The Plant Cell, Vol. 16, 2601–2613, October 2004, www.plantcell.org © 2004 American Society of Plant Biologists

Divergent Roles of a Pair of Homologous Jumonji/ Zinc-Finger–Class Transcription Factor Proteins in the Regulation of Arabidopsis Flowering Time

Bosl Noh,[a,b,1] Seung-Hee Lee,[a] Hyun-Jin Kim,[a] Gibum Yi,[a,b] Eun-Ah Shin,[a,b] Mirha Lee,[a] Kyung-Ja Jung,[a] Mark R. Doyle,[c] Richard M. Amasino,[c] and Yoo-Sun Noh[a,b,c,1]

[a] Kumho Life and Environmental Science Laboratory, Puk-Gu, Kwangju 500-712, Korea
[b] Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea
[c] Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706-1544

Flowering in *Arabidopsis thaliana* is controlled by multiple pathways, including the photoperiod pathway and the *FLOWERING LOCUS C* (*FLC*)-dependent pathway. Here, we report that a pair of related jumonji-class transcription factors, EARLY FLOWERING 6 (ELF6) and RELATIVE OF EARLY FLOWERING 6 (REF6), play divergent roles in the regulation of Arabidopsis flowering. ELF6 acts as a repressor in the photoperiod pathway, whereas REF6, which has the highest similarity to ELF6, is an *FLC* repressor. Ectopic expression studies and expression pattern analyses show that ELF6 and REF6 have different cellular roles and are also regulated differentially despite their sequence similarities. Repression of *FLC* expression by REF6 accompanies histone modifications in *FLC* chromatin, indicating that the transcriptional regulatory activity of this class of proteins includes chromatin remodeling. This report demonstrates the in vivo functions of this class of proteins in higher eukaryotes.

INTRODUCTION

Flowering is a major developmental switch in plants and involves a transition from vegetative to reproductive growth. Because reproductive success of plants could be significantly affected by flowering time, each plant species has developed optimum strategies for its flowering time regulation.

Molecular genetic studies have identified four major floral regulatory pathways in *Arabidopsis thaliana* (for reviews, see Mouradov et al., 2002; Simpson and Dean, 2002). Two of these pathways that are involved in the interpretation of environmental cues are the photoperiod and vernalization pathways. The photoperiod pathway regulates flowering in response to daylength. In Arabidopsis, long days promote flowering (Koornneef et al., 1998). Thus, mutations in photoperiod pathway genes (e.g., *gigantea* [*gi*], *constans* [*co*], *fd*, *fe*, *cryptochrome 2/fha* [*cry2*], *flowering locus t* [*ft*], and *fwa*) blind Arabidopsis such that it does not recognize long days as an inductive photoperiod, resulting in delayed flowering in long days but not in short days. *GI* and *CRY2* act upstream of *CO*, which mediates between the circadian clock and the control of flowering time (Suarez-Lopez et al., 2001). *FT* is an early target gene of CO (Samach et al., 2000), and *FT* mRNA levels correlate with CO protein levels (Valverde et al., 2004).

Vernalization confers competence to flowers after an extended cold treatment, thus preventing premature flowering in the fall (Michaels and Amasino, 2000; Henderson et al., 2003). The winter-annual habit is conferred by dominant alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). FLC is a MADS box transcription factor that represses flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* expression is increased to levels that delay flowering by FRI, a novel protein with two coiled-coil domains (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). A large portion of the promotion of flowering by vernalization is achieved through the down-regulation of *FLC* mRNA levels. However, some portion of the vernalization effect is also achieved through *FLC*-independent pathways as evidenced from the vernalization responsiveness of an *flc* null mutant (Michaels and Amasino, 2001).

A major role of the autonomous pathway is to repress *FLC* expression. Arabidopsis plants with mutations in autonomous-pathway genes (*fca*, *fld*, *fpa*, *fve*, *fy*, and *ld*) have elevated levels of *FLC* expression and mimic the late-flowering behavior of *FRI*-containing accessions (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999). Like *FRI*-containing accessions, the elevated expression of *FLC* and the late flowering of the autonomous-pathway mutants are rapidly repressed by vernalization. Unlike photoperiod pathway late-flowering mutants, autonomous-pathway mutants are delayed in flowering under all daylengths but still respond to photoperiod by flowering earlier in long days than in short days (e.g., Mouradov et al., 2002; Simpson and Dean, 2002).

[1] To whom correspondence should be addressed. E-mail noh@kkpc.com or bnoh@kkpc.com; fax 82-62-972-5085.
The authors 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) are: Bosl Noh (bnoh@kkpc.com) and Yoo-Sun Noh (noh@kkpc.com).
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.104.025353.

The gibberellin (GA) pathway acts as a default floral promotion pathway in noninductive short days. Mutations that reduce GA levels or GA perception (e.g., *ga1* or *gai*) cause an extreme delay in flowering in short days but have a minor effect in long days (Wilson et al., 1992). GA-induced floral promotion is mediated by *LEAFY* (*LFY*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Blázquez and Weigel, 2000; Moon et al., 2003).

Floral regulatory signals generated in the photoperiod, *FLC*-dependent, and GA-dependent pathways are integrated by a group of genes called floral integrators. So far, three genes that function as floral integrators have been identified: *FT*, *SOC1*, and *LFY* (reviewed in Simpson and Dean, 2002). *FT* and *SOC1* are able to integrate floral regulatory signals generated in the photoperiod and *FLC*-dependent pathways. *SOC1* is also involved in the integration of GA-dependent floral promotion signals (Moon et al., 2003). *LFY* integrates photoperiodic and GA-dependent signals through discrete *cis* elements in the promoter (Blázquez and Weigel, 2000).

Current understanding of the floral regulation pathways mentioned above has largely come from studies on mutations that cause late flowering (Mouradov et al., 2002; Simpson and Dean, 2002). However, many mutations that cause early flowering have also been described (for review, see Sung et al., 2003). Many of these mutations have been placed in the defined floral regulatory pathways, whereas others have not. One class of early-flowering mutations (e.g., *elf3*, *elf4*, and *toc1*) affects the photoperiodic floral regulatory pathway by disrupting circadian rhythms. Another class of early-flowering mutations (e.g., *elf5*, *pie1*, *esd4*,

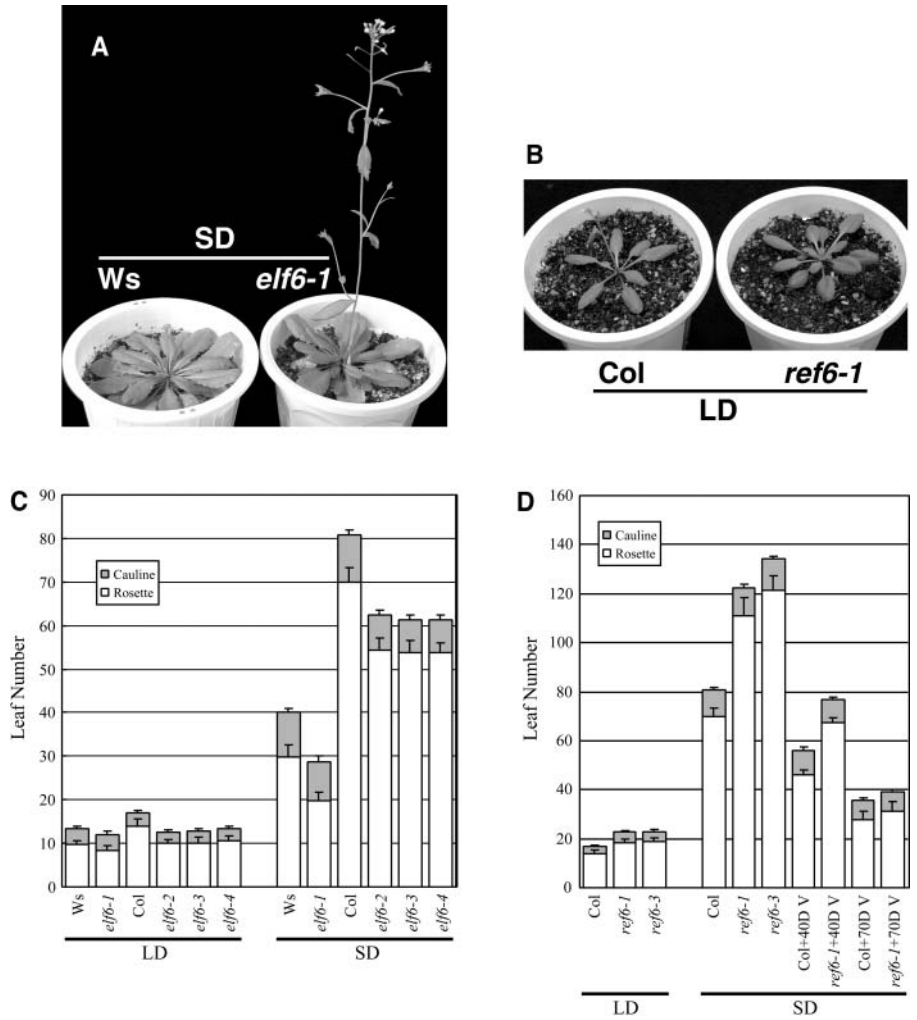

Figure 1. Early Flowering of *elf6* and Late Flowering of *ref6* Mutants Compared with the Wild Type.

(A) Wild-type (Ws) and *elf6-1* plants grown for 49 d under SD.

(B) Wild-type (Col) and *ref6-1* plants grown for 32 d under LD.

(C) Flowering time of *elf6* mutants. Wild-type and *elf6* mutants were grown under LD or SD, and their flowering times were measured as the number of rosette and cauline leaves formed by the primary meristem. Data shown are means ± SD of at least 12 plants for each genotype and treatment (see **[C]** and **[D]**).

(D) Flowering time of *ref6* mutants. Flowering time was measured as described in **(C)**. Col and *ref6-1* mutants were treated with vernalization as previously described (Noh and Amasino, 2003) for 40 (+40D V) or 70 d (+70D V).

and *efs*) causes a decrease in *FLC* expression, especially in genetic backgrounds in which *FLC* is highly expressed. However, these mutations also appear to affect *FLC*-independent floral regulation as the mutants flower earlier than an *flc* null mutant (Soppe et al., 1999; Reeves et al., 2002; Noh and Amasino, 2003; Noh et al., 2004).

In fact, many other early-flowering mutations do not fall into any of the defined floral regulatory pathways and appear to affect multiple flowering and developmental pathways (Sung et al., 2003). The genes affected by many of these mutations encode chromatin remodeling factors (e.g., *LHP1*/*TFL2*, *EMF1*, *EMF2*, *FIE*, *CLF*, and *EBS*) (Sung et al., 2003). Lesions in these genes often lead to the premature derepression of genes far downstream in floral regulatory pathways such as *FT* or floral meristem or floral organ identity genes such as *AP1* and *AG*. The early flowering caused derepression of such downstream genes is expected to be epistatic to lesions that delay flowering in multiple flowering pathways.

Here, we report the identification of a pair of homologous genes, *EARLY FLOWERING 6* (*ELF6*) and *RELATIVE OF EARLY FLOWERING 6* (*REF6*) that are involved in the regulation of flowering time in Arabidopsis. *ELF6* and *REF6* encode nuclear proteins with jumonji (Jmj) and zinc-finger (ZnF) domains and are most homologous to each other. Despite their structural similarities, *ELF6* and *REF6* have divergent roles in floral regulations: *ELF6* acts as an upstream repressor in the photoperiodic flowering pathway, whereas *REF6* is an *FLC* repressor. Spatial expression patterns of *ELF6* and *REF6* are also different and reflect their roles as repressors of the photoperiodic and the *FLC*-dependent floral regulatory pathways, respectively. Repression of *FLC* mRNA expression by REF6 involves histone modifications in *FLC* chromatin, suggesting a role of Jmj-domain proteins as chromatin remodelers. Therefore, our results demonstrate the in vivo functions of Jmj-domain proteins in higher eukaryotes as well as the identification and characterization of novel floral regulators in Arabidopsis.

RESULTS

Mutations in *ELF6* Cause Early Flowering, whereas Mutations in *REF6* Cause Late Flowering

The *elf6* mutant was identified in a screen for early-flowering mutants as described previously (Noh and Amasino, 2003; Noh et al., 2004). *elf6-1* displayed an earlier floral transition compared with the wild-type Wassilewskija (Ws) in long days (LD; 16 h of light and 8 h of dark) as well as in noninductive short days (SD; 8 h of light and 16 h of dark; Figures 1A and 1C). The early-flowering phenotype of *elf6-1* segregated in a recessive manner (data not shown). Except for the early-flowering phenotypes, *elf6* mutants displayed normal development of all organs throughout the entire life cycle, suggesting that *ELF6* might play a specific role in flowering time regulations. Because *elf6-1* was isolated from a T-DNA insertion population, an effort to obtain the sequence flanking T-DNA insertion sites was made (see Methods) and resulted in the identification of a T-DNA insertion at the 3′ end of *At5g04240* (Figure 2). To confirm that the loss of *At5g04240* leads to the early-flowering phenotype of *elf6*, three more T-DNA insertion alleles of *At5g04240* in the Columbia (Col) background (*elf6-2*, *-3*, and *-4*; Figure 2) were obtained and analyzed for flowering time. Homozygous mutants of all three Col alleles also showed an earlier floral transition compared with the wild-type Col both in LD and SD (Figure 1), confirming that *At5g04240* is indeed *ELF6*. The difference in flowering time in LD between *elf6* mutants and the wild type was not large but was statistically significant (Table 1).

Similarity searches using *ELF6* against Arabidopsis genome databases showed that *At3g48430* is the gene having the highest similarity to *ELF6*. Thus, we named *At3g48430 RELATIVE OF EARLY FLOWERING 6* (Figure 3). To address the function of *REF6*, three T-DNA insertion alleles of *REF6* in the Col background (*ref6-1*, *-2*, and *-3*; Figure 2) were obtained and analyzed for phenotypes. Unlike *elf6* mutants, all three *ref6*

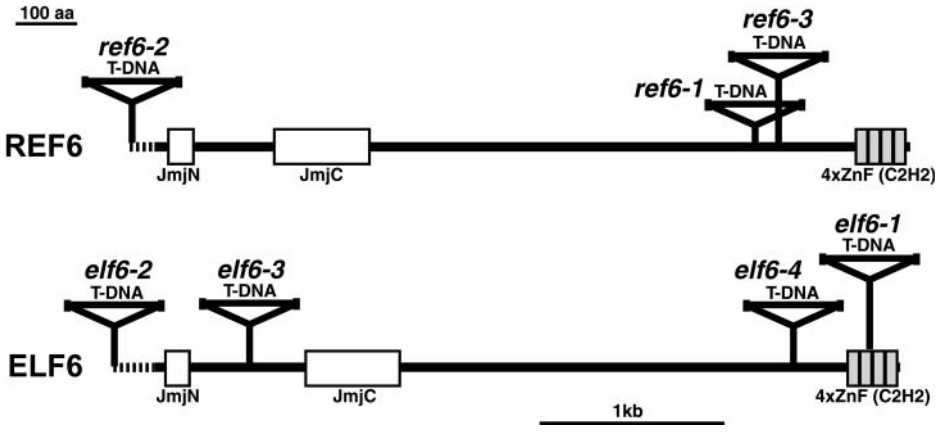

Figure 2. Domains of REF6 and ELF6 and the T-DNA Insertion Sites in *ref6* and *elf6* Mutants.

Domains predicted by the SMART (http://smart.embl-heidelberg.de/) program are indicated. Lines indicate interdomain regions. T-DNA insertion sites on the genomic sequences of *REF6* and *ELF6* are marked on the corresponding positions of their translated protein products with their allele numbers. Intergenic or 5′ untranslated regions are indicated as dotted lines. aa, amino acids.

Table 1. Student's *t* Test for the Flowering Time of *elf6* and *ref6* Mutants in LD

Statistical Significance	Ws versus *elf6-1*	Col versus *elf6-2*	Col versus *elf6-3*	Col versus *elf6-4*	*ref6-1* versus Col	*ref6-3* versus Col
t Value	4.44780	7.98659	7.52869	6.98178	9.10342	9.74121
P Value	0.00008	0.00000	0.00000	0.00000	0.00000	0.00000

mutants showed recessive late-flowering phenotypes both in LD and SD (Figures 1B and 1D; Col and *ref6-2* flowered with 11.8 ± 1.0 and 15.1 ± 1.1 rosette leaves, respectively, in LD). The flowering time difference between *ref6* mutants and the wild type in LD was not large but statistically significant (Table 1). Late-flowering mutants in the photoperiodic floral regulatory pathway show late-flowering phenotypes in LD but not in SD, and their late-flowering phenotypes are not effectively suppressed by vernalization (e.g., Koornneef et al., 1998). However, late-flowering mutants in the *FLC*-dependent pathways display late-flowering phenotypes both in LD and SD, and these late-flowering phenotypes are suppressed effectively by vernalization (e.g., Michaels and Amasino, 2000). Therefore, the photoperiod-independent late-flowering phenotypes of *ref6* mutants suggested a role of *REF6* in the *FLC*-dependent pathways. Consistent with this, late-flowering phenotypes of *ref6* mutants were also effectively suppressed by vernalization (Figure 1D). The difference in flowering time between wild-type and *ref6* mutants was reduced significantly after 40 d of vernalization, and *ref6* mutants flowered at a similar time as the wild type after 70 d of vernalization.

The rate of leaf initiation was identical in *elf6-2*, *ref6-1*, and Col in SD (Table 2), indicating that the altered flowering phenotype of *elf6* or *ref6* mutants was caused by the earlier or delayed transition of the shoot apical meristem from vegetative to reproductive phase.

ELF6 and *REF6* Encode Jmj/ZnF-Class Proteins That Are Most Homologous to Each Other

To identify the open reading frames of *ELF6* and *REF6*, *ELF6* and *REF6* full-length cDNAs were cloned by RT-PCR. One large open reading frame was found within each cDNA that was predicted to encode 1340 amino acids of ELF6 or 1360 amino acids of REF6.

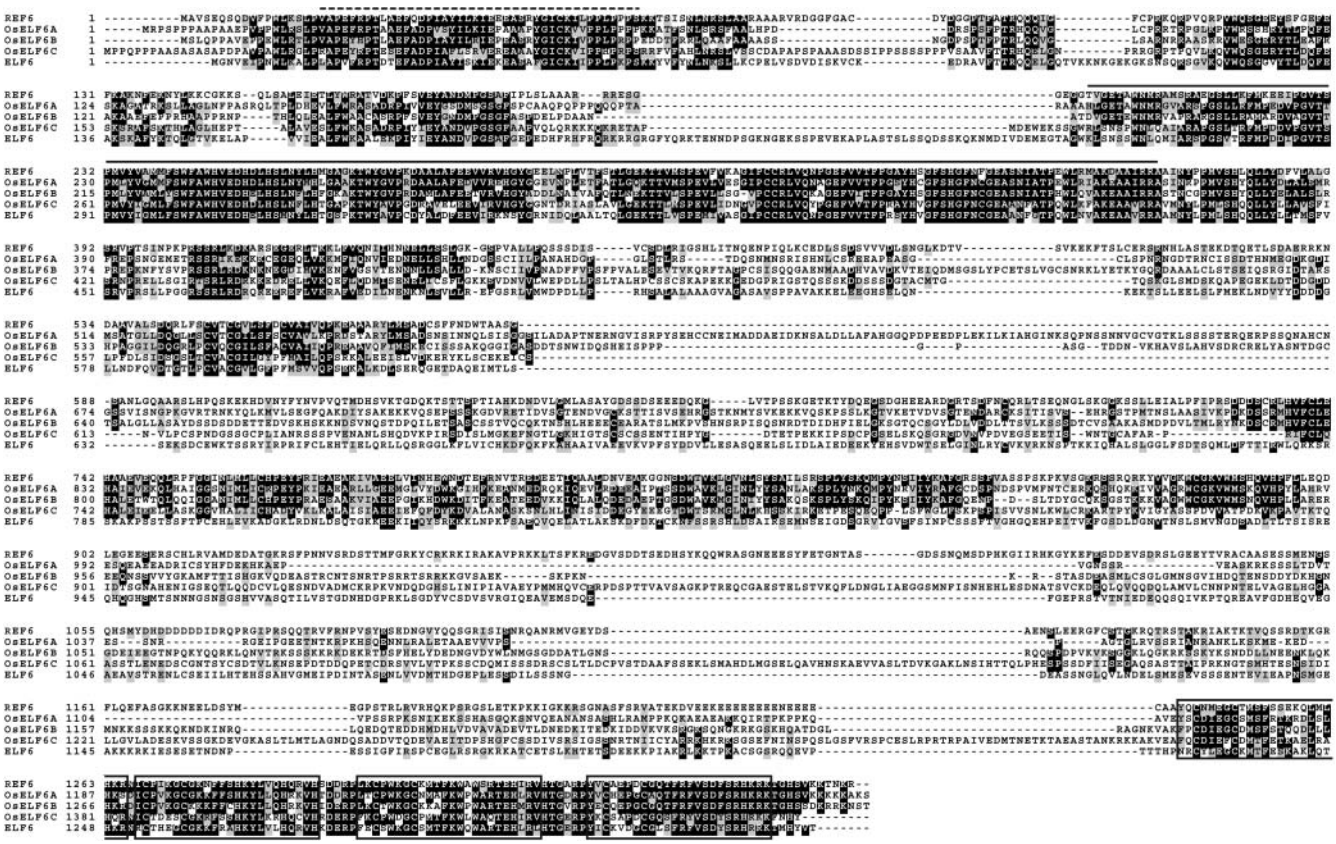

Figure 3. Sequence Comparison of REF6 with ELF6 and Their Three Rice Homologs (OsELF6A, OsELF6B, and OsELF6C).

JmjN and JmjC domains are marked by the dotted and solid lines, respectively. Four ZnF domains are marked by boxes. REF6, ELF6, OsELF6A, and OsELF6B sequences were derived from full-length cDNA sequences of their transcripts. OsELF6C sequence was derived from a protein sequence predicted by the gene prediction program GENSCAN (http://genes.mit.edu/GENSCAN.html).

Table 2. Leaf Initiation Rate of *elf6* and *ref6* Mutants Compared with the Wild Type

DAP	Genotype	LN
20	Col	6.0 ± 0.6
	elf6-2	6.2 ± 0.7
	ref6-1	6.1 ± 0.7
27	Col	11.1 ± 0.9
	elf6-2	11.0 ± 1.0
	ref6-1	11.2 ± 0.9
34	Col	17.1 ± 1.0
	elf6-2	17.3 ± 1.1
	ref6-1	17.0 ± 1.0
41	Col	24.2 ± 1.5
	elf6-2	24.3 ± 1.4
	ref6-1	24.4 ± 1.4

Values shown are means ± SD of rosette leaf numbers (LN) of wild-type (Col), *elf6-2*, or *ref6-1* plants grown in SD. Visible leaves were scored at 20, 27, 34, and 41 d after planting (DAP). Twelve plants were scored for each genotype.

The cDNA and predicted protein sequences of *ELF6* and *REF6* were different from the annotated sequences because of the misprediction of splicing sites, and the correct cDNA and protein sequences were deposited in GenBank.

Both ELF6 and REF6 contain jumonji N (JmjN) and jumonji C (JmjC) domains and four copies of C2H2-type ZnF domains (Figures 2 and 3). Significant sequence similarities between ELF6 and REF6 or between these two proteins and other proteins in the database are detected only in these domains. Clissold and Ponting (2001) categorized numerous eukaryotic proteins containing JmjC domains into seven groups based on sequence similarities. ELF6 and REF6 belong to the Group 1 proteins in which JmjC domains accompany JmjN domains and various classes of DNA binding or protein–protein interaction motifs such as PHD, ARID/BRIGHT, and ZnF domains. Because of such domain organizations, the Group 1 proteins are also called the Jmj family of transcription factors and have been implicated in gene transcription or chromatin remodeling (Balciunas and Ronne, 2000), although the biochemical activities of JmjN and JmjC domains have not yet been demonstrated. The Group 1 type domain organization of ELF6 and REF6 suggests that ELF6 and REF6 might also be involved in transcriptional gene regulation.

In addition to ELF6 and REF6, Arabidopsis has seven additional putative JmjN/JmjC-containing proteins that belong to Group 1 (At5g46910, At2g34880, At1g08620, At4g20400, At1g63490, At1g30810, and At2g38950). ELF6 and REF6 are the most similar to each other among these nine members. Sequence similarity among these nine proteins is confined only to the JmjN/JmjC domains. The seven proteins other than ELF6 and REF6 contain different types of DNA binding or protein–protein interaction motifs from C2H2-type ZnF at their C termini, suggesting divergent biological roles between ELF6/REF6 and the other seven members.

Despite of the phylogenic distance between Arabidopsis and rice (*Oryza sativa*), it has been reported that these two plant species share key components in their flowering time regulation (Hayama et al., 2003; Izawa et al., 2003; Tadege et al., 2003; Lee et al., 2004). This prompted us to examine if the rice genome has genes homologous to *ELF6* or *REF6* by similarity searches against the databases of rice full-length cDNAs and rice genome sequences at the National Institute of Agrobiological Sciences (http://www.dna.affrc.go.jp/). Three rice genes were identified to have significant and comparable sequence similarities to *ELF6* or *REF6* and are named *OsELF6A*, *OsELF6B*, and *OsELF6C* (Figure 3). The predicted protein sequences of the OsELF6 proteins show that the domain organization of ELF6 and REF6 is also conserved in these rice proteins. Whether these *OsELF6* genes play similar roles in rice flowering time regulation either to those of *ELF6* or *REF6* in Arabidopsis remains to be determined.

REF6 Acts as an *FLC* Repressor, whereas *ELF6* Is Involved in the Regulation of Photoperiodic Flowering

Photoperiod-independent late flowering and the effective suppression of the late-flowering phenotype by vernalization of *ref6* mutants (Figure 1) suggested a role of *REF6* in the regulation of *FLC*. To address this at the molecular level, we examined *FLC* expression in *ref6* mutants. *FLC* mRNA level was increased in *ref6-1* and *ref6-3*; however, there was no detectable difference in *CO* mRNA level between the mutants and the wild type (Figure 4A). To address the role of *REF6* in *FLC* regulation more directly, a double mutant between *ref6-1* and an *FLC* null mutant (*flc-3*; Michaels and Amasino, 1999) was generated and evaluated for flowering time. *ref6-1 flc-3* double mutants flowered at a similar time to either wild-type Col or *flc-3* single mutants (Figure 4B), demonstrating that *REF6* indeed acts as an *FLC* repressor. Similarly, the late-flowering phenotype of *ref6* was also largely suppressed by a mutation in *ELF5*, a gene required for high levels of *FLC* expression (Noh et al., 2004; Figure 4B).

However, double mutants between *elf6* and *elf5* flowered earlier than either single mutant (Figure 4C). This result showed that *ELF6* does not act in a linear pathway to *ELF5* and suggested the possibility that *ELF6* functions in an *FLC*-independent pathway. To evaluate the role of *ELF6*, a series of double mutants were generated between *elf6-2* and a variety of late-flowering mutants. *elf6* partially suppressed the late-flowering phenotypes induced by mutations in the *FLC*-dependent pathway genes (*ref6*, *ld*, or *fpa*) or by the presence of *FRI*, resulting in intermediate flowering phenotypes in the double mutants (Figure 4D). *FLC* transcript level in *elf6 ld* or *elf6 fpa* double mutants was not reduced compared with that in *ld* or *fpa* single mutants (Figure 4E), demonstrating that the partial suppression of the late-flowering phenotypes of these single mutants by the *elf6* mutation did not result from the downregulation of *FLC*. However, double mutants between *elf6* and the late-flowering mutants in the photoperiodic flowering pathway (*co* and *gi*) flowered at the same time as the single photoperiodic late-flowering mutants (i.e., the *co* and *gi* mutations are epistatic to the *elf6* mutation) (Figure 4D). Taken together, these results indicate that *ELF6* does not function in the same pathway as the *FLC*-dependent pathway but rather functions as an upstream repressor of the photoperiodic flowering pathway. The late-flowering phenotype of *soc1* but not *ft* mutants was partially suppressed by the *elf6* mutation (Figure 4D). These results suggest the possibility that

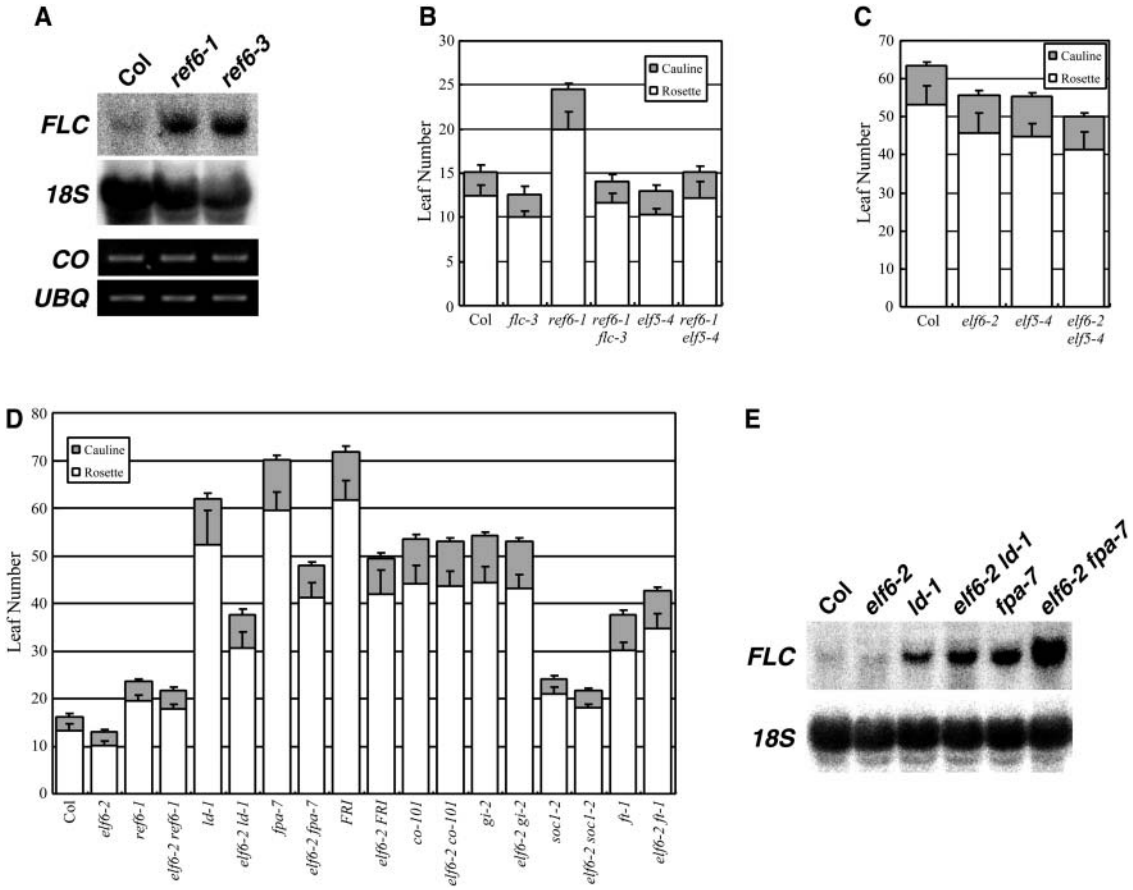

Figure 4. *REF6* Acts as an *FLC* Repressor, whereas *ELF6* Plays a Role in the Photoperiodic Floral Regulatory Pathway.

(A) Increased *FLC* expression in *ref6* mutants. RNA was isolated from 10-d-old seedlings of Col, *ref6-1*, and *ref6-2* grown under continuous light. The blot was probed first with *FLC* and then reprobed with 18S ribosomal DNA (18S) as a loading control. *CO* expression was studied by RT-PCR analyses. The RNA used for the RNA gel blot analyses was also used for the RT-PCR. *Ubiquitin* (*UBQ*) expression was analyzed as a control for the RT-PCR.
(B) Suppression of *ref6*-mediated late flowering by *flc* and *elf5* mutations. Wild-type (Col) and each single or double mutant was grown under LD, and their flowering times were measured as the number of rosette and cauline leaves. Data shown are means ± SD of at least 12 plants per genotype (see **[C]** and **[D]**).
(C) Earlier flowering of *elf6 elf5* double mutants compared with *elf6* and *elf5* single mutants. Flowering time was measured under SD as described in **(B)**.
(D) Double mutant analyses between *elf6* and a variety of late-flowering mutants. Flowering time was measured under LD.
(E) *FLC* transcript level is not affected by a mutation in *elf6*. RNA was isolated from 10-d-old seedlings of each genotype grown under LD. The blot was probed first with *FLC* and then reprobed with 18S ribosomal DNA (18S) as a loading control.

the two floral integrators are regulated differentially by the *FLC*-dependent and the photoperiodic pathways and that the regulation of *FT* is more dependent on the photoperiodic pathway than on the *FLC*-dependent pathway.

Overexpression of *REF6* Activates Floral Integrators, *FT* and *SOC1*, in an *FLC*-Independent Manner

Genetic analyses using the loss-of-function mutants of *REF6* and *ELF6* showed they act as an *FLC* repressor and a negative regulator in the photoperiodic flowering pathway, respectively (Figure 4). We were also interested in testing the effects of the overexpression of these genes. Genomic clones of *REF6* and *ELF6* were fused to the strong 35S promoter of *Cauliflower mosaic virus* and introduced into wild-type Ws. *35S:ELF6* did not cause detectable changes in flowering time in wild-type Ws, although it could rescue the early-flowering phenotype of *elf6* mutants (data not shown). However, 11 out of 18 transgenic lines containing the *35S:REF6* showed a strong early-flowering phenotype both in LD and SD compared with wild-type Ws (Figures 5A and 5B). No other notable developmental alterations were observed in the transgenic plants except for the fact that a few of these transgenic lines showed growth condition–dependent upward-curling phenotypes in their first and second rosette leaves. *REF6* mRNA level was increased dramatically in the two early-flowering *REF6* overexpression lines (*REF6OE6* and *REF6OE7*) tested (Figure 5C). The early-flowering phenotypes of the *35S:REF6* plants were more obvious in SD than in LD. However, a photoperiod response was retained in the *35S:REF6* plants as evidenced by earlier flowering in LD than in SD.

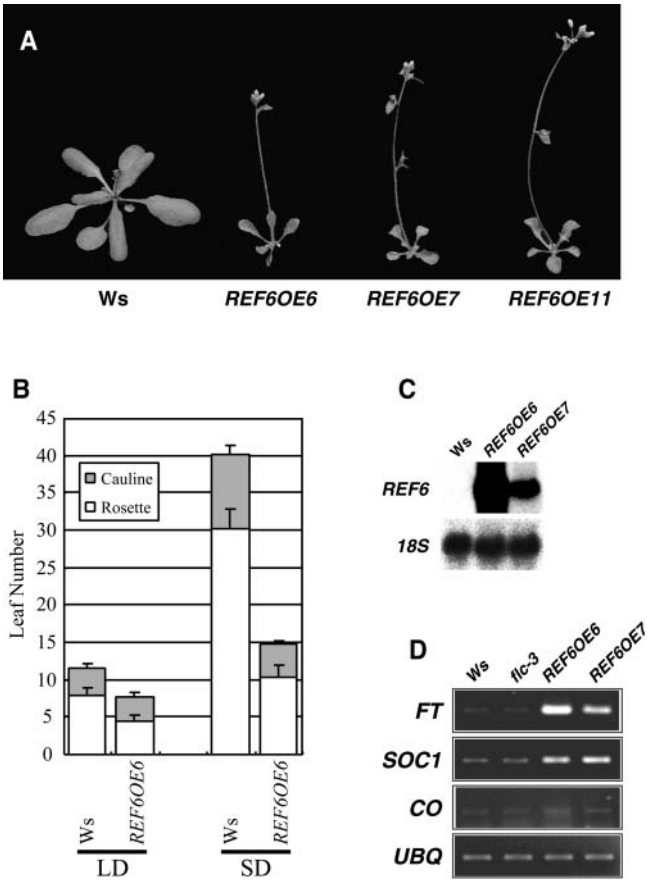

Figure 5. *REF6* Overexpression Causes Early Flowering by Increasing the Expression of *FT* and *SOC1*.

(A) Early flowering of three *REF6* overexpression lines (*REF6OE6*, *REF6OE7*, and *REF6OE11*). Representative plants of Ws and the three *REF6* overexpression lines grown in LD at the time of initiation of Ws flowering are shown.

(B) Flowering time of a *REF6* overexpression line in LD and SD. Ws and *REF6OE6* plants were grown under LD and SD. Data shown are means ± SD of at least 12 plants for each genotype and treatment.

(C) *REF6* mRNA expression in Ws and *REF6* overexpression lines. RNA was isolated from 10-d-old seedlings of Ws, *REF6OE6*, and *REF6OE7* grown under continuous light. The blot was probed first with *REF6* and then reprobed with 18S ribosomal DNA (18S) as a loading control.

(D) Increased expression of *FT* and *SOC1* but not *CO* in *REF6* overexpression lines. RT-PCR analyses of *FT*, *SOC1*, and *CO* in Ws, *flc-3* in Ws, *REF6OE6*, and *REF6OE7*. RNA was isolated from 10-d-old seedlings grown under continuous light. *Ubiquitin* (*UBQ*) expression was analyzed as a control.

One hypothesis to explain the strong early-flowering phenotype of *REF6* overexpressors could be that *REF6* overexpression might reduce the *FLC* expression, which in turn increases the expression of downstream floral integrators, *FT* and *SOC1*. However, this hypothesis is not likely because an *flc* null mutant (*flc-3*) in Ws flowers at a similar time as wild-type Ws (Noh et al., 2004). Therefore, the early-flowering phenotype of the *REF6* overexpressors is likely to be caused by an *FLC*-independent mechanism. To examine the effect of *REF6* overexpression on the photoperiodic flowering pathway and downstream floral regulators, we studied the expression of *FT*, *SOC1*, and *CO* by RT-PCR in two *REF6* overexpressors in comparison with that in wild-type Ws and *flc-3* in Ws. There was no detectable difference in *FT*, *SOC1*, and *CO* mRNA levels between wild-type Ws and *flc-3* in Ws (Figure 5D), consistent with their flowering time behaviors. However, the two *REF6* overexpressors showed increased expressions of *FT* and *SOC1* but not *CO* compared with wild-type Ws and *flc-3* in Ws. In conclusion, overexpression of *REF6* causes increases in *FT* and *SOC1* mRNA levels in an *FLC*-independent manner that leads to the early-flowering phenotype.

Distinctive Expression Patterns of *REF6* and *ELF6*

Structural similarity but difference in biological role between REF6 and ELF6 raises interesting questions on their molecular functions. These two proteins may have different cellular functions despite their structural similarity. Overexpression studies described above indicate that the differences in biological functions between REF6 and ELF6 come at least partially from differences in their cellular activities. In addition, REF6 and ELF6 may have different expression patterns that reflect their different in vivo roles. The expression patterns of *REF6* and *ELF6* were evaluated by analysis of fusions of the *REF6* and *ELF6* upstream and coding regions to the reporter gene β-*glucuronidase* (*GUS*; Figure 6A). Because a transcriptional terminator from *nopaline synthase* (*NOS-T* in Figure 6A) instead of the genes' own 3′ cognate sequences was used for the fusion constructs, possibilities of additional regulation by the 3′ untranslated region of *REF6* or *ELF6* could not be tested in our experiments. Nevertheless, these constructs were capable of complementing *ref6* and *elf6* mutant phenotypes, respectively, indicating that the fusion proteins are functional (data not shown).

In seedlings, *REF6* expression was highest in the shoot apical meristem region and primary and secondary root tips (Figures 6B to 6E), but lower expression was also observed in the cotyledons, leaves, and root axis, especially along vascular tissues (Figures 6B and 6E). This expression pattern of *REF6* is similar to that of *FLC* and other genes affecting *FLC* expression (e.g., Aukerman et al., 1999; Michaels and Amasino, 2000; Schomburg et al., 2001; Macknight et al., 2002; He et al., 2003; Noh and Amasino, 2003; Noh et al., 2004), consistent with the function of *REF6* as an *FLC* repressor. Nuclear localization of the REF6:GUS fusion protein was observed in the cells of the root distal elongation region (Figures 6F and 6G), and this is in agreement with the possible role of REF6 as a transcriptional regulator.

By contrast, the *ELF6* expression pattern in seedlings was quite different from that of *REF6*. Overall, *ELF6* was expressed at very low levels, but its highest expression was observed in cotyledons and leaves (Figures 6H and 6I). There was no detectable *ELF6* expression in the shoot apical meristem or in root tips. This expression pattern of *ELF6* is similar to the expression pattern of photoperiodic flowering genes, *CO* and *FT* (Takada and Goto, 2003), consistent with its role as an upstream repressor of the photoperiodic flowering pathway.

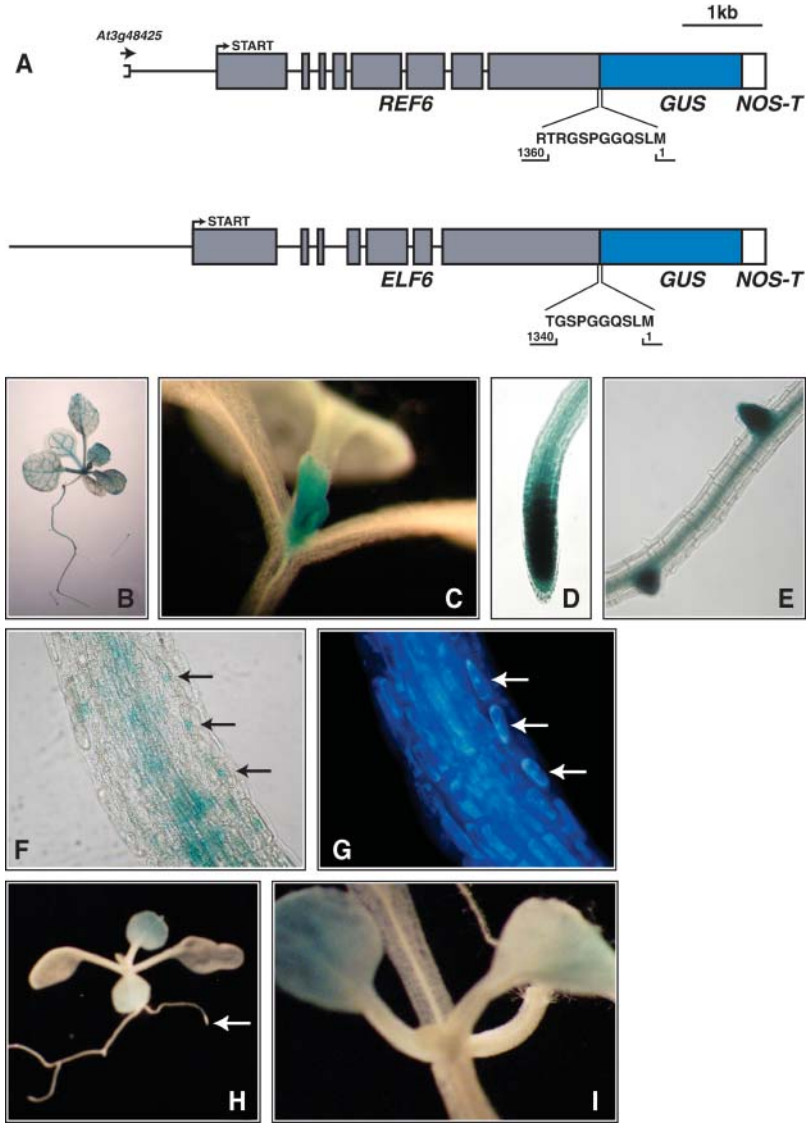

Figure 6. Expression Pattern of REF6 and ELF6.

(A) Schematic representation of the *REF6*:*GUS* and *ELF6*:*GUS* translational fusion constructs. The translated *REF6* and *ELF6* exons are indicated by gray boxes. Lines indicate introns or intergenic sequences. The linker sequence between the last amino acid of REF6 or ELF6 and the first amino acid of GUS is shown. Numbers indicate amino acid positions in REF6, ELF6, or GUS.
(B) to **(F)** Histochemical GUS staining of transgenic Arabidopsis containing the *REF6*:*GUS* fusion.
(B) Seventeen-day-old whole seedling grown under continuous light.
(C) to **(E)** Shoot apical meristem region **(C)**, primary root tip **(D)**, and developing secondary root tips **(E)** of a 10-d-old seedling grown under continuous light.
(F) Nuclear localization of the REF6:GUS in the cells of the root distal elongation region of a 10-d-old seedling grown under continuous light. Arrows in **(F)** and **(G)** indicate cells with clear nuclear localization of REF6:GUS.
(G) Staining of the root tissue shown in **(F)** with 4',6'-diamidino-2-phenylindole hydrochloride to visualize nuclei.
(H) and **(I)** Histochemical GUS staining of transgenic Arabidopsis containing the *ELF6*:*GUS* fusion.
(H) Ten-day-old whole seedling grown under continuous light. Arrow indicates a root tip.
(I) Magnification of the shoot apical meristem region of the seedling shown in **(H)**.

Therefore, in addition to the difference in cellular functions between REF6 and ELF6, different spatial expression patterns can also be ascribed to the divergence in their biological roles.

REF6 Represses *FLC* Transcription through Chromatin Modifications

Recently, it has been shown that transcription of *FLC* is regulated by multiple proteins through chromatin remodeling (He et al., 2003; Ausín et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004). Because REF6 acts as an *FLC* repressor (Figure 4) and has domains suggesting a role in chromatin remodeling (Figure 2), we tested if the increased *FLC* mRNA expression in *ref6* mutants accompanies alterations in *FLC* chromatin structure by examining the modification of histone tails. Chromatin immunoprecipitation (ChIP) with antibodies specific to the penta-acetylated histone H4 tail followed by quantitative PCR showed that the amount of histone H4 acetylation in *FLC* chromatin is increased in *ref6-3* mutants as well as in *fld-3* mutants in which *FLC* chromatin is known to be hyperacetylated (He et al., 2003) compared with in wild-type Col (Figure 7). Hyperacetylation of histone H4 in *ref6-3* mutants was observed in the promoter region close to the translation start site (FLCII) and in the first intron (FLCIII and V1) but not in the 3′ untranslated region (U1) of *FLC*. The same type of histone H4 tail modification was observed in *fld-3* mutants (Figure 7) as reported previously (He et al., 2003). Thus, our ChIP studies show that there is a positive correlation between the level of H4 acetylation and the expression of *FLC* mRNA. This is in agreement with the general idea that H4 acetylation is a marker commonly associated with transcriptionally active loci (for review, see Eberharter and Becker, 2002). In summary, the above results indicate that the repression of *FLC* transcription by REF6, a Jmj/ZnF protein, is accompanied by histone modifications in the *FLC* chromatin.

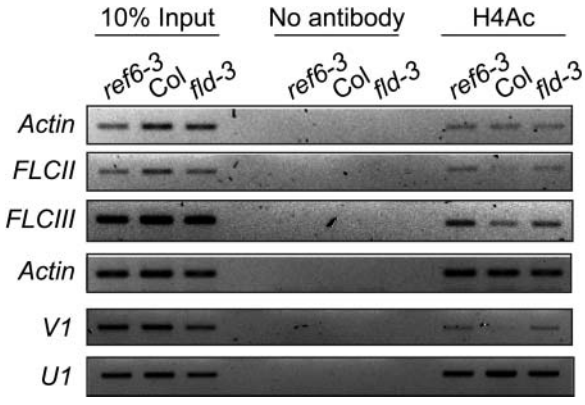

Figure 7. ChIP Using Antibody against Hyperacetylated H4.

ref6 and *fld* mutations increase the precipitation of regions II, III (He et al., 2003), and V1 (Sung and Amasino, 2004) of *FLC*. Association of U1 region (Sung and Amasino, 2004) with antibody against hyperacetylated H4 was not increased in *ref6* and *fld* mutants compared with in wild-type Col. Representative images of two independent experiments are shown.

DISCUSSION

In this study, we report the identification of two novel floral regulators, *ELF6* and *REF6*. Interestingly, *ELF6* and *REF6* both encode nuclear proteins containing JmjN/JmjC and four copies of C2H2-type ZnF domains that are most homologous to each other in Arabidopsis despite the difference in their role in the regulation of flowering time; ELF6 acts as a repressor in the photoperiodic flowering pathway, whereas REF6 is an *FLC* repressor. The role of REF6 as an *FLC* repressor was demonstrated in multiple ways. First, the late-flowering phenotype of *ref6* mutants was effectively suppressed by vernalization (Figure 1D), a floral inductive process mediated mainly by the repression of *FLC* expression. Second, mutations in *REF6* caused increases in *FLC* mRNA level without decreases in *CO* mRNA level (Figure 4A). Third, the late-flowering phenotype of *ref6* mutants was almost fully suppressed by an *flc* null mutation (Figure 4B).

The late-flowering phenotype of photoperiod pathway mutants (*gi*, *co*, and *ft*) are epistatic to the early-flowering phenotype of *elf6* mutants (Figure 4D). Furthermore, double mutants between *elf6* and *FLC*-dependent late-flowering mutants (*ref6*, *ld*, *fpa*, and *FRI*) show intermediate flowering times without reduction of *FLC* mRNA expression compared with the single late-flowering mutants (Figures 4D and 4E). These results indicate that *ELF6* acts as a repressor upstream of the photoperiodic floral regulatory pathway. However, a molecular description of the function of *ELF6* in the regulation of photoperiodic flowering has not yet been made. Because the early-flowering phenotype of *elf6* mutants is weak and the mRNA expression of photoperiod pathway marker genes, such as *GI*, *CO*, and *FT*, is detected only by RT-PCR but not by the more quantitative method of RNA gel blot analysis, attempts to evaluate if there are differences in mRNA levels of these photoperiod pathway marker genes between wild-type and *elf6* mutants have not yet been successful (data not shown). One possibility is that *ELF6* may regulate the circadian rhythm of the genes in the photoperiodic floral regulatory pathway. Our double mutant analyses show that *ELF6* is upstream of *GI*, a gene with roles both in the circadian clock and the photoperiodic floral regulation (Fowler et al., 1999; Park et al., 1999). Therefore, it is possible that the mutations in *ELF6* may affect flowering time through differential regulation of the circadian clock. It will be of interest to test if *elf6* mutants have defective circadian rhythms and if their early-flowering phenotype is derived from circadian defects.

JmjC-domain proteins with additional domains known to be involved in protein–protein interactions or DNA binding have been implicated in the regulation of gene transcription or chromatin remodeling, although the role of the JmjC domain itself still remains enigmatic (Balciunas and Ronne, 2000; Clissold and Ponting, 2001). Lately, it was reported that Epe1, a JmjC-domain protein without a notable DNA binding or protein–protein interaction domain from *Saccharomyces pombe*, modulates heterochromatin formation, and its JmjC domain is necessary for activity (Ayoub et al., 2003). Dynamic changes in the modification pattern of histones, such as acetylation, phosphorylation, methylation, and ubiquitination, are thought to control transcriptional activity by affecting chromatin

structure and accessibility of nonhistone regulatory factors to chromatin (Strahl and Allis, 2000). Growing evidence suggests that remodeling of chromatin structure controls *FLC* transcription. The chromatin within the *FLC* locus is structurally modified during the vernalization process, and this modification results in the reduction of *FLC* transcription (Bastow et al., 2004; Sung and Amasino, 2004). It was also reported that the mutations in *FLD* and *FVE* among the autonomous-pathway floral regulatory genes cause hyperacetylation in *FLC* chromatin, leading to increased levels of *FLC* mRNA in the mutants (He et al., 2003; Ausín et al., 2004). Similarly, our ChIP studies show that the disruption of REF6 activity changes modification patterns of histone H4 tails in the *FLC* locus (Figure 7). Therefore, our results further demonstrate the chromatin remodeling-related function of a Jmj-class protein (REF6) at the target gene (*FLC*) level. FLC-like spatial expression pattern and nuclear localization of REF6 (Figure 6) also indirectly support its role as a component of chromatin-related transcriptional machinery involved in *FLC* regulation.

Along with the late-flowering phenotypes, *ref6* mutants also displayed darker green color in old leaves and stems compared with the wild type (data not shown). The darker green phenotype was observed only in *ref6* mutants but not in *elf6* mutants and was more obvious in older tissues than in younger tissues. Anthocyanin but not chlorophyll levels were significantly increased in old leaves of *ref6* mutants, suggesting that in addition to its role in floral regulations, REF6 also acts as a repressor of anthocyanin biosynthesis or accumulation. Thus, REF6 might have multiple target genes, which is in agreement with general characteristics of chromatin modifiers.

At this moment, the biochemical roles of REF6 in histone modification are not clear. FLD and FVE are homologous to the components of HDAC complexes in human and other organisms; however, no HDAC component has been found to be homologous to REF6. REF6 might be involved in the recruitment or in the stabilization of the binding of a HDAC complex to *FLC* chromatin through interactions with one of the components in the HDAC complex. Hyperacetylation of histone H4 in *ref6* mutants might increase the accessibility of nonhistone regulatory proteins to *FLC* chromatin that are required for orchestrating highly complex processes of transcription leading to increased levels of *FLC* expression followed by late flowering.

Difference in biological role between *REF6* and *ELF6* is indicated by two lines of evidence. First, ectopic expression of *REF6* but not *ELF6* causes early flowering by an *FLC*-independent induction of downstream floral activators, *FT* and *SOC1* (Figure 5), indicating that REF6 and ELF6 exert different cellular functions. Second, the spatial expression patterns of these two genes are also quite different (Figure 6); *REF6* and *ELF6* resemble those of *FLC* and photoperiod pathway genes (*CO* and *FT*), respectively. The expression pattern of *REF6* or *ELF6* is consistent with the genetic role each plays as an *FLC* or photoperiodic flowering-pathway repressor. Therefore, both different cellular functions and differential expression patterns of REF6 and ELF6 might contribute to the different biological roles of these two proteins. Pairs of closely related protein having opposite effects on flowering time have been reported. FT and TFL are similar to each other. However, one acts as a floral activator, whereas the other is a floral repressor (Kobayashi et al., 1999). In another case, SVP and AGL24 act as a floral repressor and activator, respectively, despite a high level of sequence similarity between them (Michaels et al., 2003). It would be of interest to find a domain or domains that are responsible for the different cellular roles between REF6 and ELF6 as well as to find a regulatory element responsible for their differential expression patterns.

The *FT* and *SOC1* activation-mediated early flowering of *REF6* overexpressors (Figure 5) was surprising. A similar observation was made when *VRN1*, which allows stable *FLC* repression after vernalization, was overexpressed (Levy et al., 2002). Similar to the *FLC*-independent activation of *FT* and *SOC1* in *REF6* overexpressors, *VRN1* overexpression also caused the activation *FT* and *SOC1* in an *FLC*-independent manner. Despite the activation of downstream floral integrators, both *REF6* and *VRN1* overexpressors maintained photoperiod sensitivity as evidenced by their earlier flowering in LD than in SD. The retention of photoperiod sensitivity of *REF6* and *VRN1* overexpressors combined with the *CO*-independent activation of *FT* and *SOC1* in *REF6* overexpressors (Figure 5D) suggest that *REF6* and *VRN1* do not promote flowering through the photoperiod pathway. *FT* and *SOC1* integrate flowering signals generated through *FLC* and *CO*. GA mainly exerts its floral regulatory effects in SD through the activation of *SOC1* and *LFY* but not *FT* (Blázquez and Weigel, 2000; Moon et al., 2003). Vernalization induces flowering mainly through the repression of *FLC* transcription, although it also has a marginal activation effect on *SOC1* and *FT* in an *flc* null mutant background (Moon et al., 2003). Therefore, *FLC*- and *CO*-independent induction of *FT* and *SOC1* in *REF6* overexpressors might be mediated through a mechanism yet to be discovered. Recently, floral regulatory functions of a few *FLC* homologs (*FLM*, *MAF2*, etc.) have been reported (Scortecci et al., 2001, 2003; Ratcliffe et al., 2003). Therefore, it would be of interest to test if the strong induction of *FT* and *SOC1* in *REF6* overexpressors is mediated through these *FLC* homologs.

METHODS

Plant Materials and Growth Conditions

elf6-1 in the Ws background was isolated from the BASTA population of the Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis/). *elf6* and *ref6* T-DNA insertion lines in the Col background were obtained either from the SALK collection (http://signal.salk.edu/; *elf6-2*, *elf6-3*, *ref6-1*, and *ref6-2*, which are SALK_018830, SALK_074694, SALK_001018, and SALK_122006, respectively) or from the Torrey Mesa Research Institute (*elf6-4* and *ref6-3*, which are SAIL371D8 and SAIL747A7). The following mutants are in the Col background and described previously: *flc-3* (Michaels and Amasino, 1999), *elf5-4* (Noh et al., 2004), *ld-1* (Lee et al., 1994a), *fpa-7* (Michaels and Amasino, 2001), *FRI* (Lee et al., 1994b), *co-101* (Takada and Goto, 2003), *gi-2* (Park et al., 1999), *soc1-2* (Moon et al., 2003), and *ft-1* (Lee et al., 2000). All plants were grown under $\sim$100 μE m^{-2} s^{-1} cool white fluorescent light at 22°C. Plants were vernalized as described previously (Noh and Amasino, 2003).

T-DNA Flanking Sequence Analyses

The sequence flanking the T-DNA of *elf6-1* was obtained by thermal asymmetric interlaced PCR (Liu et al., 1995) as described by Schomburg et al. (2003).The T-DNA borders of *elf6* and *ref6* alleles were defined by

sequencing PCR products obtained using a T-DNA border primer and a gene-specific primer. SALKLB1 (5′-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3′) or SAILLB3 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′) was used as a T-DNA border primer for the mutants obtained from the SALK collection or from the Torrey Mesa Research Institute, respectively.

ChIP

ChIP was performed as described by Gendrel et al. (2002) using ~10 to 12-d-old seedlings. Briefly, seedlings were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching cross-linking. Chromatin was isolated and sonicated to ~0.5 to 1 kb. Antihyperacetylated histone H4 (Upstate Biotechnology, Lake Placid, NY) was added to the chromatin solution precleared with salmon sperm DNA/Protein A agarose beads (Upstate Biotechnology). After subsequent incubation with salmon sperm DNA/Protein A agarose beads, immunocomplexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immunocomplexes were removed by the incubation with proteinase K followed by phenol/chloroform extraction. DNA was recovered by ethanol precipitation. The amount of immunoprecipitated *FLC* chromatin was determined by quantitative PCR on four different regions of *FLC* locus as previously reported (FLCII and FLCIII [He et al., 2003]; V1 and U1 [Sung and Amasino, 2004]). The sequences of primer pairs for each PCR reaction are as follows: FLCII, CH1 (5′-CTGCGACCATGATAGATACATGAGA-3′) and HIS2 (5′-TTCACTCAACAACATCATCGAGCACG-3′); FLCIII, CH2 (5′-GTTCTCAATTCGCTTGATTTCTAGTTTTT-3′) and CH3 (5′-GGCCCGACGAAGAAAAAGGTAGATAGGC-3′); V1, V1F (5′-ATAGATTTGCCTCATATTTATGTGATTG-3′) and V1R (5′-TATTTCTTACATGAAGACAAGTGTTGTGG-3′); U1, U1F (5′-ACAAAAGGTTGATGAACTTTGTACCT-3′) and U1R (5′-CCGACATAGGCCCAAAAAACCCATGC-3′).

Histochemical GUS Assays

For the construction of the *ELF6*:*GUS* translational fusion construct, 7.4-kb genomic fragment of *ELF6* containing 2.3-kb 5′ upstream region and the entire coding region was generated by PCR amplification using ELF6GUS-1 (5′-agcggatccAAACTGTTCATCACCCTCTTCATCCACAAG-3′) and ELF6GUS-2 (5′-gccggatccTGTGACATAGTGCATGGTTTTACGTCTGT-3′) as primers. The resulting PCR product was digested with *Bam*HI and ligated to pPZP211G (Noh et al., 2001) at the *Bam*HI site. The *REF6*:*GUS* translational fusion construct was generated by PCR amplification of 6 kb of the *REF6* genomic DNA fragment containing 1.2-kb 5′ upstream region and the entire coding region using REF6GUS-1 (5′-gaccccgggAGTGATCATTGTCCGGTTACACTCGAGCTT-3′) and REF6GUS-2 (5′-gccactagtCCTTTTGTTGGTCTTCTTAACCGAATGACC-3′) as primers. After restriction digestion with *Sma*I-*Spe*I, the PCR product was ligated to pPZP211-GUS (Noh and Amasino, 2003) and digested with *Pst*I-*Xba*I (*Pst*I site was blunted with T4 DNA polymerase [New England Biolabs, Beverly, MA] after the digestion). In primer sequences, restriction sites for cloning are underlined, and sequences corresponding to the *ELF6* or *REF6* are in capital letters. *elf6-4* or *ref6-3* mutant plants were transformed using *Agrobacterium tumefaciens* strain ABI containing the *ELF6*:*GUS* or the *REF6*:*GUS* construct, respectively, by infiltration (Clough and Bent, 1998). Histochemical GUS staining was performed as described by Schomburg et al. (2001) using transgenic lines selected as previously described (Noh and Amasino, 2003).

RNA Gel Blot Analyses

Total RNA was isolated from 10-d-old seedlings by TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. For RNA gel blot analyses, 40 μg of total RNA was separated by denaturing formaldehyde-agarose gel electrophoresis as described by Sambrook et al. (1989). The *FLC* probe was a cDNA fragment lacking the conserved MADS domain sequences. The *REF6* probe was a 2.5-kb *REF6* cDNA fragment generated by PCR using REF6CS-3 (5′-CCTGAAGTATTTGTTAAAGC-3′) and REF6-1 (5′-CCTCCATGTTACATTGGTATGCTGCACATT-3′) as primers. As a control for the quantity of RNA loaded, blots were also probed with 18S rDNA.

RT-PCR Analyses

Reverse transcription was performed with Superscript II (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions using 2.5 μg of total RNA isolated as described above. Quantitative PCR was performed on first strand DNA with ExTaq polymerase (TaKaRa Bio, Otsu, Shiga, Japan) using the following primers as previously described by Cerdan and Chory (2003) and Michaels et al. (2003): *FT* (5′-GCTACAACTGGAACAACCTTTGGCAAT-3′ and 5′-TATAGGCATCATCACCGTTCGTTACTC-3′), *CO* (5′-AAACTCTTTCAGCTCCATGACCACTACT-3′ and 5′-CCATGGATGAAATGTATGCGTTATGGTTA-3′), *SOC1* (5′-TGAGGCATACTAAGGATCGAGTCAG-3′ and 5′-GCGTCTCTACTTCAGAACTTGGGC-3′), and *UBIQUITIN* (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′).

Overexpression of *ELF6* and *REF6*

For the overexpression of *ELF6* or *REF6*, 5.89- or 5.81-kb genomic DNA containing the entire coding region and the 3′ untranslated region of *ELF6* or *REF6* was generated by PCR amplification using ELF6OE-1 (5′-cgggatccATGGGTAATGTTGAAATTCCGAATTGGCT-3′) and ELF6OE-2 (5′-cgctcgagCTCAGCAACAGTGCGAATCCATGGTCAAGG-3′) for *ELF6* or REF6OE-1 (5′-ggactagtATGGCGGTTTCAGAGCAGAGTCAAGATGTG-3′) and REF6OE-2 (5′-agggggcccCCTCCATTAACTTCTTCTTCTAGTTTCCCA-3′) for *REF6*; restriction sites for cloning are underlined, and sequences corresponding to the *ELF6* or *REF6* genomic DNA are in capital letters. The resulting PCR product was digested with *Bam*HI-*Sac*I or *Spe*I-*Sma*I and ligated to pPZP211-GUS (Noh and Amasino, 2003) digested with *Bam*HI-*Sac*I or *Xba*I-*Sac*I (*Sac*I site was blunted with T4 DNA polymerase [New England Biolabs] after the digestion), respectively, generating the transcriptional fusion between the 35S promoter of *Cauliflower mosaic virus* and either *ELF6* or *REF6*. Transformation of *elf6-4* and *ref6-3* with *A. tumefaciens* carrying the corresponding fusion construct and selection of the transgenic lines were performed as described above.

Sequence Analyses

Genes were predicted with GenScan (Burge and Karlin, 1997). Protein sequences were analyzed with SMART (Schultz et al., 2000), PSORT (Nakai and Kanehisa, 1992), and ψ-BLAST (Altschul et al., 1997). Protein sequence alignments were generated using ClustalW (Thompson et al., 1994). cDNA was synthesized, cloned, and sequenced as described (Noh and Amasino, 2003). For the *ELF6* or *REF6* cDNA cloning, ELF6OE-1 (5′-cgggatccATGGGTAATGTTGAAATTCCGAATTGGCT-3′) and ELF6-GUS-2 (5′-gccggatccTGTGACATAGTGCATGGTTTTACGTCTGT-3′) or REF6F (5′-ggctcgagATGGCGGTTTCAGAGCAGAGTCAA-3′) and REF6R (5′-cgactagtCCTTTTGTTGGTCTTCTTAACCG-3′) were used as gene-specific primers for cDNA amplification, respectively; restriction sites for cloning are underlined, and sequences corresponding to the *ELF6* or *REF6* genomic DNA are in capital letters. *OsELF6A* and *OsELF6B* cDNA sequences correspond to the rice full-length cDNA clone J023001N18 (accession number AK068952) and J013002J08 (accession number AK065251) of KOME (http://cdna01.dna.affrc.go.jp/cDNA/).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY664499 (*REF6*) and AY664500 (*ELF6*).

ACKNOWLEDGMENTS

We thank Pill-Soon Song for careful reading of the manuscript. We are grateful to the Salk Institute Genome Analysis Laboratory and Torrey Mesa Research Institute of Syngenta for providing knockout pools containing *elf6* and *ref6* alleles. This work was supported by a grant from the Molecular and Cellular BioDiscovery Research Program (M1-0311-09-0000) from the Ministry of Science and Technology of Korea to Y.-S.N. and by a grant from the Korea Science and Engineering Foundation to the Environmental Biotechnology National Core Research Center (R15-2003-012-01002-0) to B.N. and Y.-S.N. Work in R.M.A.'s lab was supported by the College of Agricultural and Life Sciences and the Graduate School of the University of Wisconsin and by Grant 0209786 from the National Science Foundation. This is Kumho Life and Environmental Science Laboratory publication 72.

Received June 23, 2004; accepted July 23, 2004.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: New generation of protein database search programs. Nucleic Acids Res. **25,** 3389–3402.

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.

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

Ayoub, N.A., Noma, K., Isaac, S., Kajan, T., Grewal, S.I.S., and Cohen, A. (2003). A novel jmjC domain protein modulates heterochromatization in fission yeast. Mol. Cell. Biol. **23,** 4356–4370.

Balciunas, D., and Ronne, H. (2000). Evidence of domain swapping within the jumonji family of transcription factors. Trends Biochem. Sci. **25,** 274–276.

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.

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

Burge, C., and Karlin, S. (1997). Prediction of complete gene structures in human genomic DNA. J. Mol. Biol. **268,** 78–94.

Cerdan, P.D., and Chory, J. (2003). Regulation of flowering time by light quality. Nature **423,** 881–885.

Clissold, P.M., and Ponting, C. (2001). JmjC: Cupin metalloenzyme-like domains in jumonji, hairless and phospholipase $A_2\beta$. Trends Biochem. Sci. **26,** 7–9.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

Eberharter, A., and Becker, P.B. (2002). Histone acetylation: A switch between repressive and permissive chromatin. EMBO Rep. **3,** 224–229.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Coupland, G., and Putterill, J. (1999). *GIGANTEA*: A circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J. **18,** 4679–4688.

Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V., and Martienssen, R.A. (2002). Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. Science **297,** 1871–1873.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M., and Shimamoto, K. (2003). Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature **422,** 719–722.

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

Henderson, I.R., Shindo, C., and Dean, C. (2003). The need for winter in the switch to flowering. Annu. Rev. Genet. **37,** 371–392.

Izawa, T., Takahashi, Y., and Yano, M. (2003). Comparative biology comes into bloom: Genomic and genetic comparison of flowering pathways in rice and *Arabidopsis*. Curr. Opin. Plant Biol. **6,** 113–120.

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.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science **286,** 1960–1962.

Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M., and Soppe, W. (1998). Genetic control of flowering time in *Arabidopsis*. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49,** 345–370.

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., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994a). Isolation of *LUMINIDEPENDENS*: A gene involved in the control of flowering time in Arabidopsis. Plant Cell **6,** 75–83.

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

Lee, S., Kim, J., Han, J.-J., Han, M.-J., and An, G. (2004). Functional analyses of the flowering time gene *OsMADS50*, the putative *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20* (*SOC1/AGL20*) ortholog in rice. Plant J. **38,** 754–764.

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.

Liu, Y.G., Mitsukawa, N., Oosumi, T., and Whittier, R.F. (1995). Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J. **8,** 457–463.

Macknight, R., Duroux, M., Laurie, R., Dijkwel, P., Simpson, G., and Dean, C. (2002). Functional significance of the alternative transcript processing of the Arabidopsis floral promoter *FCA*. Plant Cell **14,** 877–888.

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. (2000). Memories of winter: Vernalization and the competence to flower. Plant Cell Environ. **23,** 1145–1153.

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., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M., and Amasino, R.M. (2003). *AGL24* acts as a promoter of flowering in *Arabidopsis* and is positively regulated by vernalization. Plant J. **33,** 867–874.

Moon, J., Suh, S.-S., Lee, H., Choi, K.-R., Hong, C.B., Paek, N.-C., Kim, S.-G., and Lee, I. (2003). The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*. Plant J. **35,** 613–623.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: Interacting pathways as a basis for diversity. Plant Cell **14** (suppl.), S111–S130.

Nakai, K., and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14,** 897–911.

Noh, B., Angus, M., and Spalding, E.P. (2001). *Multidrug Resistance*-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell **13,** 2441–2454.

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.

Noh, Y.-S., Bizzell, C.M., Noh, B., Schomburg, F.M., and Amasino, R.M. (2004). *EARLY FLOWERING 5* acts as a floral repressor in *Arabidopsis*. Plant J. **38,** 664–672.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A., and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. Science **285,** 1579–1582.

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.

Reeves, P.H., Murtas, G., Dash, S., and Coupland, G. (2002). *early in short days 4*, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. Development **129,** 5349–5361.

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,** 1613–1616.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schomburg, F.M., Bizzell, C.M., Lee, D.J., Zeevaart, J.A.D., and Amasino, R.M. (2003). Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell **15,** 151–163.

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.

Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P., and Bork, P. (2000). SMART: A Web-based tool for the study of genetically mobile domains. Nucleic Acids Res. **28,** 231–234.

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.

Scortecci, K.C., Michaels, S.D., and Amasino, R.M. (2001). Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. Plant J. **26,** 229–236.

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.

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

Soppe, W.J.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.

Strahl, B.D., and Allis, D. (2000). The language of covalent histone modifications. Nature **403,** 41–45.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410,** 1116–1120.

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

Sung, Z.R., Chen, L., Moon, Y.H., and Lertpiriyapong, K. (2003). Mechanisms of floral repression in Arabidopsis. Curr. Opin. Plant Biol. **6,** 29–35.

Tadege, E., Sheldon, C.C., Helliwell, C.A., Upadhyaya, N.M., Dennis, E.S., and Peacock, W.J. (2003). Reciprocal control of flowering time by *OsSOC1* in transgenic *Arabidopsis* and by *FLC* in transgenic rice. Plant Biotechnol. J. **1,** 361–369.

Takada, S., and Goto, K. (2003). TERMINAL FLOWER2, an Arabidopsis homolog of HETEROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T* by CONSTANS in the vascular tissues of leaves to regulate flowering time. Plant Cell **15,** 2856–2865.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22,** 4673–4680.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science **303,** 1003–1006.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. Plant Physiol. **100,** 403–408.