PCR analysis of the *FLC* clade genes *UFC*, *CO*, and *FT*. The expression of the floral integrator *FT* is increased by *suf4* mutation. All others are not affected. Total RNAs were extracted from plants grown for 10 d under long days.

It also did not affect the expression of *CO*, a central regulator of photoperiod pathway (Figure 3C).

It was reported that vernalization suppresses *FLC* and the neighboring gene *UPSTREAM OF FLC* (*UFC*) coordinately as a cluster by chromatin modification (Bastow et al., 2004; Finnegan et al., 2004; Sung and Amasino, 2004). When checked if the *suf4* mutation reduces *UFC* expression, it was not changed by *suf4* (Figure 3C). This indicates that *SUF4* plays a specific role in the regulation of *FLC* transcription.

### *SUF4* Function Is Dependent on *FLC*

Because *suf4* and *fri* have the same phenotype, and double mutants are identical to either single mutant (Figure 1, Table 1), we wondered if *SUF4* function is dependent on *FLC* like that of

*FRI*. We compared the flowering time of the *suf4 flic-3* double mutant with those of *suf4* and *flic-3* (a null allele) single mutants (Table 1). The *flic-3* mutant flowered earlier than Col, especially in short days as reported (Michaels and Amasino, 2001). It also flowered earlier than *suf4* in Col, as *suf4* has the same phenotype with Col. The *suf4 flic-3* double mutant flowered at the same time with *flic-3* in both long days and short days (Table 1), suggesting that *SUF4* function is dependent on *FLC*. Consistently, *35S-FLC* was epistatic to *suf4*; *35S-FLC suf4* showed similar flowering time with *35S-FLC* (Table 1). This indicates that *FLC* is the major target of *SUF4* activity for flowering regulation. The phenotypes of the *suf4 vip4* double mutant confirmed this. *VIP4* is a component of the *Arabidopsis* PAF1 complex, which mediates trimethylation of H3K4 in *FLC* chromatin. The *vip4* mutation causes complete suppression of *FLC* and other *FLC*-clade genes, thus causing earlier flowering than *flic* (He et al., 2004). As expected, the *suf4 vip4* double mutant did not show further earlier flowering than *vip4* (Table 1).

### Genetic Interaction of *suf4* with Other Flowering-Time Mutants

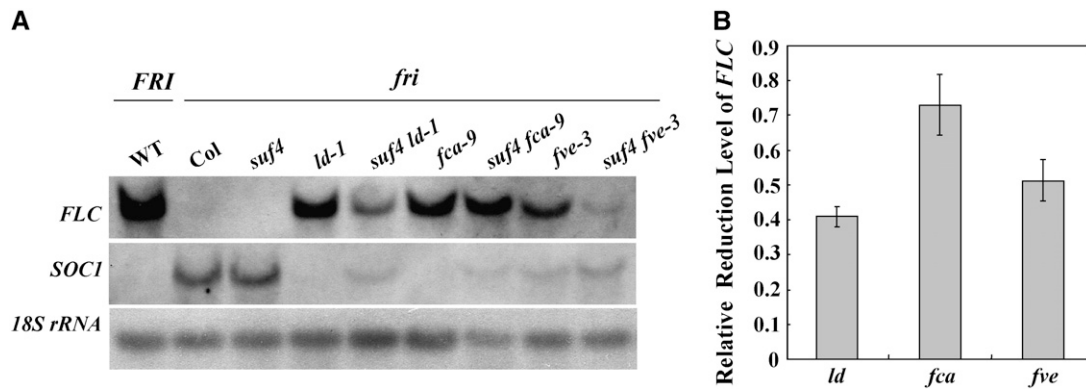
To define the role of *SUF4* in the flowering mechanism, double mutants were made that contained *suf4* and other flowering-time mutations. The photoperiod pathway mutants *co-1* and *ft-1* were crossed to *suf4*. The flowering times of *suf4 co-1* and *suf4 ft-1* were almost identical to those of the *co-1* and *ft-1* single mutants, respectively (Table 1). This is consistent with the fact that the *suf4* mutation did not affect the responsiveness to photoperiod (Figure 1B). In addition, *soc1-2*, a mutation in one of the flowering pathway integrators, was epistatic to *suf4*, indicating that *SUF4* does not have an effect downstream of *FLC* (Table 1).

It was of interest to determine whether *SUF4* interacts with autonomous pathway genes or acts independently to increase the *FLC* expression. We checked the flowering time of the double mutants of *suf4* and several autonomous pathway mutations (Table 1). The *suf4 fve-3* and *suf4 fca-9* double mutants flowered slightly earlier than the *fve-3* and *fca-9* single mutants, respectively. The most significant suppression of late flowering by *suf4* was found in *ld-1*. Such suppression resulted from decreased *FLC*; the double mutants showed a decrease of *FLC* compared with single autonomous pathway mutants (Figure 4). Consistent with the flowering phenotype, *suf4 ld-1* showed the strongest decrease in *FLC* expression (Figure 4B). However, *FLC* transcript levels in any of the double mutants were still higher than that in Col or the *suf4* single mutant (Figure 4A). Such results show that *SUF4* activity is responsible for the late-flowering phenotype of *ld*, *fve*, and *fca* at least partially.

### Cellular Localization of *SUF4*, *FRI*, and *LD*

To understand the cellular function, we determined the subcellular locations of *SUF4*, *FRI*, and *LD*. For this, genes encoding *SUF4*:green fluorescent protein (GFP), *SUF4*:red fluorescent protein (RFP), yellow fluorescent protein (YFP):*FRI*, and YFP:*LD* fusion proteins were introduced transiently into *Arabidopsis* protoplasts. *SUF4*:GFP, *SUF4*:RFP, YFP:*FRI*, and YFP:*LD* were detected in the nucleus (Figure 5). However, the subnuclear





**Figure 4.** *FLC* and *SOC1* Expression in Double Mutants of *suf4* with Autonomous Pathway Mutants.

(A) RNA gel blot analysis shows that the high *FLC* transcript level caused by *fca*, *fve*, or *ld* was partially reduced by *suf4* mutation. Total RNAs were extracted from plants grown for 10 d under long days.

(B) Relative *FLC* expression ratio of *suf4* double mutants to single autonomous pathway mutants is presented. Band intensities in (A) were quantified using Image J software. Bars show mean values  $\pm$  SD of the relative *FLC* levels in three independent RNA gel blot experiments. The level of 18S RNA was used as the internal control.

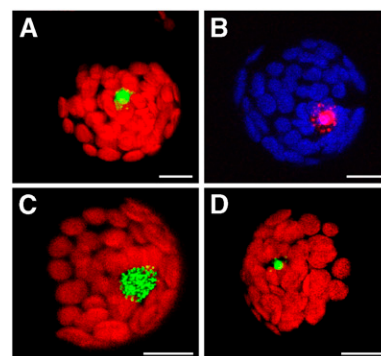
localization pattern of YFP:FRI protein was somewhat different than that of others. YFP:FRI was dispersed throughout the nucleus, and fluorescence was observed as evenly distributed speckles (Figure 5C). By contrast, SUF4:GFP, SUF4:RFP, and YFP:LD did not show such speckles (Figures 5A, 5B, and 5D).

#### Protein Interaction of SUF4 with FRI, FRL1, and LD

The single mutants of *suf4* and *fri* showed similar early flowering and similar decrease of *FLC* expression; thus, both are required for activation of *FLC*. Recently, *FRL1*, a *FRI*-related gene, has been found to be required for *FLC* activation (Michaels et al., 2004). Because FRI and FRL1 have a coiled-coil domain that may provide a protein interaction surface, and both FRI and SUF4 are localized in the nucleus, we wondered if the three proteins SUF4, FRI, and FRL1 interact with each other. To test this, the full-length proteins were fused to GAL4 DNA binding domain or to GAL4 transcriptional activation domain for yeast two-hybrid interaction analysis (Figures 6A and 6B). As a negative control, we checked the interaction of SUF4 and TERMINAL FLOWER2 (TFL2) (Figure 6A). *TFL2* encodes the nuclear protein HP1 (for heterochromatin protein 1) that acts in the photoperiod pathway upstream of *FT* and thus plays a role in a different genetic pathway than *SUF4* (Gaudin et al., 2001; Kotake et al., 2003). As expected, no interaction was detected between SUF4 and TFL2. However, the interaction analysis showed that SUF4 binds to both FRI and FRL1. In addition, SUF4 and FRI showed homodimerization activity. Notably, we did not detect FRL1 binding to FRI nor FRL1 homodimerization (Figure 6A).

The genetic interaction analysis showed that *suf4* strongly suppresses the *ld* phenotype (Table 1). Because LD encodes a homeodomain protein and is also localized in the nucleus, we tested if SUF4 interacts with LD at the protein level. As shown in Figure 6A, LD protein bound to SUF4 but not to FRI or FRL1 in yeast two-hybrid analysis. When the bait and prey were changed, the yeast cells grew slowly but confirmed the interaction of LD and SUF4 (Figure 6B). This result indicates that the protein–protein interaction is the basis of genetic suppression of *ld* by *suf4*.

We tested if the protein–protein interactions also occur in plant cells using a transient gene expression system (Fischer et al., 1999; Voinnet et al., 2003). The *Agrobacterium tumefaciens* cells harboring empty vector, SUF4:MYC alone, and SUF4:MYC with FRI:HIS, FRL1:HA, LD:HA, or ARP6:FLAG fusion constructs were infiltrated into tobacco leaves, and then total proteins were extracted 2 d after infiltration for coimmunoprecipitation tests (Figure 6C). Protein extracts were immunoprecipitated with anti-MYC antibody for empty vector or SUF4:MYC-infiltrated tissues, anti-HIS antibody for SUF4:MYC with FRI:HIS coinfiltrated tissues, anti-HA antibody for SUF4:MYC with LD:HA or FRL1:HA coinfiltrated tissues, and anti-FLAG antibody for SUF4:MYC with ARP6:FLAG, respectively. The precipitated proteins were then analyzed by protein gel blots using each antibody. As expected, a negative control,



**Figure 5.** Nuclear Localization of SUF4:GFP, SUF4:RFP, YFP:FRI, and YFP:LD Proteins in *Arabidopsis* Protoplasts.

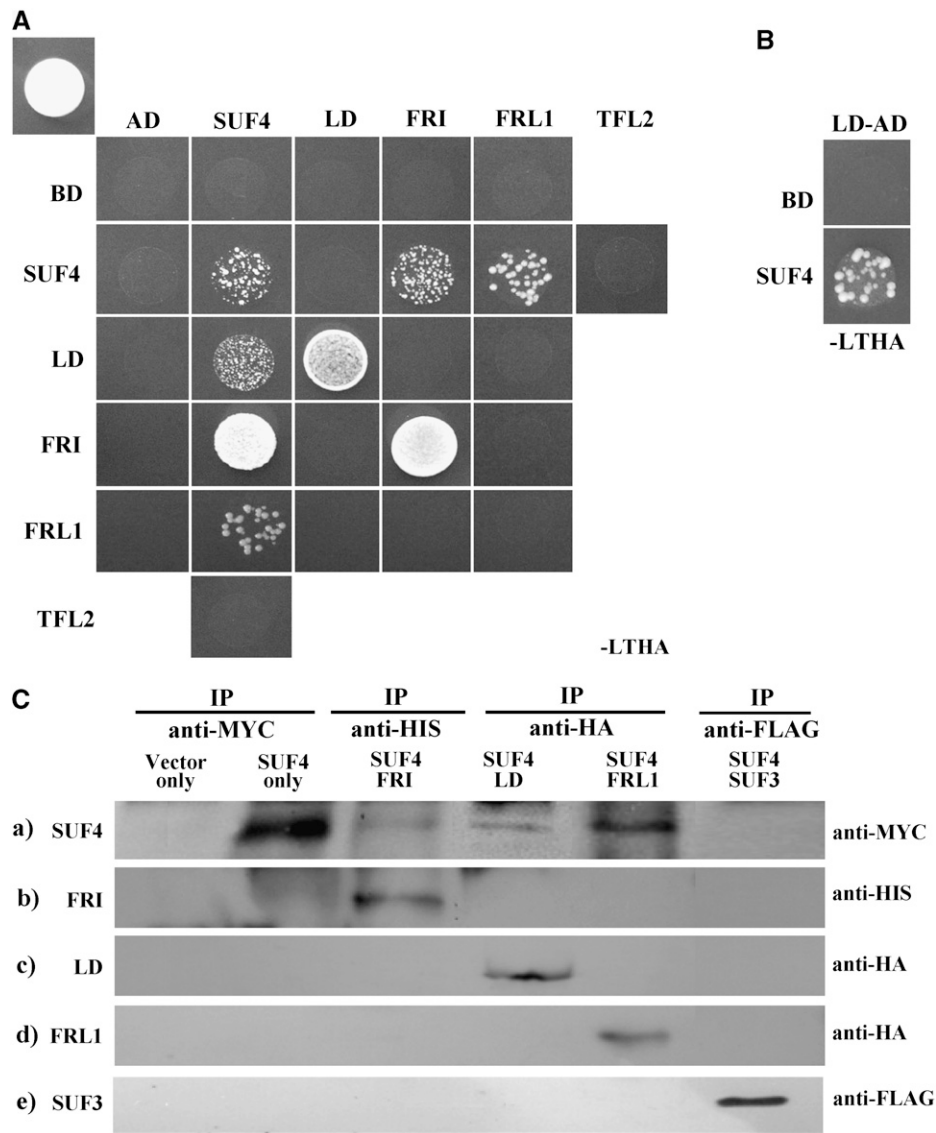
Chloroplasts appear red or blue (pseudocolor). GFP or YFP fluorescence is green, and RFP is red. All are projections. Bars = 10  $\mu$ m.

(A) Protoplast expressing SUF4:GFP.

(B) Protoplast expressing SUF4:RFP.

(C) Protoplast expressing YFP:FRI.

(D) Protoplast expressing YFP:LD.



**Figure 6.** Interactions among FRI, FRL1, LD, and SUF4 Proteins.

**(A)** Yeast two-hybrid interaction analysis. A positive control harboring p53 in pGBKT7 and T-antigen in pGADT7 is shown at top left. The interaction between SUF4 and TFL2 is used as a negative control. Plate was incubated at 22°C for 6 d.

**(B)** Yeast cells harboring LD in GADT7 and SUF4 in pGBKT7 grew slowly, so they were visualized after growing for 12 d.

**(C)** Coimmunoprecipitation analysis after *SUF4:MYC* with *FRI:HIS*, *LD:HA*, *FRL1:HA*, or *ARP6:FLAG* were transiently expressed in tobacco. Vector only is for the MYC tag vector. Total proteins were extracted 2 d after infiltration with vector only, *SUF4:MYC* (SUF4 only), *SUF4:MYC* and *FRI:HIS* (SUF4 FRI), *SUF4:MYC* and *LD:HA* (SUF4 LD), *SUF4:MYC* and *FRL1:HA* (SUF4 FRL1), and *SUF4:MYC* and *ARP6:FLAG* (SUF4 ARP6) and then immunoprecipitated with anti-MYC antibody (lanes 1 and 2), anti-HIS antibody (lane 3), anti-HA antibody (lanes 4 and 5), and anti-FLAG antibody (lane 6). The immunoprecipitates were separated by a 9% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-MYC (a), anti-HIS (b), anti-HA (c and d), and anti-FLAG antibody (e).

*SUF4:MYC* with *ARP6:FLAG* coinfiltrated tissues, did not show coimmunoprecipitation of SUF4 and ARP6 (Figure 6C). By contrast, SUF4:MYC was detected in the anti-HIS or anti-HA immunoprecipitates repeatedly, demonstrating the direct physical interactions of SUF4 with FRI, FRL1, and LD in plant cells. Therefore, this suggests that FRI, FRL1, and SUF4 form a protein complex, and LD interacts with SUF4 for the regulation of *FLC*.

#### SUF4 Binds to the Chromatin of the *FLC* Promoter Region

The zinc finger motif in SUF4 is well known for binding to DNA. Therefore, we addressed the question of whether SUF4 interacts with *FLC* by chromatin immunoprecipitation (ChIP) assay, a method used to detect the physical interaction of a transcription factor with DNA (Johnson and Bresnick, 2002). For the ChIP



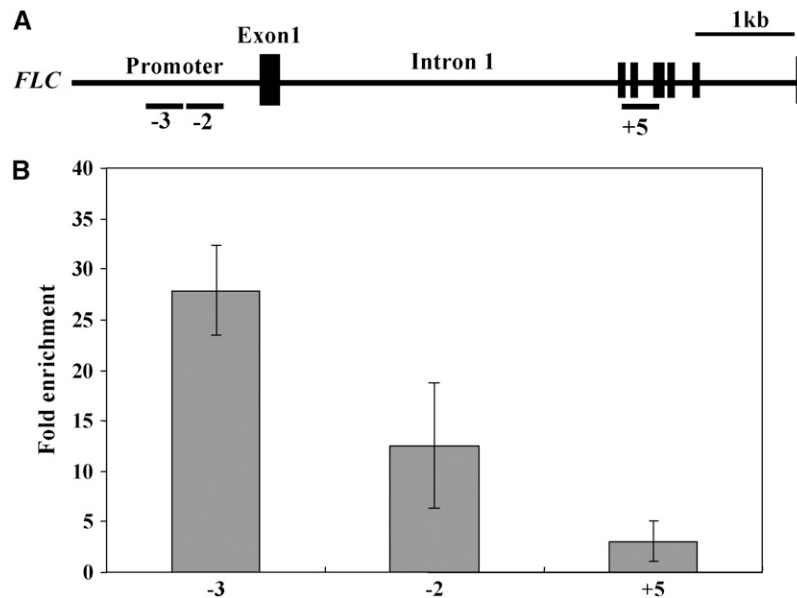
assay, we generated *35S-SUF4 $\alpha$ :MYC* transgenic lines in which the *35S-SUF4 $\alpha$ :MYC* protein was detected in expected size by protein gel blot analysis (data not shown). After immunoprecipitation with anti-MYC antibody, enrichment of *FLC* promoter region was detected by real-time quantitative PCR (Figure 7). Compared with the control of the wild type, a region of *FLC* promoter  $\sim$ 545 to 850 bp upstream from the transcription initiation site, detected by the *FLC*-3 primer set, was highly enriched in *35S-SUF4 $\alpha$ :MYC* (Figure 7). This region corresponds to the location of the positive *cis*-element in the *FLC* promoter reported previously (Sheldon et al., 2002). By contrast, reduced fold enrichment was detected in the promoter region of 248 to 560 bp upstream (*FLC*-2 region) close to the *FLC*-3 region. The *FLC*+5 region downstream of the first intron (Figure 7; Sheldon et al., 2002) showed much less enrichment. This result showed that SUF4 binds to the region around *FLC*-3 in vivo, suggesting that SUF4 is recruited to the promoter region of *FLC* for transcriptional activation.

## DISCUSSION

The vernalization requirement in *Arabidopsis* is established by the elevated expression of *FLC* that is caused by the presence of *FRI* or mutations in autonomous pathway genes (Michaels and Amasino, 2001). Although many genes regulating *FLC* expression have been identified, the molecular mechanism of *FLC* activation has not been resolved. We have isolated a rapid flowering mutant, *suf4*, that completely suppresses *FRI* activity in winter-annual strains; the *suf4* mutant showed the same pheno-

type as the *fri* mutant and *suf4 fri* double mutant. The *SUF4* gene encodes a ZP207 class zinc finger protein, of which a mammalian homolog was suggested as a transcription factor (Pahl et al., 1998). Our results showed that the SUF4 protein interacts with *FRI* and *FRL1* and binds to the *FLC* promoter in vivo. These results strongly suggest that SUF4 forms a protein complex with *FRI* and *FRL1* and functions as a transcriptional activator of *FLC*.

The *SUF4* gene encodes a protein with typical features of ZP207 class zinc finger proteins: a potential nuclear localization signal and two C2H2-type zinc finger domains that are spaced by two amino acids and a Pro-rich domain (Pahl et al., 1998; Taguchi et al., 1998; Bergqvist et al., 2006). ZP207 class zinc finger proteins are found in diverse organisms, including yeast, *Caenorhabditis elegans*, fruitfly, mouse, and human, and thus are highly conserved evolutionarily. However, the biological function of this class has not been demonstrated from any of the organisms. Although C2H2-type zinc finger modules are used for such a variety of functions as RNA packaging and protein-protein interaction, the most common role is to serve as DNA binding domains within transcription factors (Klug and Schwabe, 1995; Englbrecht et al., 2004). Specific high-affinity DNA binding requires a minimum of two fingers (Klug and Schwabe, 1995), but it has been reported that a single zinc finger is also capable of binding to DNA (Pedone et al., 1996). Pro-rich domains have also been found in many transcription factors, such as AP-2 and CTF/NF-1, and are involved in transcriptional activation (Williams and Tjian, 1991; Williamson, 1994). Indeed, SUF4 protein is localized in the nucleus (Figure 5), binds to the *FLC* promoter region in vivo



**Figure 7.** SUF4 Binding to the *FLC* Promoter Region.

Wild-type and *35S-SUF4 $\alpha$ :MYC* transgenic seedlings grown under short days were used for ChIP assay. Antibodies raised against MYC were used for immunoprecipitation, and ChIP products from wild-type and *35S-SUF4 $\alpha$ :MYC* transgenic seedlings were amplified by quantitative real-time PCR with primers in (A) to detect the enrichment of the *FLC* promoter region (B). *ACTIN* was used as an internal control to normalize the fold enrichment. Values represent means  $\pm$  SE from three independent ChIP experiments.

where positive regulatory element is located (Figure 7), and plays a role in transcriptional activation of *FLC* at least by genetic analysis.

Our results show that *FLC* is the major target of *SUF4* activity. The *suf4* mutation caused the decrease of *FLC* expression but not the expression of any of the *FLC*-clade floral repressors. It also did not affect the expression of *UFC*, a *FLC* neighboring gene, which is coordinately regulated with *FLC* by vernalization (Finnegan et al., 2004). In addition, both the *flc* mutation and *35S-FLC* were completely epistatic to *suf4*. Consistently, the *vip4* mutant that shows complete suppression of *FLC* was epistatic to *suf4*. Altogether, our results strongly suggest that the major function of *SUF4* is the activation of *FLC* transcription.

Two classes of genes have been reported that are required for the elevated expression of *FLC*. One class is required in both *FRI*-containing winter annuals and autonomous pathway mutants, but the other class is required only in the *FRI*-containing line for *FLC* activation. The components of the *Arabidopsis* PAF1 complex and *EFS*, the homolog of Set domain methyltransferase, are included in the first class (He et al., 2004; Oh et al., 2004; Kim et al., 2005; Zhao et al., 2005). This class has three interesting features. First, *FLC* expression is completely shut down in the mutants of this class, although *Col*, a *fri* null, shows a basal level expression of *FLC*. Second, the genes in this class are required for the expression of other floral repressors, such as *FLM* and *MAF2*. Third, as a result, the mutants in this class flower earlier than *flc*. By contrast, the second class, including *FRL1* and *FES1*, affects only the ability of *FRI* to elevate *FLC* expression; thus, the mutants in this class have a similar basal level expression of *FLC* and a similar flowering phenotype with *fri* (Michaels et al., 2004; Schmitz et al., 2005). In addition, these mutants do not suppress the elevated *FLC* expression and late flowering of autonomous pathway mutants. *suf4* possesses both features of the first and second classes. Similar to the second class, the *suf4* mutant has basal level expression of *FLC* and shows similar flowering phenotype with *fri*. Similar to *FRL1* or *FES1*, *SUF4* overexpression does not cause late flowering in *Col* background (Michaels et al., 2004; Schmitz et al., 2005). Thus, this indicates that *SUF4* activity is *FRI* dependent. However, unlike the second class, the *suf4* mutation at least partially suppresses the elevated expression of *FLC* in autonomous pathway mutants. Therefore, it is likely that *SUF4* function is distinct from the first class and may link the *FRI*-mediated activation of *FLC* and the autonomous pathway-mediated repression of *FLC*.

Previously, it has been suggested that *FRI*, *FRL1*, and *FES1* produce a protein complex to activate *FLC*, but the interactions among *FRI*, *FRL1*, and *FES1* have not been detected by yeast two-hybrid analysis (Schmitz et al., 2005). Our interaction analysis gives the answer for such discrepancy because *SUF4* acts as a missing link for the formation of the protein complex; *SUF4* interacts with both *FRI* and *FRL1*, although the interaction between *SUF4* and *FES1* has yet to be determined.

*SUF4* interacts not only with *FRI* but also with *LD*, a homeo-domain protein encoded by one of the autonomous pathway genes (Figure 6). Coincidentally, *suf4* not only suppresses *FRI* but also causes the strongest suppression in *ld* among autonomous pathway mutants analyzed (Table 1). In addition, *FRI* and *ld* show the strongest suppression by the *FLC-Ler* allele that has

the insertion of the transposable element in the first intron, causing transcriptional silencing through H3K9 methylation (Koorneef et al., 1994; Lee et al., 1994; Michaels et al., 2003; Liu et al., 2004). Thus, it is likely that *FRI* and *LD* activity is closely linked. Our results suggest that the molecular basis of such a link is the interactions of *SUF4* with *FRI* and *LD*.

Taken together, we propose a model for the transcriptional activation of *FLC*. In the presence of *FRI*, *SUF4* forms a protein complex consisting of *FRI*, *FRL1*, and probably *FES1* and activates *FLC* expression. In the absence of *FRI*, *SUF4* cannot maintain the protein complex and binds to *LD* instead. *LD* binding seems to cause suppression of *SUF4* activity because *Col* (i.e., the *fri* mutant) shows basal level expression of *FLC*, whereas the *ld* mutant shows strong activation of *FLC* maybe due to derepression of *SUF4*. Consistently, the *ld suf4* double mutant shows reduced expression of *FLC*.

The function of *SUF4* complex appears to be interdependent with that of the PAF1 complex homolog or *EFS*. The complete suppression of *FLC* by the mutations in the homolog of PAF1 complex or *EFS* in the presence of *SUF4* complex suggests that their activity is a prerequisite for *SUF4* activity for the transcriptional activation of *FLC* (He et al., 2004; Kim et al., 2005; Zhao et al., 2005). On the contrary, the *suf4* mutation causes reduced trimethylation of H3K4 (function of PAF1 complex homolog) and dimethylation of H3K36 (function of *EFS*) in *FLC* chromatin (see Supplemental Figure 5 online), showing that the *SUF4* activity is necessary for full activities of the PAF1 complex homolog and *EFS*. It is likely that PAF1 complex and *EFS* play a role in establishment and maintenance of the transcriptional state of *FLC*, while the *SUF4* complex plays a role in transcriptional activation of *FLC*.

In addition to *ld*, the *suf4* mutation partially suppresses other autonomous pathway mutants, such as *fve* and *fca* (Table 1). The molecular basis of such suppression is currently unknown. It may suggest that all of the autonomous pathway genes more or less affect the activity of *SUF4*. Alternatively, other autonomous pathway genes may act independently of *SUF4*; thus, they have an additive effect on *FLC* expression. Interestingly, the *fca fve suf4* triple mutant showed significantly later flowering than *FRI*-containing winter annuals (data not shown), although the *suf4* mutation partially suppresses both *fca* and *fve*. Such an additive effect supports the latter explanation. The molecular mechanism of this interaction may lead to further understanding of how the *FLC* gene is regulated.

## METHODS

### Plant Materials and Growth Conditions

The wild type used in this study was *Arabidopsis thaliana Col:FRI<sup>SF2</sup>* strain, which is a *Col* near-isogenic line described previously (Choi et al., 2005). The *suf4* T-DNA insertion line (*SALK\_056285*) was obtained from the SALK collection. Seeds were stratified on 0.65% phytoagar containing half-strength Murashige and Skoog (Plantmedia) salts for 3 d at 4°C. For vernalization, the Murashige and Skoog plates were incubated several weeks at 4°C under short-day conditions. Afterwards, plants were grown in long days (16 h light/8 h dark) or short days (8 h light/16 h dark) under cool white fluorescent lights (100  $\mu\text{mol}/\text{m}^2/\text{s}$ ) at 22°C with 60% relative humidity. Flowering time was measured by counting the number of rosette leaves from at least 20 plants.

### Mutagenesis and Cloning of *SUF4*

Fast-neutron mutagenesis and mutagenized populations of Col:*FRI*<sup>5F2</sup> strain have been described previously (Michaels and Amasino, 1999; Choi et al., 2005). *suf4* was selected among early-flowering mutants that flower as early as Col, and complementation analysis showed that it was a single allele. For the positional cloning of the *SUF4* gene, 1600 early flowering F2 progenies from the crosses between *suf4* and *Ler:FRI*<sup>5F2</sup> *FLC*<sup>5F2</sup> were obtained. Using molecular markers described by Lukowitz et al. (2000), rough mapping was obtained. Then, several SSLP and cleaved-amplified polymorphic sequence markers were made using the alignment program EditPlus 2 provided by the website ([http://www.ch.embnet.org/software/LALIGN\\_form.html](http://www.ch.embnet.org/software/LALIGN_form.html)) after extracting Col and *Ler* sequence (<http://www.arabidopsis.org/Cereon/index.jsp>). The sequences of primers for the markers made are shown in Supplemental Table 1 online.

### Plasmid Construction

To check if the three alternatively spliced transcripts could complement the early flowering of *suf4*, three differently sized cDNAs of *SUF4* were amplified by RT-PCR with forward primer (5'-GGGGGATCCATGGGTAA-GAAGAAGAAGAG-3') and reverse primer (5'-AAAGGATCCCTAAAC-GCCATCCGCCAGC-3'). The *Bam*HI fragment of each PCR product was cloned into pCambia1303-BS binary vector that contains the cauliflower mosaic virus 35S promoter and the NOS terminator (Jack et al., 1994).

For cellular localization experiments, yeast two-hybrid assays, and coimmunoprecipitation analyses, constructs were made using PCR fragments containing the open reading frame of each gene. The sequence information of primer sets for amplification of each cDNA and the proper vector for the plasmid construction are presented in Supplemental Table 2 online.

### Analysis of Gene Expression

RNA extraction and RNA gel blot analyses were performed as described previously (Choi et al., 2005). For the *SUF4*-specific probe, the digoxigenin-labeled mRNA probe prepared from pGEM-T Easy vector containing the  $\beta$  form of *SUF4* transcript was used. For RT-PCR, the primers *SUF4* forward (5'-TTCCTGGAGTCTGTTAG-3') and *SUF4* reverse (5'-GAGCATCATCAAGTG-3') were used. For quantification of GUS activity, MUG assay was performed as described (Blazquez et al., 1997) using 10 plants for each genotype. This assay was repeated three times.

### Protoplast Transient Expression Assay

*Arabidopsis* protoplasts were prepared as described (Sheen, 2002). The protoplasts expressing the GFP, RFP, and YFP fusion proteins were observed with a confocal laser scanning microscope equipped with an argon/krypton laser (Bio-Rad) as described (Choi et al., 2005). The resulting green and red images were overlaid and processed using Confocal Assistant 4.02 (Todd Clark Brejle) and Adobe Photoshop 6.0.

### Yeast Two-Hybrid Analysis

The vectors and yeast strains (Matchmaker GAL4 Two-Hybrid System 3) were obtained from Clontech. Yeast two-hybrid assay was performed according to the manufacturer's instructions. The appropriate plasmids were cotransformed into yeast strain AH109 using the lithium acetate method and selected on SD (synthetic drop) medium lacking Leu and Trp. After 4 d of incubation at 30°C, yeast cells were spotted on the selection plates containing SD medium lacking Leu, Trp, Ade, and His. These plates were incubated at 22°C until yeast cells were grown to form colonies.

### ChIP Assays

For ChIP, we generated the 35S-*SUF4* $\alpha$ -MYC transgenic line, which was made by the introduction of binary vector pKH34 into Col by the vacuum infiltration method. Wild-type and 35S-*SUF4* $\alpha$ -MYC seedlings grown under short-day conditions for 8 d were used for ChIP experiments. The procedures were followed according to the manufacturer's guide (Upstate). All experiments were done using triplicate biological samples. The antibody against MYC tag was used for immunoprecipitation, and ChIP products from wild-type and 35S-*SUF4* $\alpha$ -MYC seedlings were used for amplification of *FLC* genomic fragments by quantitative real-time PCR with the following primers: *FLC*-3 forward (5'-AAGAAATCTTAAATGTCC-3') and *FLC*-3 reverse (5'-TCGTTTATTGTGTACCATTCC-3'), *FLC*-2 forward (5'-ATTGCA-GAAAGAACCTCCAC-3') and *FLC*-2 reverse (5'-CTATTGCCATATGTG-TGGAC-3'), *FLC*+5 forward (5'-TGAAGCTCATGAAAGAGCGTT-3'), and *FLC*+5 reverse (5'-CAAGGTGTTCTCCAGTTGAA-3').

### Quantitative Real-Time PCR

Quantitative RT-PCR was performed as described with the use of SYBR-green probes (Leibfried et al., 2005). PCR product accumulation was monitored on an ABI PRISM 7300 sequence detection system (Applied Biosystems). *ACTIN* was used as an internal endogenous control to normalize the amount of target DNA. The wild type was used as a nonspecific binding control against 35S-*SUF4* $\alpha$ -MYC. All reactions were run in triplicates. The copy number of genomic fragments of *FLC* was calculated according to the 2 <sup>$\Delta\Delta$ Ct</sup> method (Livak and Schmittgen, 2001).

### Transient Expression in Tobacco and Coimmunoprecipitation Assay

All constructs were incorporated into the binary vector pCGN18 under the 35S promoter. Overnight culture (OD<sub>600</sub> of 0.5 to 1) of *Agrobacterium tumefaciens* transformed with these constructs was resuspended in 10 mM MgCl<sub>2</sub> and 150 mM acetosyringone. *Nicotiana benthamiana* plants were grown in a Magenta box at 22°C under short days until they had six leaves, and the youngest leaves >1 cm in length were infiltrated with *Agrobacterium* (Llave et al., 2000). The infiltrated plants were grown for 2 d in long days, and leaves were harvested and frozen in liquid nitrogen. Total proteins of each sample were prepared by grinding leaves in liquid nitrogen and extracting with 1 mL/three leaves extracting buffer containing 10 mM HEPES, pH 7.5, 10% glycerol (v/v), 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100 (v/v), 1 mM PMSF, and 2  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin A. The extract was centrifuged for 20 min at 13,000 rpm, and the supernatant was transferred to a new tube. The supernatant was precleared with 1/20 volume of protein A agarose beads (Upstate) for 1 h at 4°C. Each supernatant was then immunoprecipitated with anti-HA, anti-HIS, anti-MYC, and anti-FLAG according to proteins at 4°C overnight, followed by incubation with 1/10 volume of beads. After brief centrifugation, beads were washed twice each with buffer A (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) and buffer B (buffer A without NaCl), and then 45  $\mu$ L of SDS loading buffer was added. Protein gel blot analysis with anti-MYC antibody was performed to detect coimmunoprecipitated *SUF4*-MYC protein.

### Accession Numbers

*Arabidopsis* Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *SUF4* (At1g30970), *FRI* (At4g00650), *FRL1* (At5g16320), *LD* (At4g02560), and *FLC* (At5g10140).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Positional Cloning of *SUF4* and Deduced Amino Acid Sequence.

**Supplemental Figure 2.** Transcript Level of *SUF4* in *SUF4* Over-expression Lines.

**Supplemental Figure 3.** *SUF4* Expression in the Mutants of RNA Binding or Processing Genes.

**Supplemental Figure 4.** *SUF4* Expression in the Flowering Time Mutants.

**Supplemental Figure 5.** ChIP Analysis of Histone H3 Modifications at the *FLC* Locus.

**Supplemental Table 1.** SSLP and CAPS Markers Used in This Study.

**Supplemental Table 2.** Sequences of Primers Used for the Plasmid Construction.

## ACKNOWLEDGMENTS

We thank the ABRC for providing SALK\_056285 seeds; I. Hwang for p326-GFP, p326-RFP, and NLS-RFP; S. Michaels for sharing unpublished data; and R. Amasino for critical reading of the manuscript. This work was supported partially by the Korea Ministry of Science and Technology under the National Research Laboratory Program (2006-01952), Grant PF0330403-00 from the Plant Diversity Research Center, Grant R02-2003-000-10020-0 from the Basic Research Program of the Korea Science and Engineering Foundation, and a grant from the Korea Science and Engineering Foundation through the Plant Metabolism Research Center, Kyung Hee University. S.K., K.C., and C.P. were supported by the Brain Korea 21 program. This article is dedicated to I.L.'s beloved father, J.-B. Lee, who passed away while this article was being written.

Received June 23, 2006; revised October 17, 2006; accepted November 2, 2006; published November 30, 2006.

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