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

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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. 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 OVEREXPRESSSION 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 fcl 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...
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 and Amasino, 1999; Choi 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, frit, 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<sup>FRI</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>FRI</sup> was crossed to Ler:FRI<sup>FRI</sup>FLC<sup>SIP2</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 ± 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of Rosette Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Col:FRI</strong>&lt;sub&gt;GF2&lt;/sub&gt; background in LD</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>55.6 ± 4.03</td>
</tr>
<tr>
<td>fri</td>
<td>10.9 ± 0.72</td>
</tr>
<tr>
<td>suf4</td>
<td>10.5 ± 1.12</td>
</tr>
<tr>
<td>3SS-FLC</td>
<td>61.5 ± 6.56</td>
</tr>
<tr>
<td>3SS-FLC suf4</td>
<td>58.7 ± 7.45</td>
</tr>
<tr>
<td>vip4</td>
<td>7.4 ± 0.78</td>
</tr>
<tr>
<td>suf4 vip4</td>
<td>7.8 ± 0.62</td>
</tr>
<tr>
<td><strong>Col</strong> background in LD</td>
<td></td>
</tr>
<tr>
<td>Col</td>
<td>10.9 ± 0.72</td>
</tr>
<tr>
<td>suf4 fri</td>
<td>10.3 ± 0.83</td>
</tr>
<tr>
<td>fcl-3</td>
<td>9.3 ± 1.06</td>
</tr>
<tr>
<td>suf4 fcl-3</td>
<td>9.1 ± 1.10</td>
</tr>
<tr>
<td>fve-3</td>
<td>25.4 ± 1.71</td>
</tr>
<tr>
<td>suf4 fve-3</td>
<td>18.9 ± 2.08</td>
</tr>
<tr>
<td>fca-9</td>
<td>40.2 ± 2.48</td>
</tr>
<tr>
<td>suf4 fca-9</td>
<td>32.4 ± 3.08</td>
</tr>
<tr>
<td>id-1</td>
<td>42.7 ± 3.22</td>
</tr>
<tr>
<td>suf4 ld-1</td>
<td>25.8 ± 2.45</td>
</tr>
<tr>
<td>soc1-2</td>
<td>15.0 ± 0.92</td>
</tr>
<tr>
<td>suf4 soc1-2</td>
<td>15.8 ± 1.49</td>
</tr>
<tr>
<td>ft-1</td>
<td>25.2 ± 4.02</td>
</tr>
<tr>
<td>suf4 ft-1</td>
<td>29.5 ± 3.40</td>
</tr>
<tr>
<td>co-1</td>
<td>17.5 ± 2.00</td>
</tr>
<tr>
<td>suf4 co-1</td>
<td>19.6 ± 2.61</td>
</tr>
<tr>
<td><strong>Col</strong> background in SD</td>
<td></td>
</tr>
<tr>
<td>Col</td>
<td>67.2 ± 1.52</td>
</tr>
<tr>
<td>suf4 fri</td>
<td>68.7 ± 3.72</td>
</tr>
<tr>
<td>fcl-3</td>
<td>45.4 ± 2.75</td>
</tr>
<tr>
<td>suf4 fcl-3</td>
<td>45.1 ± 2.71</td>
</tr>
</tbody>
</table>

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.

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 3SS 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
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-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).
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; 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, 3SS-FLC was epistatic to suf4; 3SS-FLC suf4 showed similar flowering time with 3SS-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 id-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 id-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 id, 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
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,
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.

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

(A) Yeast two-hybrid interaction analysis. A positive control harboring p53 in pGBK7 and T-antigen in pGAD77 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 GAD77 and SUF4 in pGBK7 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 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 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 (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:FRIF2 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/m2/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>8F2</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 F<sub>2</sub> progenies from the crosses between suf4 and Ler:FRI<sup>8F2</sup> FLC<sup>SI1</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.chembnet.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′-GGGGGATCCATGGTAA-GAAGAAGAGAG-3′) and reverse primer (5′-AAAGGATCCCTAAGAC-GCCATCCTCCAGC-3′). The BamHI 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′-TTCTGGAGAGTGTGGTAG-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 Confoocal Assistant 4.02 (Todd Clark Breijie) 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<sub>MYC</sub> transgenic line, which was made by the introduction of binary vector pKH34 into Col by the vacuum infiltration method. Wild-type and 35S-SUF4<sub>MYC</sub> 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<sub>MYC</sub> seedlings were used for amplification of FLC genomic fragments by quantitative real-time PCR with the following primers: FLC-3 forward (5′-AGAAATCTTAAGATGTC-3′) and FLC-3 reverse (5′-TCGTTATTGTGTTACCATC-3′), FLC-2 forward (5′-ATTGCA-GAAAGACCTCCACG-3′) and FLC-2 reverse (5′-CATTTGCGCATATGGTGACG-3′), FLC-1:5 forward (5′-TGAACTCATGAAAGCGGTT-3′), and FLC-1:5 reverse (5′-CAAGGTGTTCCTCCAGTGA-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 non-specific binding control against 35S-SUF4<sub>MYC</sub>. All reactions were run in triplicates. The copy number of genomic fragments of FLC was calculated according to the 2<sup>-△△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, 150 mM NaCl, 0.1% Triton X-100, 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 pre-cleared 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 (At5g10140), I (At4g16320), 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.

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SUF4 Activates FLC, a Floral Repressor 2997


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