The Plant Cell, Vol. 18, 2985–2998, November 2006, www.plantcell.org © 2006 American Society of Plant Biologists

SUPPRESSOR OF FRIGIDA4, Encoding a C2H2-Type Zinc Finger Protein, Represses Flowering by Transcriptional Activation of *Arabidopsis FLOWERING LOCUS C* [W]

Sanghee Kim,[a] Kyuha Choi,[a] Chulmin Park,[a] Hyun-Ju Hwang,[a] and Ilha Lee[a,b,1]

[a] National Research Laboratory of Plant Developmental Genetics, Department of Biological Sciences, Seoul National University, Seoul 151-742, Korea

[b] Plant Metabolism Research Center, Kyung Hee University, Suwon 449-701, Korea

***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* (L*er*) 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

[1] To whom correspondence should be addressed. E-mail ilhalee@snu.ac.kr; fax 82-2-872-1993.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ilha Lee (ilhalee@snu.ac.kr).

[W] 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 *frl1* 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*, *frl1*, 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*^{SF2}) 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*^{SF2} was crossed to L*er*:*FRI*^{SF2}*FLC*^{SF2}, which was obtained by six backcrosses of San Feliu-2 to L*er* (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

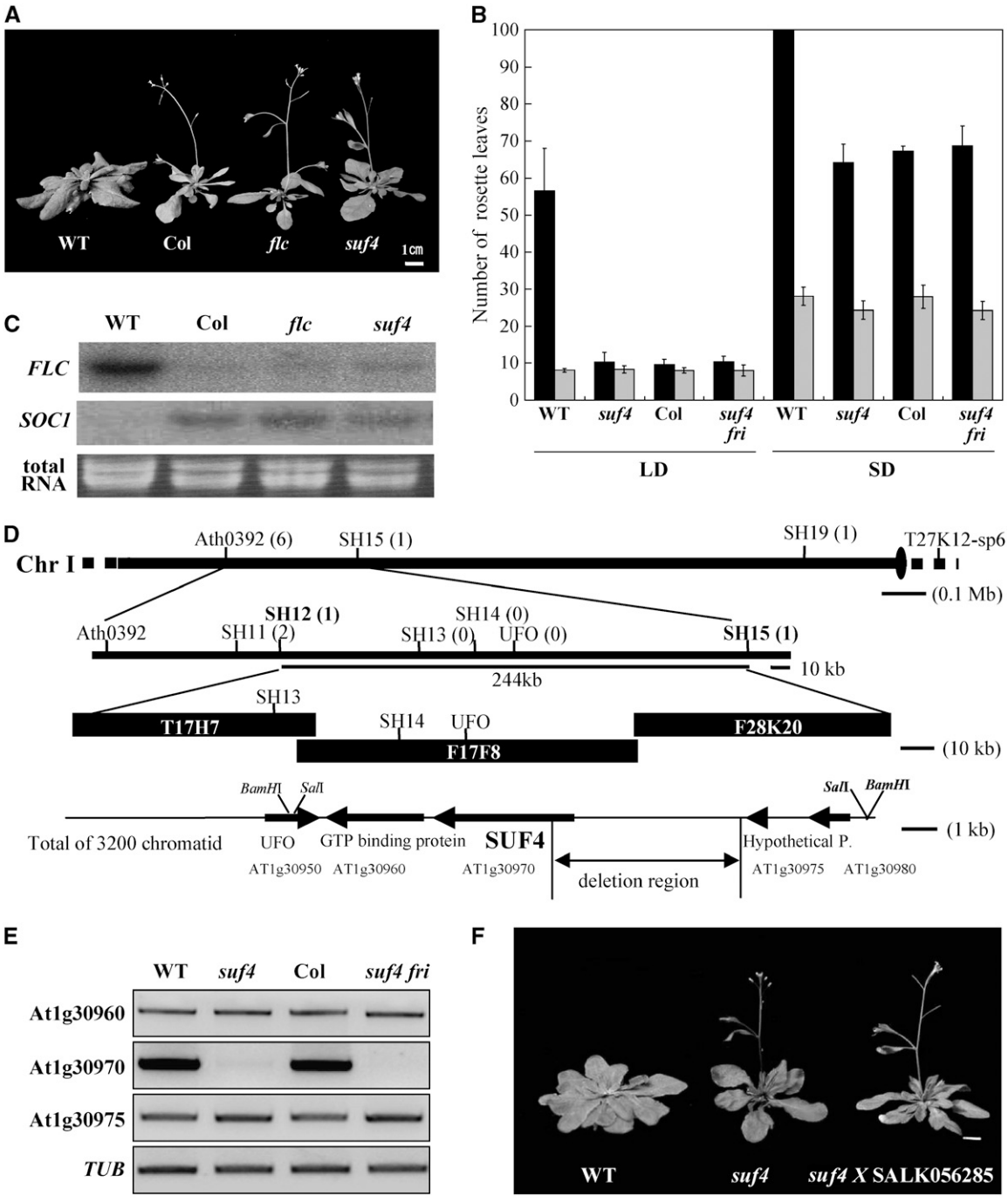

Figure 1. Mutant Characteristics and Positional Cloning of *SUF4*.

(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 ± 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:*FRI*^{SF2} background in LD	
Wild type	55.6 ± 4.03
fri	10.9 ± 0.72
suf4	10.5 ± 1.12
35S-FLC	61.5 ± 6.56
35S-FLC suf4	58.7 ± 7.45
vip4	7.4 ± 0.78
suf4 vip4	7.8 ± 0.62
Col background in LD	
Col	10.9 ± 0.72
suf4 fri	10.3 ± 0.83
flc-3	9.3 ± 1.06
suf4 flc-3	9.1 ± 1.10
fve-3	25.4 ± 1.71
suf4 fve-3	18.9 ± 2.08
fca-9	40.2 ± 2.48
suf4 fca-9	32.4 ± 3.08
ld-1	42.7 ± 3.22
suf4 ld-1	25.8 ± 2.45
soc1-2	15.0 ± 0.92
suf4 soc1-2	15.8 ± 1.49
ft-1	25.2 ± 4.02
suf4 ft-1	29.5 ± 3.40
co-1	17.5 ± 2.00
suf4 co-1	19.6 ± 2.61
Col background in SD	
Col	67.2 ± 1.52
suf4 fri	68.7 ± 3.72
flc-3	45.4 ± 2.75
suf4 flc-3	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 homologous 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 α, β, and γ, respectively. The sequencing of individual RT-PCR products showed that β and γ forms were derived from incomplete RNA processing; the last three introns remained in the γ form, whereas the last intron remained in the β 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 γ form was higher than the α 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 α, β, or γ in *suf4* showed similar late flowering as the wild type (Figure 2E). When the transgenic lines overexpressing the γ form and the β form were analyzed by RT-PCR, only the smallest transcript was overexpressed (see Supplemental Figure 2 online). Thus, the smallest α form is most likely functional, and the β and γ transcripts are intermediate forms that have not completed the splicing process. When α, β, or γ 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

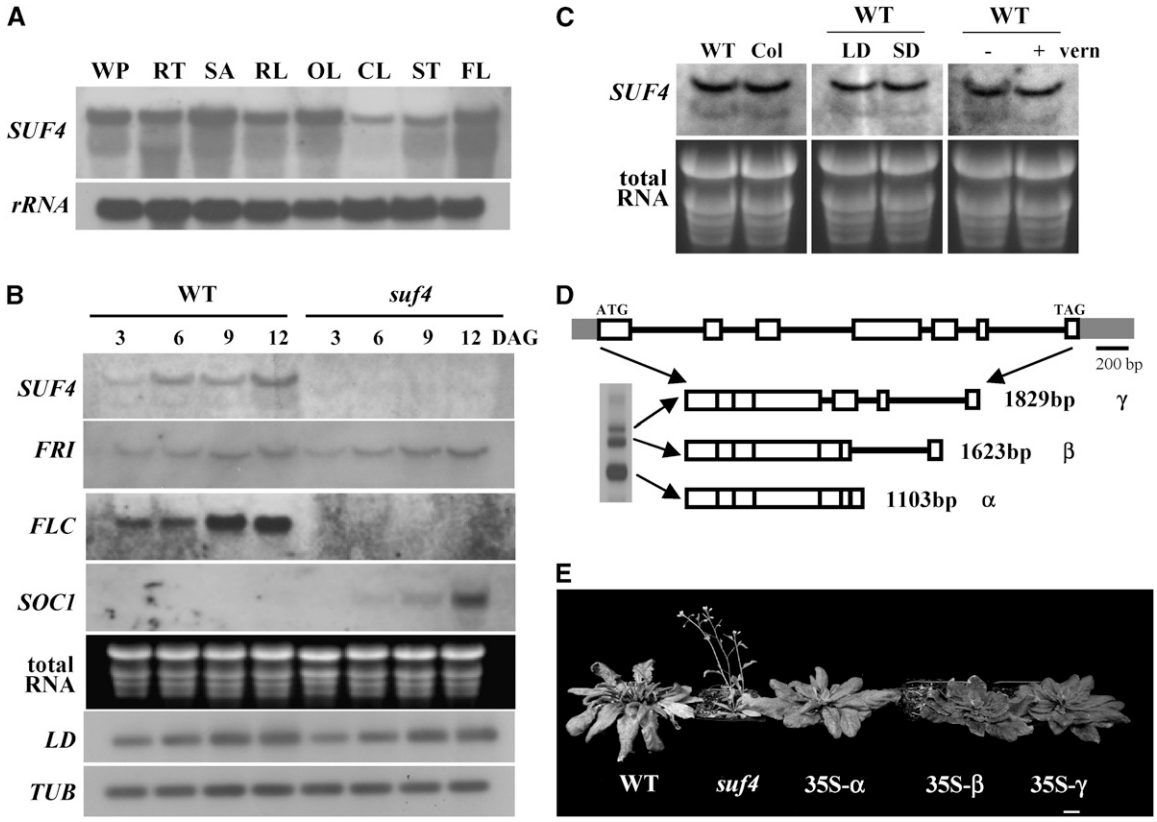

Figure 2. Expression Pattern of *SUF4*.

(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*-β-*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).

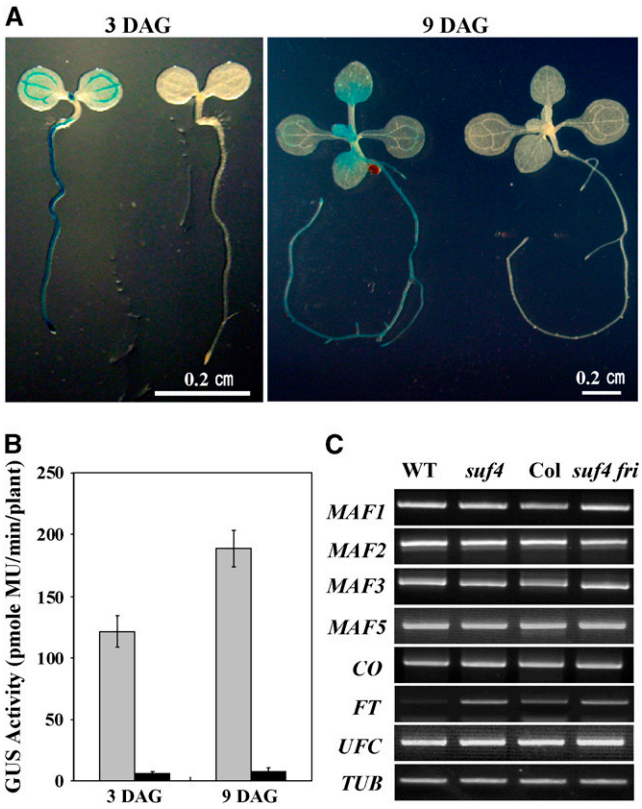

Figure 3. Effect of *suf4* on the Expression of *FLC* and Other Flowering Time Genes.

(A) *FLC* expression is suppressed in *suf4* mutants. In each panel, left is *FLC-GUS* in *flc-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 flc-3* (gray bars). Bars indicate means ± 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 flc-3* double mutant with those of *suf4* and *flc-3* (a null allele) single mutants (Table 1). The *flc-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 flc-3* double mutant flowered at the same time with *flc-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 *flc* (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

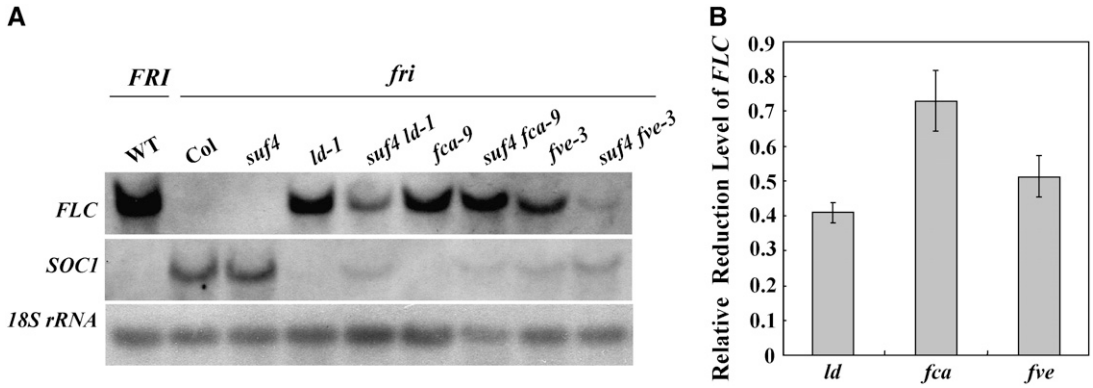

Figure 4. *FLC* and *SOC1* Expression in Double Mutants of *suf4* with Autonomous Pathway Mutants.

(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 shows mean values ± 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,

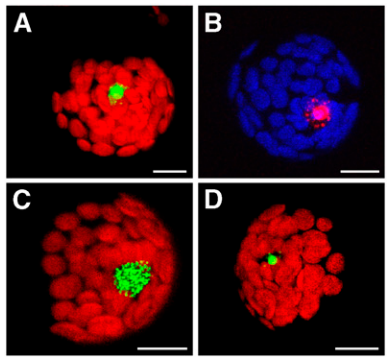

Figure 5. Nuclear Localization of SUF4:GFP, SUF4:RFP, YFP:FRI, and YFP:LD Proteins in *Arabidopsis* Protoplasts.

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

(A) Protoplast expressing SUF4:GFP.

(B) Protoplast expressing SUF4:RFP.

(C) Protoplast expressing YFP:FRI.

(D) Protoplast expressing YFP:LD.

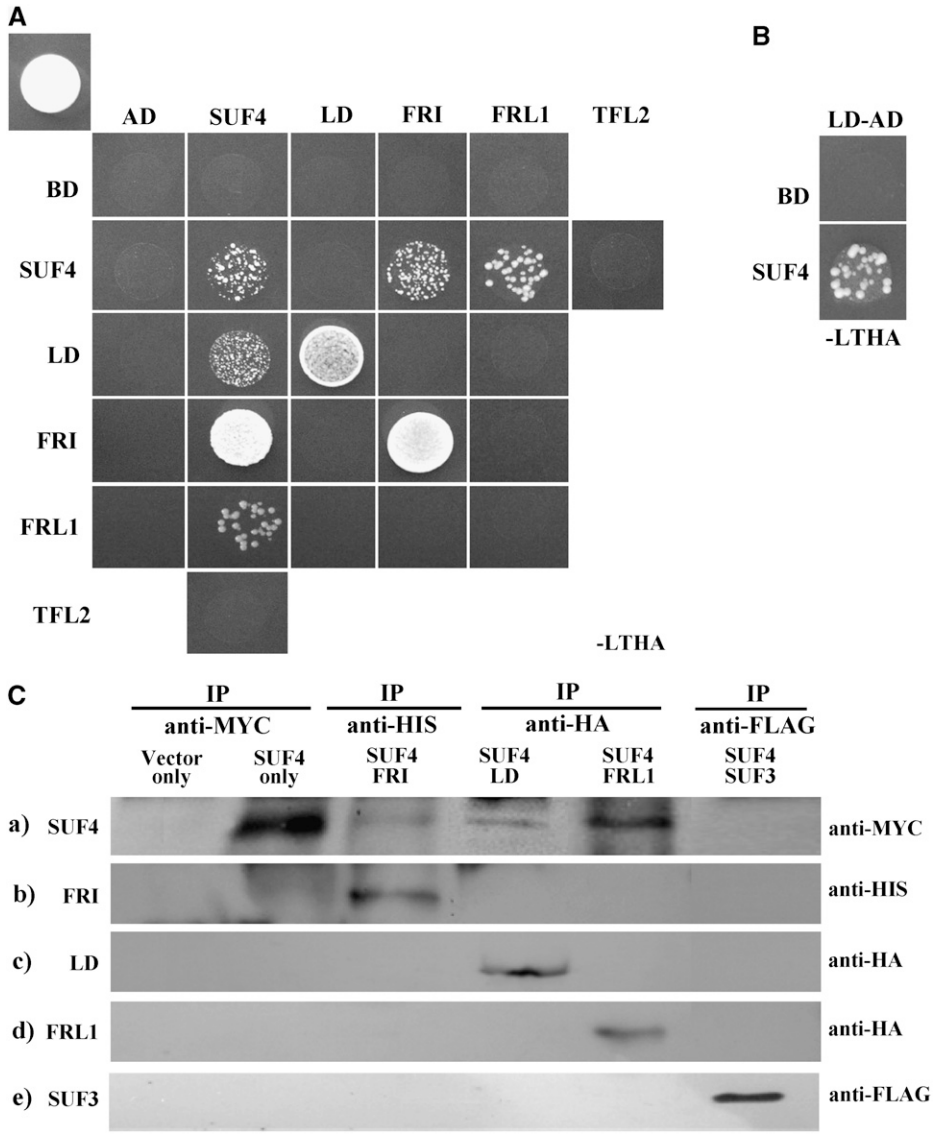

Figure 6. Interactions among FRI, FRL1, LD, and SUF4 Proteins.

(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-SUF4α:MYC* transgenic lines in which the 35S-SUF4α: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 ~545 to 850 bp upstream from the transcription initiation site, detected by the *FLC-3* primer set, was highly enriched in *35S-SUF4α: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 phenotype 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

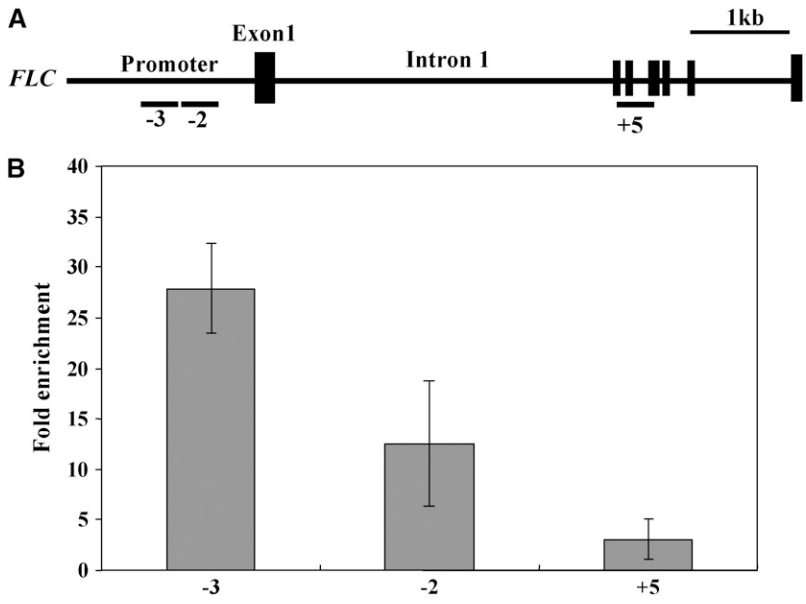

Figure 7. SUF4 Binding to the *FLC* Promoter Region.

Wild-type and *35S-SUF4α-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-SUF4α-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 ± 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 homeodomain 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*-L*er* allele that has the insertion of the transposable element in the first intron, causing transcriptional silencing through H3K9 methylation (Koornneef 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*^{SF2} 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 μmol/m^2/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*^{SF2} 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 L*er:FRI*^{SF2} *FLC*^{SF2} 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) after extracting Col and L*er* 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′-GGGGGATCCATGGGTAAGAAGAAGAAGAG-3′) and reverse primer (5′-AAAGGATCCCTAAAACGCCATCCGCCCAGC-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 β form of *SUF4* transcript was used. For RT-PCR, the primers SUF4 forward (5′-TTCCTGGAGTCTGTTAG-3′) and SUF4 reverse (5′-GAGCATCATCATCAAGTG-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 Brelje) 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-SUF4α-MYC* transgenic line, which was made by the introduction of binary vector pKH34 into Col by the vacuum infiltration method. Wild-type and *35S-SUF4α-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-SUF4α-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′-TCGTTTATTGTGTTACCATTC-3′), FLC-2 forward (5′-ATTGCAGAAAGAACCTCCAC-3′) and FLC-2 reverse (5′-CTATTGCCATATGTGTGGAC-3′), FLC+5 forward (5′-TGAACTCATGAAAGAGGCGTT-3′), and FLC+5 reverse (5′-CAAGGTGTTCCTCCAGTTGAA-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-SUF4α-MYC*. All reactions were run in triplicates. The copy number of genomic fragments of *FLC* was calculated according to the $2^{\Delta\Delta Ct}$ 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_{600} of 0.5 to 1) of *Agrobacterium tumefaciens* transformed with these constructs was resuspended in 10 mM $MgCl_2$ 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 μg/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 μL 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* Overexpression 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.

REFERENCES

Aukerman, M.J., Lee, I., Weigel, D., and Amasino, R.M. (1999). The Arabidopsis flowering-time gene LUMINIDEPENDENS is expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates LEAFY expression. Plant J. **18,** 195–203.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat. Genet. **36,** 162–166.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004). Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature **427,** 164–167.

Bergqvist, M., Brattstrom, D., Brodin, D., Lindkvist, A., Dahlman-Wright, K., Dreilich, M., Wagenius, G., and Paulsson-Karlsson, Y. (2006). Genes associated with telomerase activity levels in esophageal carcinoma cell lines. Dis. Esophagus **19,** 20–23.

Blazquez, M., Soowal, L., Lee, I., and Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis.* Development **124,** 3835–3844.

Blázquez, M.A., and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. Nature **404,** 889–892.

Chanvivattana, Y., Bishop, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development **131,** 5263–5276.

Choi, K., Kim, S., Kim, S.Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.G., Michaels, S., and Lee, I. (2005). SUPPRESSOR OF FRIGIDA3 encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in Arabidopsis. Plant Cell **17,** 2647–2660.

Deal, R.B., Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. (2005). The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of FLOWERING LOCUS C expression and repression of flowering in Arabidopsis. Plant Cell **17,** 2633–2646.

Englbrecht, C.C., Schoof, H., and Bohm, S. (2004). Conservation, diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. BMC Genomics **5,** 39.

Finnegan, E.J., Sheldon, C.C., Jardinaud, F., Peacock, W.J., and Dennis, E.S. (2004). A cluster of Arabidopsis genes with a coordinate response to an environmental stimulus. Curr. Biol. **14,** 911–916.

Fischer, R., Vaquero-Martin, C., Sack, M., Drossard, J., Emans, N., and Commandeur, U. (1999). Towards molecular farming in the future: Transient protein expression in plants. Biotechnol. Appl. Biochem. **30,** 113–116.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, G., and Grandjean, O. (2001). Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis.* Development **128,** 4847–4858.

Gazzani, S., Gendall, A.R., Lister, C., and Dean, C. (2003). Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. Plant Physiol. **132,** 1107–1114.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001). The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in *Arabidopsis.* Cell **107,** 525–535.

He, Y., Doyle, M.R., and Amasino, R.M. (2004). PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis.* Genes Dev. **18,** 2774–2784.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science **302,** 1751–1754.

Jack, T., Fox, G.L., and Meyerowitz, E.M. (1994). Arabidopsis homeotic gene *APETALA3* ectopic expression: Transcriptional and posttranscriptional regulation determine floral organ identity. Cell **76,** 703–716.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science **290,** 344–347.

Johnson, K.D., and Bresnick, E.H. (2002). Dissecting long-range transcriptional mechanisms by chromatin immunoprecipitation. Methods **26,** 27–36.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.S., Michaels, S., and Amasino, R. (2005). Establishment of the vernalization-responsive, winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. Plant Cell **17,** 3301–3310.

Klug, A., and Schwabe, J.W. (1995). Protein motifs 5. Zinc fingers. FASEB J. **9,** 597–604.

Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. Plant J. **6,** 911–919.

Kotake, T., Takada, S., Nakahigashi, K., Ohto, M., and Goto, K. (2003). *Arabidopsis TERMINAL FLOWER 2* gene encodes a heterochromatin protein 1 homolog and represses both *FLOWERING LOCUS T* to regulate flowering time and several floral homeotic genes. Plant Cell Physiol. **44,** 555–564.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. **16,** 2893–2905.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000). The *AGAMOUS-LIKE 20* MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. **14,** 2366–2376.

Lee, I., and Amasino, R.M. (1995). Effect of vernalization, photoperiod, and light quality on the flowering phenotype of Arabidopsis plants containing the *FRIGIDA* gene. Plant Physiol. **108,** 157–162.

Lee, I., Michaels, S.D., Masshardt, A.S., and Amasino, R.M. (1994). The late-flowering phenotype of *FRIGIDA* and *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of Arabidopsis. Plant J. **6,** 903–909.

Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature **438,** 1172–1175.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002). Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science **297,** 243–246.

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Kim, J., Hong, C.B., Kim, H.J., and Park, C.M. (2004). A new Arabidopsis gene, FLK, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. Plant Cell **16,** 731–740.

Liu, J., He, Y., Amasino, R., and Chen, X. (2004). siRNAs targeting an intronic transposon in the regulation of natural flowering behavior in Arabidopsis. Genes Dev. **18,** 2873–2878.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods **25,** 402–408.

Llave, C., Kasschau, K.D., and Carrington, J.C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc. Natl. Acad. Sci. USA **97,** 13401–13406.

Lukowitz, W., Gillmor, C.S., and Scheible, W.R. (2000). Positional cloning in Arabidopsis. Plant Physiol. **123,** 795–805.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89,** 737–745.

Martin-Trillo, M., Lazaro, A., Poethig, R.S., Gomez-Mena, C., Pineiro, M.A., Martinez-Zapater, J.M., and Jarillo, J.A. (2006). EARLY IN SHORT DAYS 1 (ESD1) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates FLC accumulation in Arabidopsis. Development **133,** 1241–1252.

Michaels, S.D., and Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949–956.

Michaels, S.D., and Amasino, R.M. (2001). Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell **13,** 935–941.

Michaels, S.D., Bezerra, I.C., and Amasino, R.M. (2004). FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. Proc. Natl. Acad. Sci. USA **101,** 3281–3285.

Michaels, S.D., He, Y., Scortecci, K.C., and Amasino, R.M. (2003). Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. Proc. Natl. Acad. Sci. USA **100,** 10102–10107.

Napp-Zinn, K. (1985). *Arabidopsis thaliana.* In CRC Handbook of Flowering, H.A. Halevy, ed (Boca Raton, FL: CRC Press), pp. 492–503.

Noh, Y.S., and Amasino, R.M. (2003). *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell **15,** 1671–1682.

Oh, S., Zhang, H., Ludwig, P., and van Nocker, S. (2004). A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis *FLC/MAF* MADS box gene family. Plant Cell **16,** 2940–2953.

Pahl, P.M., Hodges, Y.K., Meltesen, L., Perryman, M.B., Horwitz, K.B., and Horwitz, L.D. (1998). ZNF207, a ubiquitously expressed zinc finger gene on chromosome 6p21.3. Genomics **53,** 410–412.

Pedone, P.V., Ghirlando, R., Clore, G.M., Gronenborn, A.M., Felsenfeld, G., and Omichinski, J.G. (1996). The single-Cys_2-His_2 zinc finger domain of the GATA protein flanked by basic residues is sufficient for high-affinity specific DNA binding. Proc. Natl. Acad. Sci. USA **93,** 2822–2826.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., and Riechmann, J.L. (2003). Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. Plant Cell **15,** 1159–1169.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., and Riechmann, J.L. (2001). Regulation of flowering in *Arabidopsis* by an *FLC* homologue. Plant Physiol. **126,** 122–132.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis.* Science **288,** 1613–1618.

Schmitz, R.J., Hong, L., Michaels, S., and Amasino, R.M. (2005). FRIGIDA-ESSENTIAL 1 interacts genetically with FRIGIDA and FRIGIDA-LIKE 1 to promote the winter-annual habit of *Arabidopsis thaliana.* Development **132,** 5471–5478.

Schomburg, F.M., Patton, D.A., Meinke, D.W., and Amasino, R.M. (2001). FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. Plant Cell **13,** 1427–1436.

Scortecci, K., Michaels, S.D., and Amasino, R.M. (2003). Genetic interactions between *FLM* and other flowering-time genes in *Arabidopsis thaliana.* Plant Mol. Biol. **52,** 915–922.

Sheen, J. (2002). A transient expression assay using *Arabidopsis* mesophyll protoplasts. http://genetics.mgh.harvard.edu/sheenweb/.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The *FLF* MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11,** 445–458.

Sheldon, C.C., Conn, A.B., Dennis, E.S., and Peacock, W.J. (2002). Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. Plant Cell **14,** 2527–2537.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (2000). The molecular basis of vernalization: The central role of *FLOWERING LOCUS C* (*FLC*). Proc. Natl. Acad. Sci. USA **97,** 3753–3758.

Simpson, G.G., and Dean, C. (2002). *Arabidopsis*, the Rosetta stone of flowering time? Science **296,** 285–289.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., and Dean, C. (2003). FY is an RNA 3′-end processing factor that interacts with FCA to control the Arabidopsis floral transition. Cell **113,** 777–787.

Soppe, W.J., Bentsink, L., and Koornneef, M. (1999). The early-flowering mutant efs is involved in the autonomous promotion pathway of *Arabidopsis thaliana.* Development **126,** 4763–4770.

Sung, S., and Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature **427,** 159–164.

Taguchi, E., Muramatsu, H., Fan, Q.W., Kurosawa, N., Sobue, G., and Muramatsu, T. (1998). Zep: A novel zinc finger protein containing a large proline-rich domain. J. Biochem. (Tokyo) **124,** 1220–1228.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. **33,** 949–956.

Williams, T., and Tjian, R. (1991). Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev. **5,** 670–682.

Williamson, M.P. (1994). The structure and function of proline-rich regions in proteins. Biochem. J. **297,** 249–260.

Zhang, H., and van Nocker, S. (2002). The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of *FLOWERING LOCUS C.* Plant J. **31,** 663–667.

Zhao, Z., Yu, Y., Meyer, D., Wu, C., and Shen, W.H. (2005). Prevention of early flowering by expression of FLOWERING LOCUS C requires methylation of histone H3 K36. Nat. Cell Biol. **7,** 1256–1260.