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FLOWERING LOCUS C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering

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Winter-annual ecotypes of Arabidopsis are relatively late flowering, unless the flowering of these ecotypes is promoted by exposure to cold (vernalization). This vernalization-suppressible, late-flowering phenotype results from the presence of dominant, late-flowering alleles at two loci, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). In this study, we report that *flc* null mutations result in early flowering, demonstrating that the role of active *FLC* alleles is to repress flowering. *FLC* was isolated by positional cloning and found to encode a novel MADS domain protein. The levels of *FLC* mRNA are regulated positively by *FRI* and negatively by *LUMINIDEPENDENS. FLC* is also negatively regulated by vernalization. Overexpression of *FLC* from a heterologous promoter is sufficient to delay flowering in the absence of an active *FRI* allele. We propose that the level of *FLC* activity acts through a rheostat-like mechanism to control flowering time in Arabidopsis and that modulation of *FLC* expression is a component of the vernalization response.

INTRODUCTION

The transition of shoot apical meristems from vegetative growth to flowering is a major developmental switch in the plant life cycle. The timing of floral initiation is critical for reproductive success, and many plant species have evolved multiple pathways to regulate flowering time. These pathways monitor both the developmental state of the plant and environmental cues such as photoperiod and temperature.

In Arabidopsis, which is a facultative long-day plant, studies of induced mutations have identified several loci that delay flowering when mutated and other loci that cause early flowering when mutated (Koornneef et al., 1998; Levy and Dean, 1998). The late-flowering mutants define genes that promote flowering, whereas the early-flowering mutants define genes that inhibit the transition to flowering. Physiological analyses of the effects of such mutations support a model in which these genes operate in parallel flowering pathways. For example, one group of late-flowering mutants (e.g., *constans*, *fd*, *fe*, *fha*, *ft*, *fwa*, and *gigantea*) exhibits little difference in flowering time when grown in short days compared with long days. Thus, these mutants define genes whose products promote flowering in a pathway that responds to inductive photoperiods (Koornneef et al., 1998; Pineiro and Coupland, 1998). In another group of late-flowering mutants (e.g., *fca*, *fpa*, *fve*, *fy*, and *luminidependens* [*ld*]), flowering is delayed under inductive (long-day) conditions and even more severely delayed during short days. Thus, this group retains a photoperiod response. Mutants in this group also exhibit a flowering response to extended exposure to cold (vernalization); the late-flowering phenotype of these mutants is suppressed by vernalization. The pathway in which these genes act is referred to as the autonomous or constitutive pathway to indicate that this pathway acts independently of photoperiod (Koornneef et al., 1998; Levy and Dean, 1998). The suppression of autonomous pathway mutants by exposure to cold indicates that a vernalization pathway can bypass blocks to the autonomous pathway.

The mutational analyses described above to identify flowering-time genes have been largely conducted in early-flowering ecotypes of Arabidopsis. However, many other Arabidopsis ecotypes are relatively late flowering when grown under inductive long-day conditions, and they become early flowering only after vernalization (Napp-Zinn, 1979; Sanda and Amasino, 1996a). Thus, the flowering behavior of these late-flowering ecotypes is similar to the flowering behavior of early-flowering ecotypes that have late-flowering mutations in autonomous pathway genes. Genetic analyses demonstrate that the difference in flowering behavior between late- and early-flowering ecotypes is due to allelic variation at one or both of two loci, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Napp-Zinn, 1979; Burn et al., 1993b; Clarke and Dean, 1994; Koornneef et al., 1994; Lee et al., 1994b). Late-flowering ecotypes contain dominant alleles of *FRI* and *FLC* that suppress flowering, whereas early-flowering ecotypes contain recessive *fri* and/or *flc* alleles. Thus, *FRI* and *FLC* act synergistically to delay flowering, and the effects of *FRI* and *FLC* are suppressed by vernalization (Lee and Amasino, 1995). These genes, therefore, have been critical

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in the evolution of the vernalization-responsive, winter-annual habit of certain Arabidopsis ecotypes (Napp-Zinn, 1979; Levy and Dean, 1998).

In this study, we report the molecular cloning of *FLC* and the effect of *flc* null mutants on flowering. *FLC* encodes a novel MADS domain protein, and the levels of *FLC* mRNA are controlled by *FRI* and other genes in the autonomous flowering pathway. Furthermore, overexpression of *FLC* from a heterologous promoter is sufficient to delay flowering. We propose that the level of FLC activity acts by using a rheostat-like mechanism to control flowering time in Arabidopsis.

RESULTS

Analysis of *FLC* Mutants

Naturally occurring late flowering in Arabidopsis is caused primarily by the interaction of two genes, *FRI* and *FLC.* The late-flowering phenotype conferred by *FRI* is suppressed in the Landsberg *erecta* (L*er*) ecotype of Arabidopsis due to the recessive allele of the *FLC* gene (*flc*-L*er*) present in L*er* (Koornneef et al., 1994; Lee et al., 1994b). Similarly, late flowering caused by mutations in *LD* is also suppressed by *flc*-L*er* (Koornneef et al., 1994; Lee et al., 1994b). To study the nature of the L*er* allele of *FLC* and to determine specifically whether the ability to suppress the late-flowering phenotype of both *FRI* and mutations in *ld* could be conferred by *flc* loss-of-function mutations, we obtained additional *flc* alleles. In one screen, late-flowering *ld* plants (*ld-3*) were mutagenized with ethyl methanesulfonate (EMS), and one early-flowering M_2 plant was isolated that in complementation tests proved to contain a mutation allelic to the recessive allele of *FLC* found in the L*er* background. This allele was designated *flc-1.*

A second screen was performed using the late-flowering line homozygous for the late-flowering *FRI* allele from the ecotype San Feliu-2 (SF2) (*FRI*-SF2) in the Columbia (Col) background described in Figure 1A. Ninety-thousand seeds were treated with 5 to 6 krad of fast-neutron radiation, and 300,000 M_2 plants were screened for early flowering. Because *FLC*-Col and *FRI*-SF2 synergistically interact to cause late flowering, it was likely that mutations in either gene could cause early flowering. Early-flowering plants were crossed to two "tester" lines to determine whether mutations were present in *FRI* or *FLC.* One tester was homozygous for the *FRI*-SF2 in the L*er* background (*FRI-SF2* in L*er*), and the other was homozygous for the SF2 allele of *FLC* in the L*er* background. Because these alleles of *FRI* and *FLC* behave dominantly, M_2 plants containing a lesion in *FRI* would be early flowering when crossed to the *FLC*-SF2–containing line and late flowering when crossed to the *FRI*-SF2–containing line. Conversely, plants with a mutation in *FLC* would be early flowering when crossed to the *FRI*-containing line and late flowering when crossed to the *FLC*-containing line. From this screen, four *flc* alleles (designated *flc-2* to *flc-5*) and three *fri* alleles were isolated.

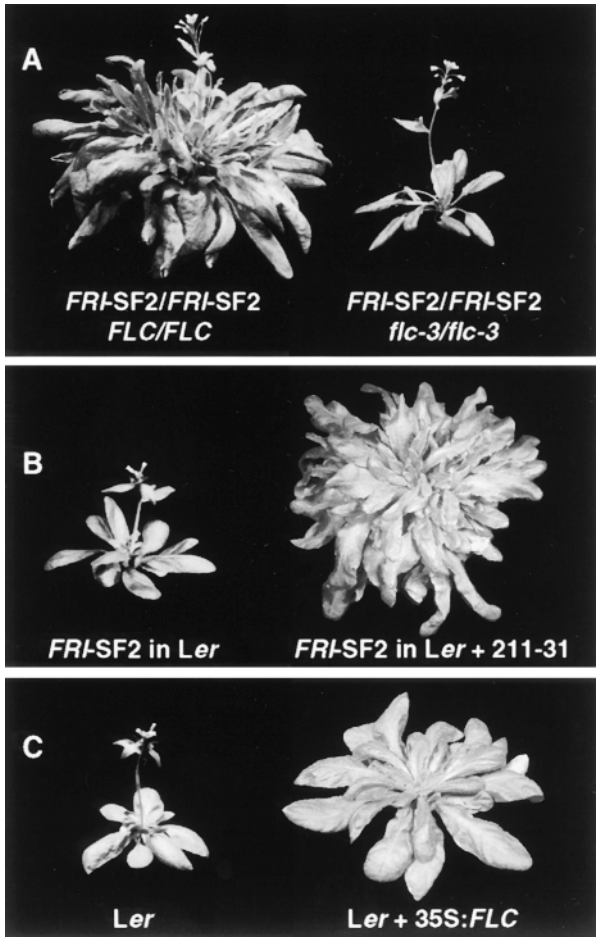

Figure 1. Flowering Phenotype of Lines Described in This Study.

(A) Homozygous *FRI*-SF2 in the Col background (left) and a fast-neutron-induced *flc* allele in the same genetic background (right).
(B) Homozygous *FRI*-SF2 in the L*er* background (left) and introduction of a genomic clone containing a late-flowering allele of *FLC* into a *FRI*-SF2 in L*er* (right).
(C) L*er* wild type (left) and introduction of constitutively expressed *FLC* into the L*er* background (right).

The effect of all the *flc* mutations on flowering time is similar to that of the early-flowering allele of *FLC* found in L*er* (Koornneef et al., 1994; Lee et al., 1994b). These *flc* alleles do not interact with *FRI*, and the extreme late flowering of *FRI*-SF2 in Col is eliminated in the presence of *flc* mutations, as shown in Figure 1A and Table 1. All *flc* alleles behave indistinguishably and represent the null phenotype (the lesions in these alleles are described below). Similarly, *FRI* mutants also become nearly identical in flowering time to Col, suggesting that Col contains a nonfunctional allele of *FRI.* Thus, our mutational analysis confirms the models de-

veloped from work with naturally occurring early- and late-flowering alleles of *FRI* and *FLC*: *FRI* and *FLC* interact synergistically to delay flowering, and a loss of function in either gene causes the late-flowering phenotype to be eliminated.

Isolation of FLC by Positional Cloning

To generate a segregating population useful for high-resolution mapping and positional cloning of *FLC*, we crossed the F_1 plants generated from a cross of L*er* to Col to the tester line described above containing the *FRI*-SF2 allele in L*er.* This line contains a late-flowering allele of *FRI* but also contains the *flc*-L*er* allele and is therefore early flowering. The progeny of the cross of the F_1 plants to *FRI*-SF2 in L*er*, which segregate 1:1 for late and early flowering, are late flowering when *FLC*-Col is present due to the interaction between *FRI* and *FLC*, but early flowering in the presence of *flc*-L*er.* Test-cross progeny (4500 plants) were screened with the microsatellite markers nga158 and nga151 (Bell and Ecker, 1994), which previously had been shown to flank *FLC.* Plants containing recombination events between nga158 and nga151 were then tested with a third microsatellite marker nga249, which revealed that *FLC* resided in the interval between nga249 and nga151.

The region between nga249 and nga151 was contained within four yeast artificial chromosome (YAC) clones (see Methods). To generate additional markers, we determined the DNA sequence of YAC end clones and designed primers to amplify the corresponding sequences from L*er* and Col. These DNAs from L*er* and Col were sequenced to identify single-nucleotide changes, which then were used to create derived cleaved amplified polymorphic sequence markers (Michaels and Amasino, 1998; Neff et al., 1998). Markers derived from the left and right ends of YAC CIC1B8 detected recombination events on either side of *FLC*, demonstrating that *FLC* resided in the 620-kb interval spanned by CIC1B8.

A group of 13 BAC, TAC, and P1 clones have been identified by the Kazusa Arabidopsis genome project that span CIC1B8. These clones have insert lengths of ~70 to 100 kb and were used as probes on DNA blots with EcoRV-digested DNA from plants containing fast-neutron-induced *flc* mutations. Two overlapping clones, K6M1 and MYB9, shown in Figure 2A, detected several deleted bands in *flc-2.* Random 10- to 20-kb fragments of K6M1 and MYB9 resulting from partial digestion with Sau3A1 were used to create a library in the binary vector pPZP211 (Hajdukiewicz et al., 1994), and individual clones from this library were used to transform the *FRI*-SF2 in L*er* line. The library was constructed with DNA from the Col background, which contains a late-flowering allele of *FLC.* Thus, *FRI*-SF2 in L*er* plants transformed with a construct containing the Col allele of *FLC* will be late flowering. One of the clones from this library, 211-31, produced T_1 plants that were very late flowering. More than one-third of the plants underwent senescence without flowering after 8 months of growth (Figure 1B).

Sequencing revealed three putative genes in 211-31 with similarity to MADS box transcription factors, a major pollen allergen, or *ETHYLENE INSENSITIVE3* (Figure 2A). To determine which gene represented *FLC*, we examined the three candidate genes from two additional fast-neutron *flc* alleles, *flc-3* and *flc-4*, and one EMS-generated allele, *flc-1.* Both of the fast-neutron alleles showed polymorphisms in bands resulting from the MADS box transcription factor. Determination of the DNA sequence of *flc-1*, *flc-3*, and *flc-4* revealed that all contained lesions in the first exon of the MADS box transcription factor. *flc-3* contains a 104-bp deletion that removes the start codon, and *flc-4* contains a 7-bp deletion that results in a frameshift after the first 20 amino acids. *flc-1* contains a single-base transition at the first exon–intron junction that changes the conserved GT donor site to AT and presumably disrupts splicing.

The genomic organization of the *FLC* gene is shown in Figure 2A. *FLC* is composed of seven exons, and the first exon contains the conserved MADS box domain that is the site of the lesions in *flc-1*, *flc-3*, and *flc-4.* An *FLC* cDNA was isolated from the Col background. The deduced amino acid

Table 1. Flowering Times of *FLC*- and *FRI*-Containing Plant Lines

Line	Leaf Number at Flowering[a]	Description
Col	12.2 (.40)[b]	Col wild type (fri-Col/fri-Col; *FLC*-Col/*FLC*-Col)
FRI-SF2 in Col	67.8 (4.7)	Homozygous for the SF2 allele of *FRI* in the Col background, derived from the eighth backcross[c] (*FRI*-SF2/*FRI*-SF2; *FLC*-Col/*FLC*-Col)
flc-2 in *FRI*-SF2 in Col	11.8 (.75)	Fast-neutron *flc* allele in *FRI*-SF2 in the Col background (*FRI*-SF2/*FRI*-SF2; *flc-2*/*flc-2*)
flc-3 in *FRI*-SF2 in Col	11.3 (.47)	Fast-neutron *flc* allele in *FRI*-SF2 in the Col background (*FRI*-SF2/*FRI*-SF2; *flc-3*/*flc-3*)
flc-4 in *FRI*-SF2 in Col	11.5 (.50)	Fast-neutron *flc* allele in *FRI*-SF2 in the Col background (*FRI*-SF2/*FRI*-SF2; *flc-4*/*flc-4*)
FRI-SF2 in L*er*	12.2 (.40)	Homozygous for the SF2 allele of *FRI* in the L*er* background, derived from the tenth backcross[c] (*FRI*-SF2/*FRI*-SF2; *flc*-L*er*/*flc*-L*er*)
FN235	11.8 (.63)	Fast-neutron *fri* allele isolated from *FRI*-SF2 in the Col background (*fri*/*fri*; *FLC*-Col/*FLC*-Col)

[a] Represents an average of at least five plants.
[b] Numbers within parentheses represent one standard deviation.
[c] See Methods for more information on derivation.

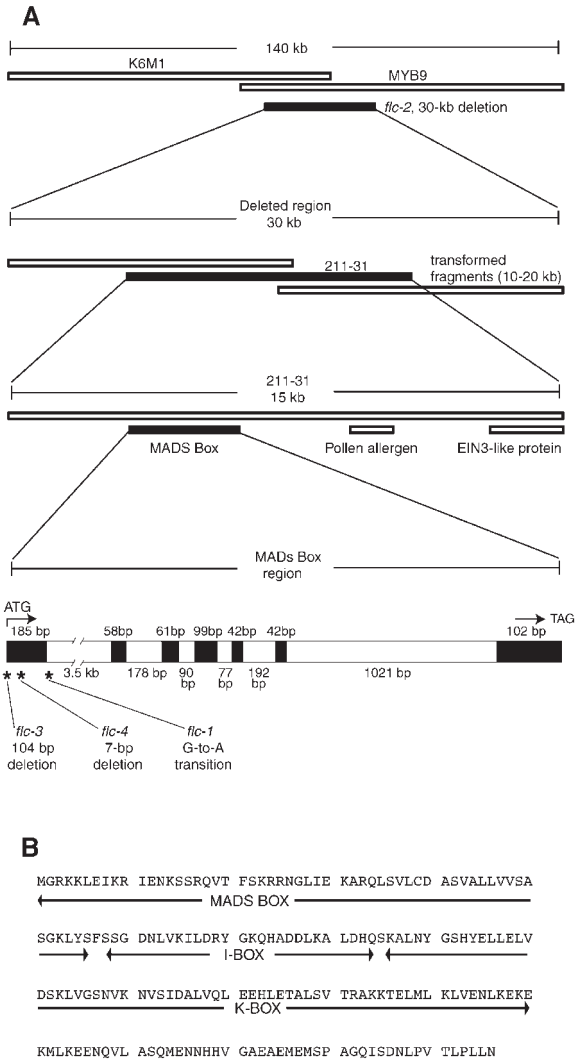

Figure 2. Cloning and Characterization of the *FLC* Gene.

(A) Summary of the positional cloning of *FLC*, genomic structure, and positions of mutations. Exons are represented by filled boxes, and lengths are give above; introns are represented by open boxes, and lengths are given below. Asterisks denote the positions of mutations.

(B) The predicted amino acid sequence of *FLC,* with the MADS, I, and K domains indicated.

sequence is highly related to MADS box–containing transcription factors and is shown in Figure 2B, with the positions of the MADS, I, and K boxes noted.

Expression of *FLC*

One model for the dependence of the late-flowering phenotype of *FRI* and *ld* mutations on the presence of an active *FLC* allele is that the role of *FRI* and *LD* is to control *FLC* expression. To investigate this possibility, we performed RNA gel blot analyses to compare the steady state levels of *FLC* mRNA in shoots of wild-type Wassilewskija (Ws) and *ld-3* in the Ws background, and a line homozygous for *FRI*-SF2 in the Col background and a *fri* mutant from the Col background. The results are shown in Figure 3A. The *FLC* transcript was only detected in the late-flowering lines containing *FRI* and the *ld-3* mutation. Thus, *FRI* acts to increase the level of *FLC* mRNA, and *LD* acts to decrease the level.

The effects of developmental stage and photoperiod on the level of *FLC* mRNA were also examined in shoots of the *FRI*-SF2 in the Col line. Shoots were collected for RNA isolation from plants grown in long days at 4, 6, 9, 11, 13, 15, and 21 days after germination and from short-day-grown plants at 4, 9, 11, and 15 days after germination. These plants had not flowered and had formed ~18 leaves in long days and 10 leaves in short days. *FLC* mRNA levels were not affected

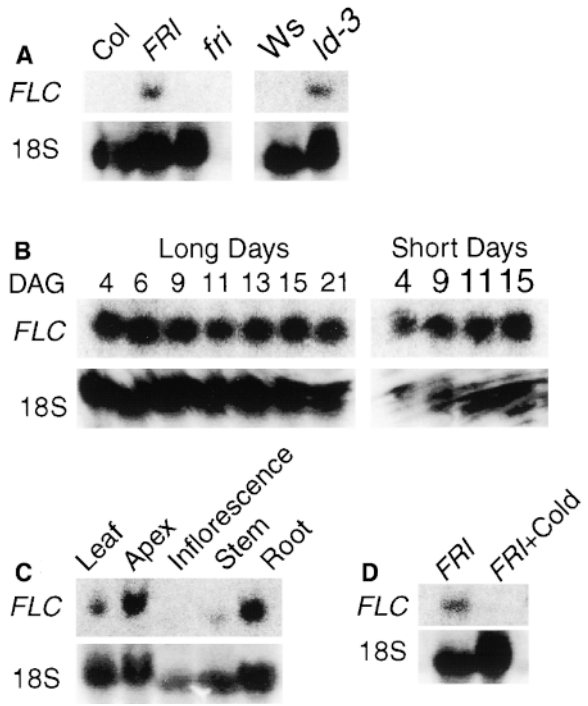

Figure 3. Expression of *FLC.*

(A) Expression of *FLC* in Col wild type (Col), *FRI*-SF2 in Col (*FRI*), a fast-neutron allele of *fri* in Col (*fri*), Ws wild type, and the *ld-3* mutant in the Ws background.

(B) Time course of *FLC* expression in RNA prepared from shoots. Time is measured as days after germination (DAG).

(C) Expression of *FLC* in various organs.

(D) Suppression of *FLC* expression by 30 days after cold treatment. Plants were grown for 10 days at 22°C before harvesting. Blots were probed with 18S rDNA as a control for loading.

by photoperiod or by plant age during vegetative development (Figure 3B).

The tissue distribution of *FLC* mRNA in *FRI*-SF2 in the Col line was examined by gel blot analysis of RNA isolated from leaves, vegetative apices, stems of flowering plants, roots, and the inflorescence (tissue from the first open flower to the shoot apical meristem) (Figure 3C). *FLC* is expressed most highly in the vegetative apex and in root tissue, but it is also detectable in leaves and stems. It is interesting that in young tissues of the inflorescence, *FLC* mRNA is not detectable, which is in sharp contrast with the high level in young tissues of the vegetative apex. This indicates that *FLC* expression is downregulated in the apex after the transition to flowering.

Effect of Vernalization on *FLC* Expression

As discussed above, the late-flowering phenotype of *FRI* and *ld* can be suppressed by loss-of-function *flc* mutations. The late-flowering phenotype of *FRI* and *ld*, in the presence of an active *FLC* allele, can also be suppressed by vernalization (Lee and Amasino, 1995). Therefore, the promotion of flowering by vernalization might involve a modulation of *FLC* expression. To explore this possibility, we determined the effect of cold treatment on *FLC* mRNA levels. Imbibed seeds of the *FRI*-SF2 in Col line were cold treated for 30 days and then transferred to soil with imbibed seeds of control plants that had not been cold treated. Shoots were collected after 10 days of growth for RNA gel blot analysis. The *FLC* transcript level was reduced after vernalization to undetectable levels (Figure 3D), similar to the difference in expression seen between *FRI*-SF2 in Col and *fri* null mutants (Figure 3A). Thus, the elimination of the late-flowering phenotype of *FRI*-SF2 in Col by vernalization is accompanied by a corresponding decline in *FLC* expression. It should also be noted that control plants, which were not cold treated and which exhibited high *FLC* expression, and the vernalized plants, which do not have detectable *FLC* expression, were both grown from seeds that were harvested from vernalized plants. This demonstrates that the reduction of *FLC* expression in vernalized plants is not meiotically stable (i.e., the decreased expression is not carried through to the next generation).

Increased *FLC* Expression Causes Late Flowering

As part of the positional cloning of *FLC* described above, the genomic clone containing *FLC*, 211-31, was transformed into a line homozygous for the SF2 allele of *FRI* in L*er*. This line normally flowers in long days after forming ~12 primary rosette leaves (Table 1; Lee et al., 1994b), but lines in which 211-31 had been introduced into *FRI*-SF2 in L*er* exhibited a severe delay in flowering due to the synergistic interaction of *FRI* with an active *FLC* allele (Figure 1B). Moreover, the severity of the late-flowering phenotype often greatly exceeded that of genetically constructed lines homozygous for the same alleles of *FRI* and *FLC*. Specifically, eight of 19 lines into which 211-31 had been introduced formed >80 rosette leaves and underwent senescence without flowering, even when grown under continuous far-red-enriched light conditions that always cause natural *FRI*/*FLC* homozygotes to flower after forming <60 rosette leaves. The extreme late flowering of some of the 211-31 transformants might have been due to position effects or multiple insertion events that cause higher levels of *FLC* expression than that found in natural *FRI*/*FLC* homozygotes.

To determine whether enhanced *FLC* expression is sufficient to cause late flowering in the absence of *FRI* or a late-flowering mutant allele such as *ld*, we introduced into L*er* the genomic coding region of *FLC* under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. (L*er* lacks FRI activity and contains functional alleles of the flowering-time genes defined by late-flowering mutants [Lee et al., 1994b; Koornneef et al., 1998].) Of the transgenic plants into which the 35S::*FLC* construct was introduced, 35% exhibited a moderate delay in flowering time (flowering after forming 10 to 20 leaves) and 35% were very late flowering (flowering after forming >45 leaves; Figure 1C). Under these growth conditions, L*er* flowers with seven or eight leaves. Thus, overexpression of *FLC* alone is sufficient to cause a significant delay in flowering time.

DISCUSSION

FLC was first identified as a suppressor of the late-flowering phenotype conferred by *FRI* and *ld* mutations. The suppressor allele was uniquely found in the L*er* ecotype of Arabidopsis (Koornneef et al., 1994). In this study, we describe the characterization of null mutants and demonstrate that suppression of the late-flowering phenotype is due to loss of *FLC* function. Thus, the role of active *FLC* alleles is to repress flowering. We have identified the *FLC* gene by positional cloning and found that it encodes a MADS domain protein. After this article was submitted to be considered for publication, Sheldon et al. (1999) reported that a DNA insertion into a MADS box gene caused delayed flowering. Sequence comparison showed that this DNA insertion creates an allele of *FLC* that dominantly delays flowering.

The MADS domain proteins comprise a large family of transcription factors in plants. In Arabidopsis, >28 family members have been identified (Riechmann and Meyerowitz, 1997), and they appear to play diverse roles in development. For example, *AGAMOUS-LIKE15* (*AGL15*) is expressed preferentially in developing embryos (Heck et al., 1995; Rounsley et al., 1995), *AGL3* is expressed in all tissues of the shoot (Ma et al., 1991), and *AGL12*, *AGL14*, and *AGL17* are expressed only in roots (Rounsley et al., 1995).

The most well characterized of the MADS domain proteins

are those involved in floral development, such as *AG*, *APETALA1* (*AP1*), *APETALA3* (*AP3*), *PISTILLATA*, and *CAULIFLOWER* (reviewed in Riechmann and Meyerowitz, 1997). Among the MADS domain genes of known function, the floral meristem identity genes *AP1* and *CAULIFLOWER* may have a biological role most similar to *FLC.* These genes act early in the development of individual flowers downstream of *FLC*'s role in floral induction. FLC exhibits amino acid sequence similarity to CAULIFLOWER (20%) and to AP1 (30%), but FLC is also 30% identical to AGL15, which is believed to not have a role in floral development. The sequence with the strongest similarity to *FLC* comes from a sequenced bacterial artificial chromosome clone (GenBank accession number AC002291), with a predicted amino acid sequence that is 58% identical to *FLC.* Thus, this gene and *FLC* appear to define a new subfamily of MADS domain proteins distinct from MADS domain proteins involved in floral development.

Based on the interactions of *FRI*, *LD*, and *FLC*, we proposed a model in which the role of *LD* is to counteract the *FLC*-mediated inhibition of flowering and the role of *FRI* is to augment this inhibition (Lee et al., 1994b). The results presented in this study are consistent with this model, shown in Figure 4, and reveal some of the molecular details. In late-flowering lines that contain dominant, late-flowering alleles of *FRI*, *FLC* mRNA levels are relatively high. In an early-flowering *fri* null mutant, *FLC* mRNA levels are below our limits of detection. *LD* acts to decrease *FLC* mRNA levels because in an *ld* null mutant, *FLC* mRNA levels are similar to those in a *FRI*-containing line. Thus, the similar flowering phenotypes of plants containing recessive "late-flowering" mutations in floral-promoting genes in the autonomous pathway and plants containing *FRI* are likely to be due, at least in part, to the increase of *FLC* expression in both situations. Indeed, we find that mutations in several genes in the autonomous pathway result in an elevation of *FLC* expression similar to that in the *ld* mutation (S.D. Michaels and R.M. Amasino, unpublished results), which provides a molecular model for the strong enhancement of the late-flowering phenotype of many autonomous pathway mutations by an active *FLC* allele (Sanda and Amasino, 1996b).

Except for *ld* and *flowering locus d* mutations, however, other autonomous pathway mutations as well as *FRI* cause a measurable late-flowering phenotype in the L*er* genetic background, which appears to lack *FLC* activity (Koornneef et al., 1991; Sanda and Amasino, 1996b). Thus, *FRI* and other autonomous pathway genes may have roles in flowering-time control in addition to the regulation of *FLC* expression, whereas *LD* and *FLD* may act solely to control *FLC* activity.

Another common feature of plants containing late-flowering mutations in autonomous pathway genes and plants containing *FRI* is the suppression of the late-flowering phenotype by vernalization (Lee and Amasino, 1995; Koornneef et al., 1998). For example, the late-flowering effect of *FRI* is completely eliminated by 40 days of cold treatment (Lee and Amasino, 1995). Our results demonstrate that vernalization eliminates the *FRI*-mediated enhancement of *FLC* expression; after cold treatment of a *FRI*-containing line, *FLC* mRNA levels were undetectable. *FLC* mRNA levels were also undetectable in an early-flowering *fri* null mutant that had not been cold treated. Thus, the vernalization suppression of *FLC* expression could be mediated through the effect of cold treatment on *FRI* activity. However, the late-flowering phenotype of an autonomous pathway mutant, such as *ld*, in a *fri* null background is also completely eliminated by vernalization; thus, we favor a model in which the vernalization pathway can directly modulate *FLC* expression rather than acting through other autonomous pathway genes such as *FRI* and *LD.*

A model for the interactions of *FLC* with *FRI* and *LD* and other autonomous pathway genes and the effect of vernalization is presented in Figure 4. In this model, *FLC* activity plays a central role in the control of flowering time in the autonomous pathway, and *FLC* activity is negatively regulated by vernalization and positively and negatively regulated by other genes in the pathway. The results of overexpression studies are also consistent with this model: overexpression of *FLC* alone (i.e., in the absence of *FRI* or *ld* mutations) is sufficient to cause late flowering and phenocopy the effect of *FRI* and *ld* mutations.

Several lines of evidence indicate that *FLC* suppresses flowering by a rheostat mechanism in which the level of *FLC* activity is proportional to the lateness to flower. For example, our previous work has demonstrated that active *FLC* alleles delay flowering in a semidominant manner in *FRI*-containing or *ld* mutant lines (Lee et al., 1994a). Furthermore, in this study, the transgenic lines in which *FLC* has been introduced under the control of its native promoter or under the control of the CaMV 35S promoter exhibit a broad range of flowering times, indicating that variation in *FLC* expression, presumably due to transgene copy number and/or position effects, has a large influence on flowering time. In fact many of these transgenic lines never flowered under conditions that readily promote flowering of lines naturally

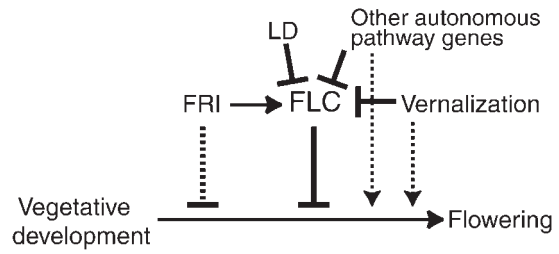

Figure 4. Model for the Interaction of *FLC*, *FRI*, *LD*, and Vernalization in the Regulation of Flowering in the Autonomous Pathway.

Boldface lines indicate interactions with FLC, and dashed lines indicate other effects that occur in the absence of FLC activity.

homozygous for late-flowering alleles of *FRI* and *FLC*, indicating that expression at levels higher than those naturally occurring in *FRI*/*FLC* homozygotes can cause a substantial further delay in flowering.

Studies of *FLC* mRNA distribution reveal that *FLC* is expressed most highly in the vegetative shoot apex and in roots. This pattern of expression is similar to that of other autonomous pathway genes such as *LD* and *FCA* (Macknight et al., 1997; Aukerman et al., 1999). The high level of expression in the vegetative shoot apex of *FRI*-containing lines is consistent with a role for *FLC* in shoot apical meristem cells to locally inhibit the floral transition. The lack of detectable *FLC* mRNA in the inflorescence apex of *FRI*-containing lines indicates that a decrease in *FLC* expression is associated with the transition to flowering, and it is possible that this decrease is necessary for the floral transition to occur. The lack of detectable *FLC* mRNA in shoots of lines that have been vernalized also suggests that a decrease in *FLC* expression may be a necessary component of the vernalization response.

A hallmark of vernalization is that the vernalized state is maintained for long periods of time and through many mitotic cell divisions after the cessation of the inductive cold treatment. We have shown that prolonged cold treatment causes *FLC* mRNA levels in the shoot to decrease to undetectable levels, and this decline is maintained after cold-treated plants are returned to optimal warm growing conditions. Another hallmark is that the vernalized state is lost in the next generation after meiosis and sexual reproduction. The decrease in *FLC* expression after cold treatment also has this feature; vernalized plants produce seeds that develop into plants in which a high level of *FLC* expression is restored.

The expression of *FLC* in leaves and roots is also consistent with a role for a stable change in *FLC* expression contributing to the maintenance of the vernalized state. Although vernalization appears to cause a change that renders the shoot apical meristem competent to flower (Lang, 1965), cells from other regions of cold-treated plants, such as leaves (Metzger, 1988) and roots (Burn et al., 1993a), can regenerate into plants in which the shoot apical meristem exhibits the vernalized state. Thus, cells other than those of the shoot apical meristem must contain a system to affect a stable change in response to cold treatment, and a decrease in *FLC* expression may represent a component of that stable change in many cell types.

METHODS

Physical Map of the *FLOWERING LOCUS C* Region

The chromosomal positions of the microsatellite markers, yeast artificial chromosomes (YACs), bacterial artificial chromosomes, transformation-competent artificial chromosomes, and P1 clones described below are available at the Kazusa Arabidopsis data opening site: http://www.kazusa.or.jp/arabi/.

Creation of Lines

The following lines used in this study have been described previously: lines homozygous for *FRIGIDA* (*FRI*)-SF2 in Landsberg *erecta* (L*er*), *FLOWERING LOCUS C* (*FLC*)-SF2 in L*er*, *FRI*-SF2 in Columbia (Col; Lee et al., 1994b), Col and *FRI* in Col (Lee and Amasino, 1995), and Wassilewskija (Ws) and *luminidependens-3* (*ld-3*) in Ws (Lee et al., 1994a).

Plant Growth Conditions

All plants were grown under cool-white fluorescent light at 22°C. Long-day conditions consisted of 16 hr of light followed by 8 hr of darkness; short-day conditions consisted of 8 hr of light followed by 16 hr of darkness. For experiments involving vernalization, imbibed seeds were incubated at 2°C on agar-solidified medium containing 0.65 g/L Peters Excel 15-5-15 fertilizer (Grace Sierra, Milpitas, CA). During cold treatment, plants were kept under short-day conditions.

RNA Gel Blot Analysis

Total RNA was isolated using RNA Isolator (Genosys Biotechnologies, The Woodlands, TX) according to the manufacturer's instructions. For RNA gel blots, 15 to 20 μg of RNA was separated by denaturing formaldehyde–agarose gel electrophoresis as described previously (Sambrook et al., 1989). RNA gel blots were probed with a ^{32}P-ATP–labeled cDNA fragment that did not contain the conserved MADS box domain. Blots were also probed with an 18S rRNA probe as a control for the quantity of RNA loaded.

Fast-Neutron and Ethyl Methanesulfonate Mutagenesis

The ethyl methanesulfonate (EMS) mutagenesis of *ld-3* seeds was conducted as follows. Eight thousand dry seeds were soaked in 0.5% EMS for 8 hr at 20°C and rinsed in eight changes of water over a 2-hr period. Seeds were then sprayed onto soil after being suspended in a solution of 0.1% agarose. Of the 8000 M_1 seeds planted, ~5000 survived to set seed and were harvested in 50 pools.

For the mutagenesis of *FRI*-SF2 in Col by fast-neutron radiation, three aliquots of 50,000 seeds each were sent to the nuclear reactor at the Plant Breeding Unit of the Food and Agriculture Organization of the United Nations and International Atomic Energy Agency Program (Seibersdorf, Austria) where they were treated with 5, 5.5, or 6 krad of radiation. Seeds were subsequently sprayed onto soil in solution of 0.1% agarose. Only small differences were seen in viability in the M_1 generation between the lots that had been exposed to different amounts of radiation and 60 to 70% of seeds germinated and survived to set seed. M_1 seeds were harvested in 98 pools.

Analysis of Fast-Neutron Lines

Fast-neutron-generated lines known genetically to contain mutations in *FLC* were used to search for lesions in the three candidate genes contained on rescuing clone 211-31. Polymerase chain reaction was

used to amplify 1- to 2-kb fragments that spanned the candidate genes. The amplified DNA was then cleaved with RsaI or TaqI to generate smaller fragments, separated on 5% nondenaturing acrylamide gels, and visualized with ethidium bromide.

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