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The *FLF* MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation

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A MADS box gene, *FLF* (for *FLOWERING LOCUS F*), isolated from a late-flowering, T-DNA–tagged Arabidopsis mutant, is a semidominant gene encoding a repressor of flowering. The *FLF* gene appears to integrate the vernalization-dependent and autonomous flowering pathways because its expression is regulated by genes in both pathways. The level of *FLF* mRNA is downregulated by vernalization and by a decrease in genomic DNA methylation, which is consistent with our previous suggestion that vernalization acts to induce flowering through changes in gene activity that are mediated through a reduction in DNA methylation. The *flf-1* mutant requires a greater than normal amount of an exogenous gibberellin (GA_3) to decrease flowering time compared with the wild type or with vernalization-responsive late-flowering mutants, suggesting that the *FLF* gene product may block the promotion of flowering by GAs. *FLF* maps to a region on chromosome 5 near the *FLOWERING LOCUS C* gene, which is a semidominant repressor of flowering in late-flowering ecotypes of Arabidopsis.

INTRODUCTION

The naturally occurring ecotypes of Arabidopsis differ in the length of their vegetative phase preceding the transition to reproductive development. Under short-day controlled growth conditions, the later-flowering ecotypes take many months to flower but will flower earlier if the germinating seeds or plantlets are exposed to a low-temperature regime (vernalization). Without low-temperature treatment, plants of the late-flowering ecotypes continue to form leaves so that at the time they produce the first flowering bolt, they have many more leaves in the rosette than do the early-flowering ecotypes.

Genetic analyses of early- and late-flowering ecotypes have identified two loci that account for most of the differences in flowering time. The products of these two loci, *FRIGIDA* (*FRI*; Napp-Zinn, 1985; Burn et al., 1993b; Lee et al., 1993) and *FLOWERING LOCUS C* (*FLC*; Clarke and Dean, 1994; Koornneef et al., 1994; Lee et al., 1994b), appear to act synergistically to cause late flowering. This behavior is inferred from data showing that ecotypes with late alleles at both loci (e.g., ecotypes Pitztal and Stockholm) have an extended vegetative phase, whereas ecotypes with a late allele at one of the loci and an early allele at the other locus (e.g., C24 and Columbia [Col]) show only a slight delay in flowering relative to ecotypes that have early alleles at both loci (e.g., Landsberg *erecta* [L*er*]). These genes have not been isolated, and nothing is known about the proteins that they encode. Whether their mode of action in repressing flowering is to directly control transcription of key flowering induction genes or whether it is more indirect remains to be determined. The late *FRI* alleles are dominant, and the late *FLC* alleles are semidominant in their action (Michaels and Amasino, 1995). The repression of flowering time by *FRI* and *FLC* ensures that flowering in the various ecotypes will occur under favorable spring temperatures.

Other genes controlling the timing of flowering in Arabidopsis have been identified by the generation of mutants (reviewed in Koornneef et al., 1998). Some of these genes have been cloned (reviewed in Levy and Dean, 1998). Approximately 12 loci at which mutations result in late flowering have been placed into three groups according to their response to environmental signals, such as vernalization and photoperiod (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Bagnall, 1992, 1993; Chandler and Dean, 1994; Lee et al., 1994a). The existence of different

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classes of late-flowering mutants combined with double mutant analyses (Koornneef et al., 1991) have led us and others to propose that there are at least three metabolic pathways capable of inducing flowering in Arabidopsis (Burn et al., 1993a; Koornneef et al., 1998; Levy and Dean, 1998): a photoperiod-responsive pathway, a vernalization-dependent pathway, and an autonomous pathway. If the autonomous pathway is blocked by late alleles of *FRI* and *FLC* or by the mutant alleles of genes normally active in this pathway, then Arabidopsis is reliant on the vernalization-dependent pathway for early flowering.

In this study, we report on a gene, *FLOWERING LOCUS F* (*FLF*), for which increased gene activity at the locus causes a severe delay in flowering. A comparison of homozygotes and heterozygotes for the late-flowering mutant allele *flf-1* has shown that the extent of the delay in flowering time correlates with the steady state level of *FLF* mRNA. Some transgenic plants overexpressing *FLF* under the control of a constitutive cauliflower mosaic virus 35S promoter also flower late, whereas plants with reduced levels of *FLF* flower early. The early-flowering plants were generated either by using a 35S::antisense construct or by insertion of the transposable element *Activator* (*Ac*) into the *FLF* gene.

We have concluded that *FLF* plays a central role in vernalization-dependent flowering because the level of the *FLF* transcript is dramatically downregulated by vernalization, and *FLF* mRNA levels are low in vernalization-nonresponsive ecotypes and mutants. The *flf-1* mutant requires a longer period of low-temperature treatment and a greater amount of a gibberellin (GA_3) to induce flowering than do other vernalization-responsive late-flowering mutants, suggesting that the *FLF* gene product may inhibit the action of GAs in the apex in promoting flowering. Consistent with our suggestion (Burn et al., 1993a; Finnegan et al., 1998) that a reduction in DNA methylation is involved in mediating the response to vernalization, *FLF* mRNA levels were reduced in plants in which an antisense methyl transferase construct reduced the level of DNA methylation. The *FLF* gene is a MADS box gene that defines a new role for this class of transcription factor in the control of flowering time. *FLF* maps to the same chromosomal region as *FLC*, and because both are semidominant repressors of flowering, it is possible that *FLF* and *FLC* are the same gene.

RESULTS

Insertion of Two Inverted Adjacent T-DNAs Produces the *flf-1* Late-Flowering Mutant

After transformation of Arabidopsis ecotype C24 with a T-DNA construct containing an *Ac* transposable element, individual T_0 plants were allowed to self, and the T_1 progeny were screened for families that flowered significantly later than did parental C24 plants. Some plants of family 14-58 flowered after 70 days compared with 30 days for the C24 control plants. Analysis of the progeny of one selfed late-flowering T_1 plant showed 53 that flowered late (flowering time $>$70 days) and 15 that flowered normally (flowering time 30 days). The late-flowering plants could be differentiated further into two classes: one that flowered between 70 and 90 days (class 1) and a second that flowered later than 150 days (class 2). Some of the plants had not flowered after a year of continuous vegetative growth. Progeny tests showed that selfed class 2 plants produced only class 2 progeny, whereas selfed class 1 plants segregated 1:2:1 for class 2, class 1, and normally flowering plants. This segregation pattern is consistent with a single semidominant mutation for lateness. We have designated the mutant locus, which was initially described in Dennis et al. (1997), *FLF*. We also note that a QTL region with a major effect on flowering time has since also been named *FLF* (Alonso-Blanco et al., 1998).

Nontransformed C24, shown in Figure 1A, and the homozygous mutant *flf-1*/*flf-1* (Figure 1B) produced leaves at a similar rate. In the *flf-1*/*flf-1* plants, after several months of growth, bolts arose from the internodes between the rosette leaves, elongated $\sim$2 to 3 cm, and formed aerial rosettes. This growth pattern gives the mutant plants a domelike appearance (Figure 1C), similar to that described for the *F* (*florens*) and *fld* (*flowering locus d*) mutants (Koornneef et al., 1994; Chou and Yang, 1998). The late-flowering phenotype is more extreme than are the phenotypes of the previously reported late-flowering mutants and ecotypes (Koornneef et al., 1991), with the possible exception of the *F* mutant (Koornneef et al.,1994).

DNA gel blot analysis of a late-flowering T_1 parent (*FLF*/*flf-1*) showed five T-DNA inserts. Figure 2A shows the segregation of these inserts in 16 of a total of 70 progeny analyzed from a selfed heterozygous T_1 plant. Only two inserts segregated with the late-flowering phenotype (bands 1 and 5). Analysis of plants containing only these two T-DNA inserts, which were obtained by backcrossing to nontransformed C24 plants, showed that the two inserts were adjacent and in inverted orientation (Figure 2B) and that no excision of the *Ac* elements had occurred.

A genomic DNA library from an *flf-1*/*flf-1* plant containing only the two linked T-DNA inserts was screened with a neomycin phosphotransferase II (*NPTII*) probe to isolate the DNA segments spanning the site of T-DNA insertion. Three overlapping clones were isolated from the left side of the T-DNA, and one clone was isolated from the right side (Figure 2C). These clones accounted for a total of 27 kb of plant DNA spanning the site of the T-DNA insertion. C24 genomic clones were isolated from a BamHI library by using probes from this defined region (probes 1 and 2; Figure 2C): clones containing a 6.5-kb segment spanning the insertion site and a 7.3-kb segment downstream of the insertion site (Figure 2D) were sequenced.

The *FLF* region was mapped to the top of chromosome 5, 4 centimorgans from restriction fragment length polymor-

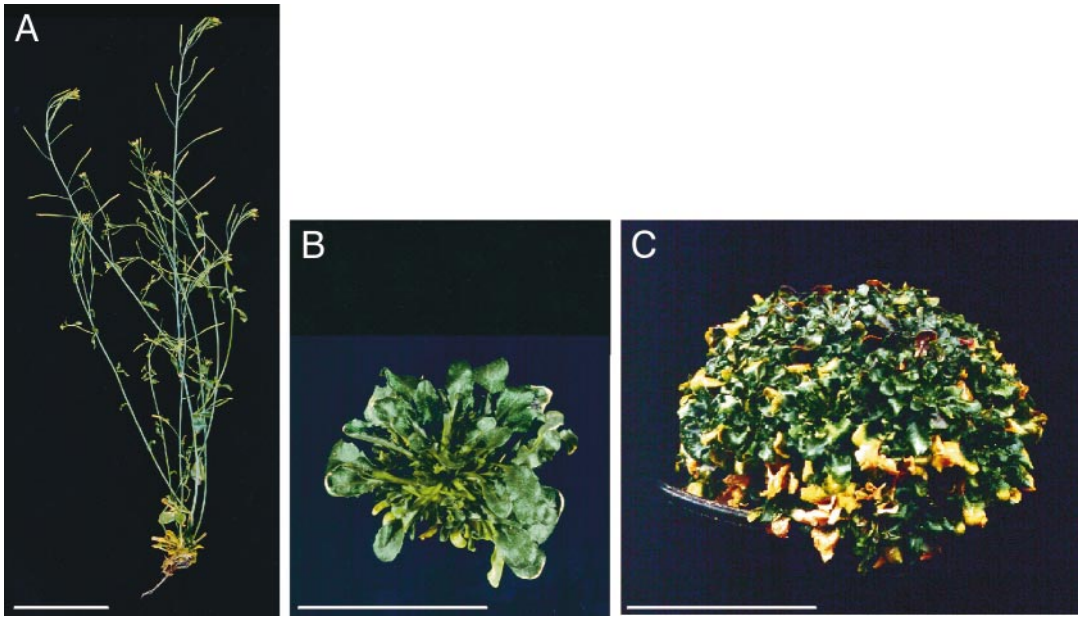

Figure 1. Late-Flowering Phenotype of the Homozygous *flf-1* Mutant.

(A) Wild-type C24.
(B) The *flf-1*/*flf-1* mutant at 70 days after germination.
(C) The *flf-1*/*flf-1* mutant at 150 days showing the dome shape caused by the aerial rosettes.
Bars = 5 cm.

phism (RFLP) marker 447, placing the gene in the vicinity of the *FLC* locus that is involved in the regulation of flower initiation (Koornneef et al., 1994; Lee et al., 1994b).

The T-DNAs Present in the *flf-1* Mutant Increase Expression of Two Genes Flanking the Insertion Site

Transcriptionally active regions near the two adjacent T-DNAs were identified by screening cDNA libraries with probes derived from plant DNA flanking the T-DNA inserts (Figure 2C, probes 2 and 3). Full-length clones were isolated, with probe 3 identifying gene A and probe 2 identifying gene B. Relative to the C24 genomic sequence, the T-DNAs had inserted between the two transcribed regions, 591 bp downstream of the polyadenylation site of gene A and 2.3 kb upstream of the start codon of gene B. The *flf-1* mutant had a 30-bp deletion immediately downstream of the insertion site, presumably a consequence of the T-DNA insertion process.

Because neither transcribed region was disrupted by the insertion of the T-DNAs, we investigated whether the expression of genes A and B was altered in the mutant. Figure 3 shows a gel blot of RNA isolated from 30-day-old C24 and heterozygous and homozygous *flf-1* plants grown under identical conditions. Antisense riboprobes specific for either gene A or gene B revealed that both genes are more highly expressed in the *flf-1* homozygote than in C24, with the 1.5-kb gene A transcript being ~10 times more highly expressed in the mutant and the 1.0-kb gene B transcript being overexpressed approximately twofold. There was an intermediate level of expression of both genes in the heterozygous mutant. These results suggest that the mutant phenotype results from overexpression of one of these two genes. We previously suggested (Dennis et al., 1997) that gene A could be *FLF*; however, we were unable to mimic the *flf-1*–conferred phenotype in transgenic plants overexpressing gene A.

Insertion of an *Ac* Element into Gene B Results in Early Flowering

In one seed lot of bulked seed from *flf-1* late-flowering plants, two early-flowering plants (M_1 plants termed *flf-2* and *flf-3*) were identified. Both plants flowered at 18 days, which is earlier than C24 that flowered at 30 days. Polymerase chain reaction (PCR) analysis using primers from within the T-DNA and from the flanking genomic sequence confirmed that these early-flowering plants were derived from the *flf-1* mutant and were not contaminants.

The two adjacent T-DNAs present in the *flf-1* mutant each contain an *Ac* element (*Ac1* and *Ac2*; Figure 2B). DNA

isolated from bulked M_2 progeny of *flf-2* and *flf-3*, digested with EcoRI, and probed with the 3′ region of *Ac* (Lawrence et al., 1993) showed that the two original *Ac* elements had remained in the T-DNAs and that a new *Ac* element was present (*Ac3*; Figure 4A). Probe 4 revealed that *Ac3* had inserted into a 2.7-kb EcoRI segment, resulting in a new 4.8-kb band (Figure 4B). The presence of a faint 2.7-kb band suggests that *Ac3* may have excised in some of the M_2 plants, resulting in the regeneration of the original-sized band. Individual early-flowering M_2 plants contained only the 4.8-kb band, indicating that these plants were homozygous for the insertion of *Ac3*. PCR using a primer from the 3′ end of *Ac* and primers near the EcoRI sites at either end of the 2.7-kb EcoRI fragment showed that *Ac3* had inserted within intron I in gene B (Figure 4C). RNA isolated from rosette leaves of early-flowering M_2 plants and from C24 and *flf-1*

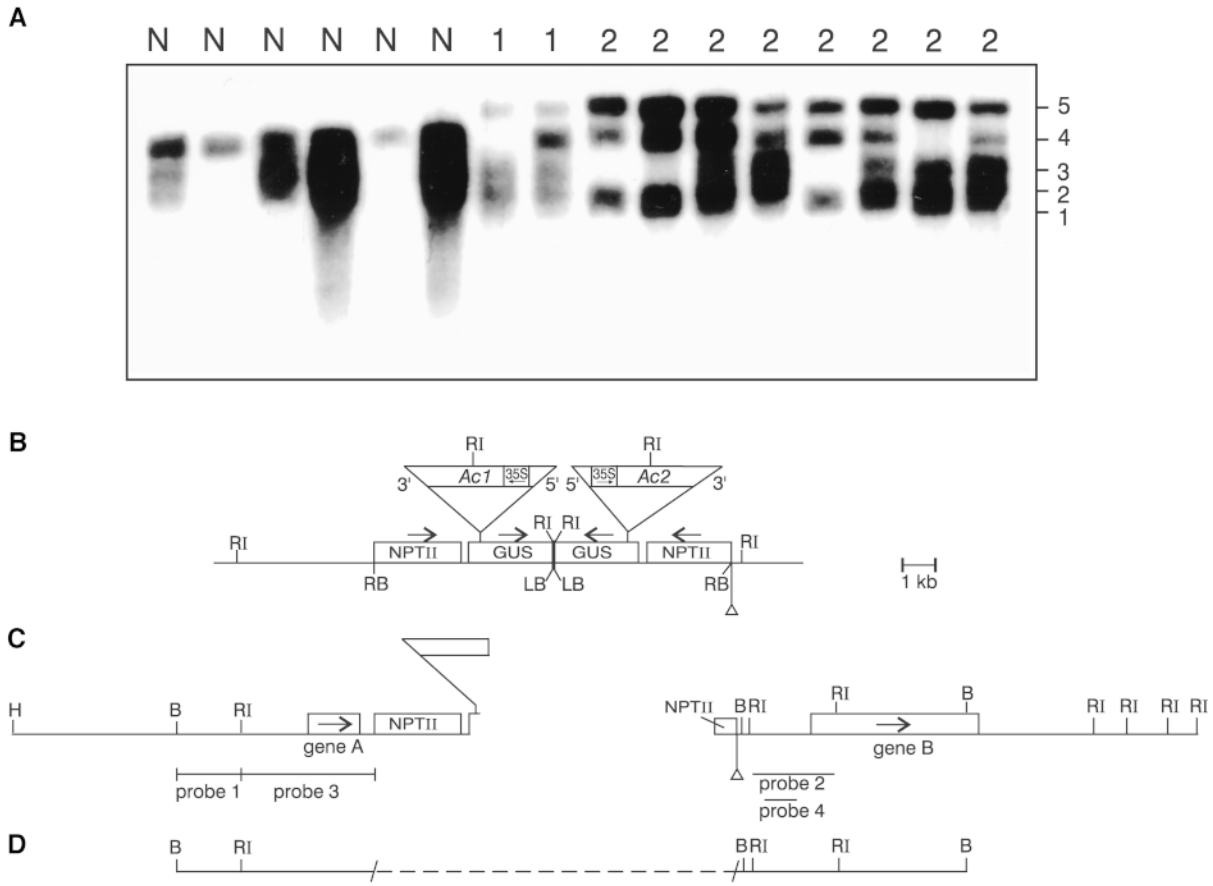

Figure 2. Segregation of T-DNA Inserts and Genomic Organization of the *FLF* Region.

(A) Genomic DNA isolated from a T_2 population segregating for normally flowering plants (N) and class 1 (1) and class 2 (2) late-flowering plants, digested with EcoRI, and probed with the *NPTII* gene. Only bands 1 and 5 segregate with the late-flowering phenotype. Numbers at right indicate bands.

(B) Map of the two T-DNA inserts linked to the *FLF* locus showing their orientation. EcoRI sites are labeled RI; LB and RB represent the left and right borders, respectively, of the T-DNA. The triangle represents the site of deletion of 30 bp to the right of the T-DNA. The arrows represent the direction of transcription of the genes. GUS, β-glucuronidase gene.

(C) Depiction of *flf-1*/*flf-1* mutant genomic clones isolated with an *NPTII* probe, spanning a 27-kb region of DNA, including genes A and B. The diagram is aligned to **(B)** to show the location of the T-DNA inserts. DNA fragments from the flanking plant DNA (probes 2 and 3) were used as probes to isolate cDNA clones. The restriction enzyme sites indicated are B, BamHI; H, HindIII; RI, EcoRI. The positions of genes A and B are shown, and their directions of transcription are indicated by arrows.

(D) The BamHI genomic clones isolated from a C24 wild-type genomic library with probes 1 (6.5-kb clone) and 2 (7.3-kb clone) **(C)**, spanning the site of the T-DNA insertions. Restriction sites are as in **(C)**. The dotted line represents the sequence present in the mutant genomic DNA but absent from the C24 genomic clones (corresponding to the T-DNA and *Ac* sequences).

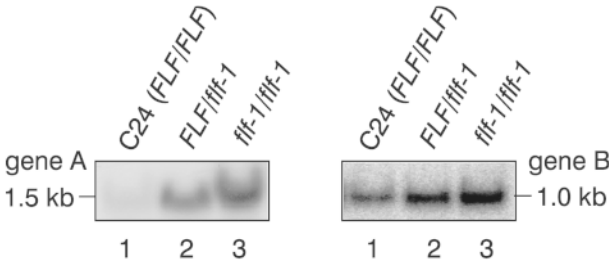

Figure 3. Expression Levels of Genes Flanking the T-DNA Insertion.

Expression of gene A and gene B transcripts in 30-day-old in vitro–grown wild-type C24 (*FLF*/*FLF*) plants (lanes 1) and *FLF*/*flf-1* heterozygous (lanes 2) and *flf-1*/*flf-1* homozygous (lanes 3) plants.

plants showed that in the early-flowering plants, the gene B transcript level was reduced to ~5% of the C24 expression level (Figure 4D), whereas there was no effect on the level of gene A expression. Presumably, the presence of *Ac3* reduces the gene B transcript level by reducing transcription of gene B, by interfering with splicing of the transcript, or by decreasing mRNA stability, resulting in the early-flowering phenotype.

Excision of *Ac3* from Intron I Causes Later Flowering and Increased Gene B Transcript Level

The flowering times of 20 progeny of the early-flowering M_1 *flf-2* plant were recorded. Fifteen M_2 plants flowered at 18 days, as did their M_1 parent; however, five plants flowered later than did their parent. The approximate days to bolting for these plants are as follows: *flf-4*, 100 days; *flf-5*, 50 days; *flf-6*, 85 days; *flf-7*, 50 days; and *flf-8*, 60 days. In the M_2 progeny of *flf-3*, 36 plants flowered at the same time as did their M_1 parent (18 days), but one plant flowered at 38 days (*flf-9*). DNA was isolated from individual plants. PCR analysis using primers derived from the intron sequence flanking the site of insertion and with primers from the 3′ end of *Ac* and the flanking intron sequence demonstrated that in the six late-bolting plants, *Ac3* had excised from one chromosome so that the plants were hemizygous for the presence of *Ac3* in intron I. Further analysis showed that the excision event in each of these plants was likely to have been independent.

Gene B expression level in the six late-bolting plants was compared with that of the *flf-1* mutant. In all six plants, the transcript level was higher than in the early-flowering parent and lower than in the homozygous *flf-1* mutant (Figures 4D and 4E), which is consistent with the late-bolting plants being heterozygous for the late allele. It seems that insertion of *Ac3* into intron I of gene B greatly reduced gene B transcript levels, resulting in early flowering, and that excision of *Ac3* from intron I restored a higher level of expression of the gene B transcript and resulted in later flowering. This provides compelling evidence that overexpression of gene B is the cause of the late-flowering phenotype in the *flf-1* mutant. Gene B is hereafter referred to as *FLF.*

Transgenic Plants Overexpressing *FLF* Have Altered Flowering Times

To check whether overexpression of the *FLF* coding sequence is sufficient to cause late flowering, we generated transgenic plants in which the *FLF* cDNA was under the control of a cauliflower mosaic virus 35S promoter (35S::*FLF*). Two of the 23 transgenic T_1 plants (the primary transformants) had a late-flowering phenotype: both plants produced leaves at a rate similar to nontransformed C24 plants and had not bolted after 100 days. Four plants bolted at approximately the same time as did C24 and 17 bolted earlier than did C24; eight of these early-flowering plants showed either full or partial sterility. Table 1 shows that a high level of *FLF* transcript was present both in the early-flowering progeny of two early-flowering T_1 plants and in the rosette leaves of two nonflowering T_1 plants. In a further experiment, we generated transgenic plants containing the same 35S::*FLF* construct in the L*er* ecotype. Of the 24 T_1 lines generated, none bolted earlier than did wild-type L*er* (20 days), nine bolted at ~20 days, three bolted at ~40 days (two of these exhibited floral abnormalities and partial sterility), and 12 had not bolted after 100 days. Rosette leaves of two nonflowering T_1 plants had a very high level of expression of the transgene (Table 1).

These data show that in L*er*, overexpression of *FLF* causes a delay in flowering, whereas in C24, *FLF* overexpression can result in a similar delay in flowering, but some plants flowered significantly earlier than did the controls. We expected that independent insertions in the different T_1 plants would result in flowering times differing in the degree of lateness compared with the parental line as a consequence of different levels of expression of the transgene; however, at this stage, we are not able to explain the early-flowering times in some C24 transgenic plants, which also have high levels of expression of the transgene.

Transgenic C24 plants also were generated with a construct in which the 3′ end of the *FLF* gene, lacking the MADS box region (see below), was placed in antisense orientation under the control of the 35S promoter. The majority of the 26 T_1 plants bolted significantly earlier than did C24 plants. The plant bolting earliest did so at 16 days. This early-flowering phenotype of the antisense plants parallels the early flowering in the *flf-2* and *flf-3* mutants, which showed a reduction in *FLF* mRNA.

The *FLF* Gene Is a New MADS Box Gene

Figure 5A depicts the structure of the *FLF* gene and shows the location of the six introns, with intron I being 3.5 kb. The

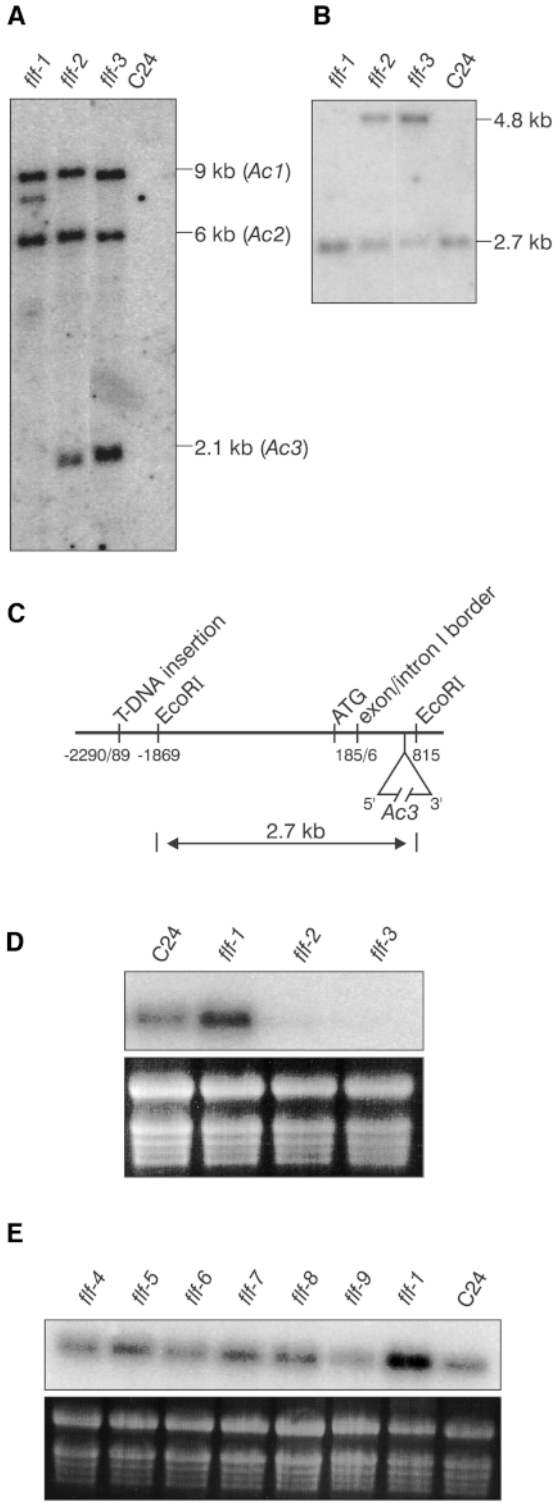

Figure 4. Transposition of *Ac3* into Intron I of the *FLF* Gene Decreases *FLF* Transcript Levels.

(A) Genomic DNA isolated from *flf-1*, bulked M_2 *flf-2*, bulked M_2 *flf-3*, and C24 plants digested with EcoRI and probed with the 3′ region of *FLF* coding sequence exhibits similarity to members of the MADS box class of transcription factors (Davies and Schwarz-Sommer, 1994) (GenBank accession numbers are AF116527 for the cDNA sequence and AF116528 for the genomic sequence). The location of the MADS box and of the I (intervening), K, and C-terminal domains are indicated in Figure 5A. The *FLF* gene is most similar to the MADS box gene *AGAMOUS-LIKE14* (Rounsley et al., 1995) in the M-I-K region, but in the I-K-C region it shows greatest similarity to *CAULIFLOWER* (Kempin et al., 1995) and *APETALA1* (Mandel et al., 1992).

FLF is expressed at different levels in a range of tissues (Figure 5B), suggesting a number of roles for the *FLF* gene. Despite a high level of *FLF* expression in roots, no obvious root phenotype was observed in the transgenic lines, *flf-1*, or the *Ac*-induced mutants. A number of the transgenic lines did exhibit reduced fertility, which appeared to be caused by a lack of pollen in C24 lines and abnormal carpels in L*er* lines. One of the main roles of MADS box genes in plant development is in specifying floral organ identity (Davies and Schwarz-Sommer, 1994); however, some MADS box genes are involved in specifying root architecture (Zhang and Forde, 1998) or are specifically expressed in vegetative tissue, suggesting a role in vegetative growth (Davies and Schwarz-Sommer, 1994).

The expression of the *FLF* gene is lower in reproductive tissues than it is in the vegetative rosette leaves. Gel blotting of RNA isolated from C24 and *flf-1* plants harvested every 10 days after sowing, until the stage at which the majority of C24 plants had bolted (50 days under the growth conditions used) showed that the expression of the *FLF* gene remained

Ac. Some of the plants from which the DNA for the *flf-1* sample was extracted contained a third T-DNA band (at ~8 kb). The presence of this third band had no effect on flowering time. The numbers at right are the lengths of the DNA fragments; also indicated is which *Ac* element is contained in each band.

(B) As given for **(A)**, except that probe 4 was used (see Figure 2C). The numbers at right are the lengths of the DNA fragments detected.

(C) Diagram of part of gene B showing the location of the *Ac3* insertion into intron I that caused the early-flowering phenotype in *flf-2* and *flf-3*. The nucleotide positions are given below, taking the A of the ATG as nucleotide 1. *Ac3* is inserted between nucleotides 746 and 747.

(D) Expression levels of gene B in rosette leaves of C24, *flf-1*, *flf-2*, and *flf-3*. The early-flowering mutants had just started to bolt, whereas the other plants remained vegetative. The ethidium bromide–stained ribosomal bands are shown as a loading control in **(D)** and **(E)**.

(E) *FLF* expression in young rosette leaves from 80-day-old *flf-4*, *flf-6*, and *flf-1* plants and 25-day-old C24 plants and in a mixture of young rosette and cauline leaves from 80-day-old *flf-5*, *flf-7*, *flf-8*, and *flf-9* plants. *flf-4* through *flf-8* are plants that are heterozygous for the early *flf-2* allele and a new *Ac*-generated allele. *flf-9* is heterozygous for the early *flf-3* allele and a new *Ac*-generated allele.

Table 1. Flowering Time of 35S::*FLF* T_1 Transgenic Plants

Plants[a]	Flowering Time (Days)[b]	Relative Expression Level of FLF mRNA[c]
C24	30	1
B2	18	>10
B5	18	>10
B11	>100	>10
B12	>100	>10
L*er*	20	ND[d]
B36	>100	>10
B45	>100	>10
flf-1	>100	2

[a] Transgenic T_1 plants (in C24: B2, B5, B11, B12; or in L*er*: B36, B45) were selected on Murashige and Skoog plates containing 50 mg/L kanamycin and grown at 23°C under fluorescent lights (16 hr of light and 8 hr of dark). Plants were then transferred to soil at 20 days. The other plants were grown under identical conditions but without selection on kanamycin.

[b] Flowering time was recorded as the number of days to stem elongation.

[c] The *FLF* transcript level was determined in early-flowering T_2 progeny for B2 and B5, from young leaves of the T_1 late-flowering plants (B11, B12, B36, and B45), and from in vitro–grown C24, L*er*, and *flf-1* plants.

[d] ND, not detectable.

approximately at the same level throughout development in these plants (Figure 5C). This suggests that if a reduction in the level of *FLF* transcript accompanies the transition to flowering, it is likely to occur in a limited number of apical cells.

Both the Level of *FLF* Expression and the Late-Flowering Phenotype of the *flf-1* Mutant Are Suppressed by Vernalization

In a number of late-flowering mutants and ecotypes, low-temperature treatment of germinating seed induces early flowering (vernalization) (Napp-Zinn, 1985), with a 4°C treatment for 21 days saturating the vernalization requirement to produce the shortest time to flower (Bagnall, 1992). The effect of low temperature on the time to flower of heterozygous and homozygous *flf-1* mutants is shown in Table 2. A 28-day 4°C treatment resulted in a substantial reduction in the flowering time; however, 8 weeks at 4°C was required to saturate the vernalization response in both *flf-1* heterozygotes and homozygotes and to reduce the flowering time to that of the C24 control. This implies that there is an interaction between *FLF* activity and a component of the vernalization-dependent flowering pathway.

Gel blotting of RNA extracted from 12-day-old C24 seedlings that were vernalized and from nonvernalized control seedlings showed a dramatic decrease in *FLF* expression in response to the cold treatment (Figure 5D). The level of the *FLF* transcript also was reduced in *flf-1* seedlings vernalized for 3 weeks (Figure 5D) but not to the low levels observed in vernalized C24, which is consistent with the only partial reduction in flowering time observed in the *flf-1* mutant that had a 4-week cold treatment. *FLF* expression also was greatly decreased in early-flowering plants in which an antisense construct of the methyl transferase gene had resulted in a very low level of DNA methylation (Finnegan et al., 1996) (Figure 5E). This suggests that DNA methylation controls *FLF* transcription either directly or indirectly.

As with other late-flowering vernalization-responsive mutants of Arabidopsis, *flf-1* plants responded to applications of GA_3 by flowering earlier; however, a single treatment of 1 μg of GA_3 on 4-week-old plants, which induced early flowering of the late-flowering *fca* mutant (Bagnall, 1992), was not sufficient to induce flowering of *flf-1*. Repeated applications of GA_3 were required to induce early flowering: 4-week-old *flf-1* homozygotes treated with 1 μg of GA_3 every second day for a total of 2 weeks flowered 2 weeks after the final GA_3 application, compared with later than 20 weeks for untreated plants. In contrast to the effect of vernalization in reducing the level of expression of the *FLF* gene, GA_3 application had no effect on the expression of the *FLF* gene in either C24 or *flf-1* seedlings (Figure 5D).

The Expression of the *FLF* Gene Is Regulated by Other Genes in the Pathways to Flowering

Figure 6A shows the expression of the *FLF* gene in five ecotypes of Arabidopsis; *FLF* is highly expressed in ecotypes that have a late allele at the *FRI* locus (Pitztal and C24; Burn et al., 1993b; Sanda and Amasino, 1995) and is particularly highly expressed in C24, but it has very low expression in ecotypes with an early *FRI* allele (Col, Wassilewskija [Ws], and L*er*; Lee et al., 1994b). L*er* lines containing late alleles of either *FRI* or *FLC* are several days late to flower (Lee and Amasino, 1995); *FLF* is expressed in the L*er* line with the late *FRI*–Sf2 (San-Feliu-2) allele and in the lines containing late *FLC*–Sf2 and *FLC*–Col alleles (Figure 6B). The high expression of the *FLF* gene in L*er* containing *FLC*–Col but not in the Col ecotype suggests that either the *FRI*–L*er* and *FRI*–Col alleles are not the same, although both have been designated as early, or that the L*er* line containing *FLC*–Col and Col differ at another locus (or loci), perhaps linked to *FLC*–Col. A Col allele at such a locus (or loci) may induce expression of the *FLF* gene in the presence of modifying genes present in the L*er* background. Alonso-Blanco et al. (1998) report that a large region on the top of chromosome 5, presumably containing multiple loci that would be linked to *FLC*, influences flowering time.

A number of late-flowering mutants in either L*er* or Ws have been classified into three classes based on their responses to vernalization and on the effects on flowering time of double mutant combinations (Koornneef et al., 1991; Lee et al., 1994a). We found that the *FLF* gene is upregulated in the

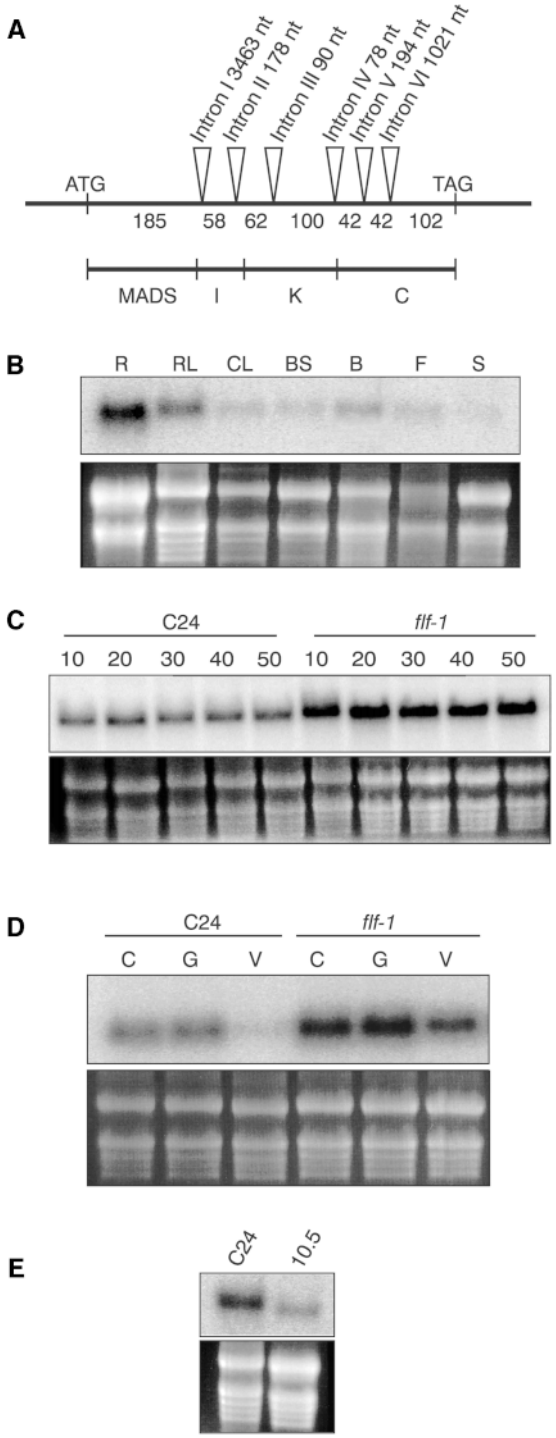

Figure 5. *FLF* Gene Structure and Expression Pattern.

(A) Genomic structure of the *FLF* gene showing the location and size of the introns and the location of the MADS box, intervening domain (I), K domain (K), and C-terminal domain (C). The numbers below the line are the size (in nucleotides) of each exon. nt, nucleotide.

(B) Distribution of *FLF* mRNA in C24 plants in the roots (R) and rosette leaves (RL) from in vitro–grown vegetative plants, in cauline leaves (CL), in bolt stems (BS), and in apex and unopened flower buds (B) from soil-grown plants with bolt stems between 1 and 5 cm. Open flowers (F) and siliques (S) were collected from older plants. The ethidium bromide–stained ribosomal bands are shown as a loading control in **(B)** to **(E)**.

(C) Expression level of *FLF* mRNA in in vitro–grown C24 or *flf-1* plants harvested every 10 days until the majority of the C24 plants were bolting (50 days under these growth conditions).

(D) Effect of vernalization and GA treatment on the *FLF* transcript in C24 and *flf-1* seedlings. RNA was isolated from 12-day-old in vitro–grown seedlings that either had no treatment (C), had been grown on media containing 10^{-5} M GA_3 (G), or had a pretreatment of 3 weeks at 4°C (V).

(E) *FLF* expression in rosette leaves of C24 and the early-flowering antisense methyl transferase line 10.5 (T_3 generation) harvested soon after bolting.

vernalization-responsive *fca*, *fve*, *fpa*, and *ld* mutants but not in the vernalization-nonresponsive late-flowering mutants *co*, *gi*, *fha*, *ft*, *fe*, and *fwa.* However, there was a slight upregulation in the vernalization-nonresponsive late-flowering mutant *fd* (Figure 6C). *FLF* expression was not altered in the early-flowering *tfl1* mutant when compared with L*er.* Two mutants that have a reduced response to vernalization are *vrn1* and *vrn2.* These mutants were isolated in an *fca* (L*er*) mutant background (Chandler et al., 1996); we did not detect any increase in expression of the *FLF* gene in *vrn1* above the L*er* level. We were not able to study *vrn2* except in combination with *fca* because of their tight linkage (Chandler et al., 1996). We found that the *fca vrn2* double mutant, which is also late flowering without a vernalization treatment relative to *fca* (Chandler et al., 1996), showed a significant increase in *FLF* expression over that found in the *fca* mutant.

DISCUSSION

The data presented in this study point to a key role for *FLF* in the switch of the apical meristem from the vegetative to reproductive mode, and they provide information on the relationship of the autonomous flowering pathway to the vernalization-inducible pathway and on interactions between genes involved in these pathways. Furthermore, our data suggest that DNA methylation controls the induction of the vernalization pathway and that *FLF* antagonizes the promotive effect of the phytohormone GA on flowering.

The *FLF* Gene Encodes a Dosage-Dependent Repressor of Flowering

Our data suggest that *FLF* encodes a protein that represses the transition to flowering in a concentration-dependent

Table 2. Flowering Time of Vernalized and Nonvernalized C24 and *flf-1* Plants

	Length of Vernalization[a]		
Plants	0 Weeks	4 Weeks	8 Weeks
C24 (*FLF*/*FLF*)	25.2 ± 0.2	13.6 ± 0.3	—[b]
FLF/*flf-1*	71.4 ± 1.2	39.3 ± 3.7	20.2 ± 0.9
flf-1/*flf-1*	>150	100.8 ± 10.7	17.6 ± 1.3

[a] Twenty seeds of the *FLF*/*flf-1* and *flf-1*/*flf-1* mutant and wild-type C24 were grown aseptically on Murashige and Skoog media in test tubes and exposed to 4°C for either 4 or 8 weeks. Nonvernalized plants were grown in soil (20 plants per 20-cm pot). All plants were then grown at 23°C under fluorescent lights (16 hr of light and 8 hr of dark). The data are presented as the average number of days (±SE) until stem elongation and exclude the period of vernalization.
[b] Dash indicates not determined.

manner. We are currently limited to mRNA data obtained from RNA gel blot analyses, and we recognize that data on the MADS box protein and its activity in the cells of the apical meristem and elsewhere in the plant will provide further information on the role of *FLF* in the induction of flowering. Data from three different sources indicate that an increase in the steady state level of *FLF* mRNA results in later flowering, and a decrease in the *FLF* transcript level results in earlier flowering. These data are consistent with the gene encoding a repressor of flowering. The *flf-1* mutant, generated by the upstream insertion of two T-DNAs, has an increased level of the *FLF* transcript and is late flowering. The homozygous mutant overexpresses the transcript twofold and takes >150 days to flower, whereas the heterozygote has an intermediate level of expression and flowers at ~70 days. Flowering time is proportional to the level of the transcript. The insertion and excision of an *Ac* element from intron I (*Ac3*) of the *FLF* gene reaffirm the correlation of transcript level and flowering time. Mutants homozygous for the insertion of *Ac3* into intron I have very low transcript levels and are very early flowering. In addition, when *Ac3* excised from the intron in one copy of the *FLF* gene, the resulting heterozygote showed both an increase in transcript level and a delay in flowering.

Data from transgenic plants support the findings from the T-DNA– and *Ac*-generated mutants. Transgenic L*er* plants overexpressing the *FLF* gene had not flowered after 100 days, and C24 transgenic plants overexpressing the *FLF* gene showed a range of flowering date behaviors extending from an unexpected early-flowering phenotype to plants that had not flowered after 100 days. Transcript levels in the early-flowering plants were comparable with those in the nonflowering transgenic plants; this result does not fit the correlation of *FLF* transcript level and flowering time. It will be important to determine protein expression levels and the distribution of *FLF* activity in the cells of the apex to explore these differences in flowering time. An antisense construct in C24 resulted in the majority of transgenic plants flowering early. The earliest flowering time was 16 days compared with 30 days for nontransgenic C24 plants.

We have not determined the mechanism for increased expression levels in the *flf-1* mutant; however, because both genes flanking the T-DNAs have greater transcriptional activity, presumably it results from an enhancer present in the T-DNA or *Ac* or from a change in chromatin structure, or both. The overexpression of both flanking genes suggests that it is unlikely that the 30-bp deletion immediately downstream of the T-DNA insertion site is the cause of altered expression of the *FLF* gene.

FLF Is Critical for Floral Induction

The late-flowering *flf-1* mutant has a dramatic early-flowering response to vernalization, placing it in the class of late-flowering mutants that are vernalization responsive, together with *fca*, *fve*, *fpa*, and *ld* (Koornneef et al., 1991; Lee et al., 1994a). The genes in this class are in the autonomous pathway to flowering; the mutant alleles block this pathway. The vernalization-dependent pathway is still active in these mutants, and the plants flower at wild-type times if vernalized. Compared with other vernalization-responsive late-flowering mutants, the *flf-1* mutant requires a longer period of low-temperature treatment to flower at the same time as C24, suggesting that the gene product also partially blocks the vernalization-dependent pathway (Figure 7). Support for a role of *FLF* in both pathways comes from the finding that vernalization-responsive genes in the autonomous pathway (*FCA*, *FPA*, *LD*, and *FRI*) and a gene in the vernalization-dependent pathway (*VRN2*) regulate the expression of the *FLF* gene, placing these genes upstream of *FLF* (Figure 7). Two of the autonomous pathway genes have been cloned; *FCA* encodes an RNA binding protein, which may have a role in post-transcriptional regulation (Macknight et al., 1997), and *LD* encodes a putative transcription factor (Lee et al., 1994a). These gene products could modulate *FLF* expression through transcriptional control or mRNA stability. FLF is a MADS box protein that may heterodimerize with other MADS box proteins or interact with other classes of proteins to mediate its repression of flowering.

The *FLF* gene plays a central role in the early-flowering response to vernalization. In C24, a 3-week cold treatment reduces flowering time by approximately one-third and results in a dramatic decrease in *FLF* transcript abundance, again providing evidence for the proportionality between transcript level and flowering time. A 3- or 4-week cold treatment is insufficient either to decrease the flowering time of the *flf-1* mutant to that of vernalized C24 plants or to fully negatively regulate *FLF* expression in these plants. The requirement for a longer period of low-temperature treatment for early flowering may be a consequence of the mechanism of removal of the *FLF* transcript or altered promoter characteristics, due to the presence of the T-DNAs and *Ac1* and *Ac2*.

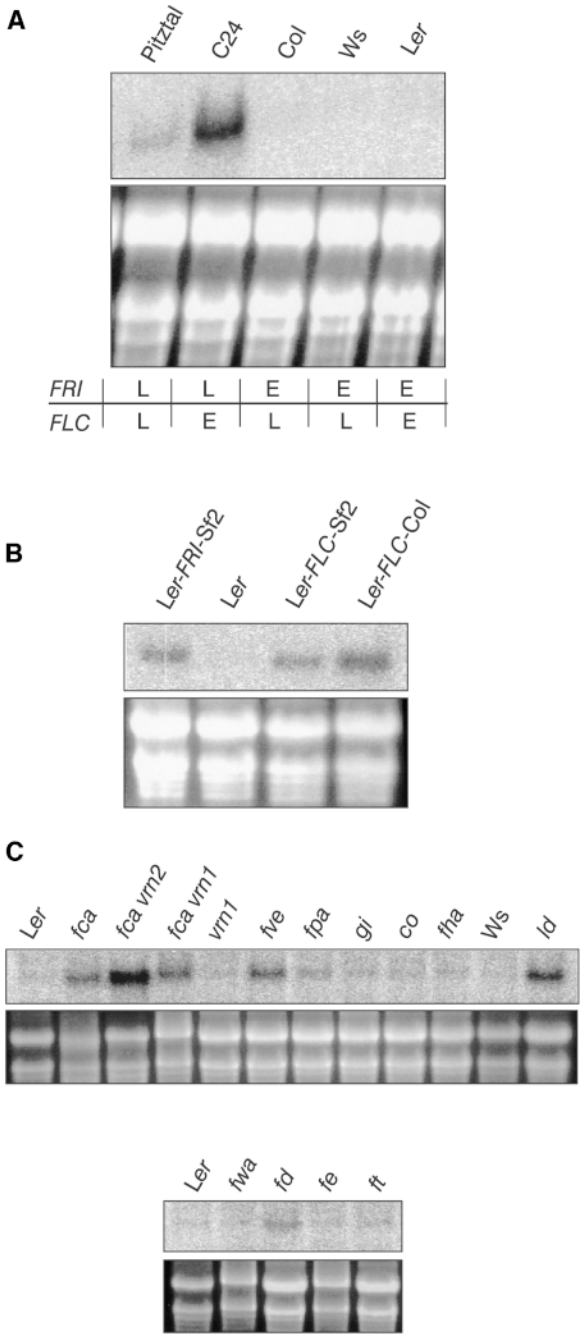

Figure 6. Expression of the *FLF* Gene in Ecotypes and Late-Flowering Mutants.

(A) *FLF* expression in 12-day-old in vitro–grown seedlings of five Arabidopsis ecotypes: Pitztal, C24, Col, Ws, and L*er.* The *FRI* and *FLC* genotypes are indicated below the gels (Lee et al., 1994b; Sanda and Amasino, 1995): E, early allele; L, late allele. Note that longer exposures revealed a very low expression in L*er*, Col, and Ws. The ethidium bromide–stained ribosomal bands are shown as a loading control in **(A)** to **(C)**.

(B) *FLF* expression in 12-day-old in vitro–grown L*er* seedlings and L*er* lines containing late alleles at either the *FRI* (*FRI*–Sf2) or the *FLC* (*FLC*–Sf2 and *FLC*–Col) locus.

(C) *FLF* expression at day 12 for in vitro–grown seedlings of late-flowering mutants in either the L*er* ecotype (*fca*, *fve*, *fpa*, *gi*, *co*, *fha*, *fwa*, *fd*, *fe*, and *ft*) or Ws ecotype (*ld*) and in two mutants with a reduced response to vernalization, either in the *fca* (L*er*) background in which they were generated (*fca vrn1* and *fca vrn2*) or in isolation (*vrn1*).

We have suggested that vernalization is mediated through demethylation of specific genes (Burn et al., 1993a; Finnegan et al., 1998). In support of this suggestion, we found that either a chemical or a genetic reduction in the level of DNA methylation largely substituted for vernalization, resulting in vernalization-responsive mutants and ecotypes flowering early (Burn et al., 1993a; Finnegan et al., 1998). We proposed that this effect may be mediated by the removal of a methylation block to the transcription of one or more key genes in the vernalization-dependent flowering pathway (Burn et al., 1993a). Our data show that the *FLF* transcript level is substantially reduced in antisense methyl transferase C24 plants that have been shown to have low levels of DNA methylation and flower early (Finnegan et al., 1998). Demethylation parallels vernalization in its effect on the *FLF* gene, with the transcript level being decreased. Contrary to our earlier model, in which we had proposed that demethylation activated transcription, data from this study show that both demethylation and vernalization downregulate the *FLF* transcript level. Downregulation may be due to the changed methylation status of the *FLF* promoter, in which, for example, a transcriptional activator may bind only to a methylated binding site, or it could be a response involving a repressor protein, which is produced from a gene upregulated by demethylation.

In our previous work, we postulated that the removal of the block to the vernalization pathway triggered GA biosynthesis in the apical meristem. In this set of experiments, we found that exogenous GA did not alter *FLF* mRNA levels in C24 (although this treatment causes C24 to flower early), suggesting that the promotive effect of GA on flowering acts downstream of *FLF* in the pathways to flowering (Figure 7). The *flf-1* mutant required repeated GA treatment to induce a flowering response, many more treatments than are required to induce a flowering response in C24 or in the vernalization-responsive late-flowering mutant *fca.* This observation suggests that the *FLF* gene product lowers the effectiveness of applied GA; the increased *FLF* mRNA level (and presumably protein product) in the *flf-1* mutant may be the cause of the need for repeated applications of GA to induce flowering.

In C24, *FLF* mRNA is present at high levels throughout much of the plant, with a reduced level in some of the tissues of the inflorescence bolt. C24 is a vernalization-responsive ecotype, with flowering time being reduced by

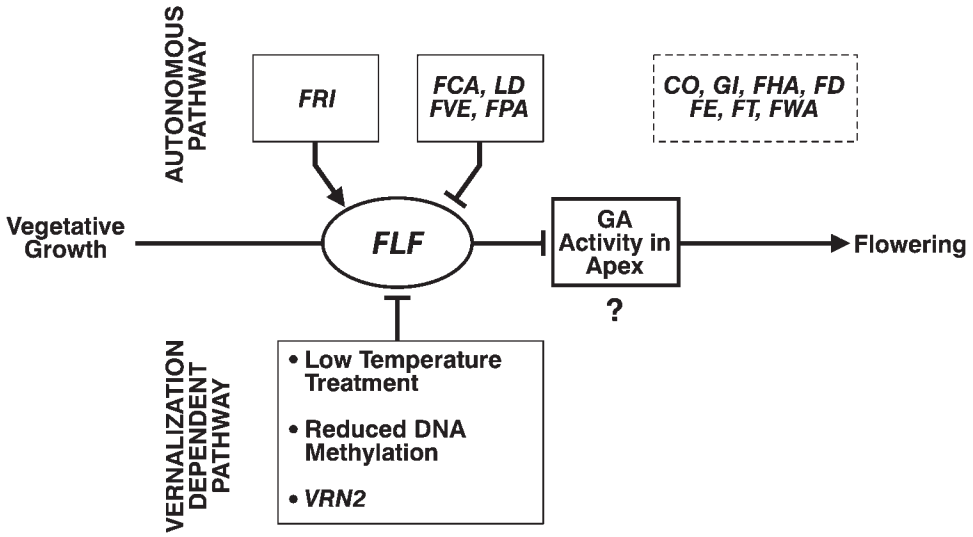

Figure 7. *FLF* Plays a Key Role in the Pathways to Flowering.

A model is shown for the transition from vegetative growth to flowering that details the integrative role of *FLF* in both the autonomous and vernalization-dependent pathways. The autonomous pathway acts to promote flowering through the action of the *FCA*, *LD*, *FVE*, and *FPA* genes by decreasing the expression of the *FLF* gene. *FRI* is a locus identified in late-flowering ecotypes. We have identified *FRI* as having a positive effect on *FLF* expression. The diagram shows that the repressive effect of *FLF* is reduced by low-temperature treatment, reduced levels of DNA methylation, or the activity of the *VRN2* gene. The *FLF* gene product may act to block the promotion of flowering by GA in the apical meristem. The genes *CO*, *GI*, *FHA*, *FD*, *FE*, *FT*, and *FWA* are not responsive to vernalization and have low *FLF* expression. The position of *FLC* has not been indicated because it may correspond to *FLF.* Gene symbols in open boxes represent genes that regulate *FLF* expression, and gene symbols in the dashed box represent genes that have very little effect on *FLF* expression.

approximately one-third in response to cold treatment. The ecotypes L*er*, Col, and Ws have low *FLF* mRNA levels and respond minimally to vernalization, with a decrease in their already early time to flower (~20 days) of only one or two days. Pitztal, a vernalization-responsive late-flowering ecotype, has a higher level of *FLF* mRNA than do L*er*, Col, and Ws, fitting the correlation between level of transcript and responsiveness to vernalization. At this stage, we cannot explain why *FLF* is expressed at such high levels in C24 plants compared with the plants of other ecotypes; the C24 ecotype may harbor a mutation in one of the genes that we have shown to negatively regulate *FLF.* We also cannot explain why a twofold increase in *FLF* transcript in the *flf-1* mutant causes such a dramatic delay in flowering. It should be noted that the RNA analysis was done at the whole-plant level, and it will be important to look at the expression of *FLF* in those cells directly involved in the flowering response.

The *FLF* Gene May Correspond to *FLC*

FLF maps to the region of chromosome 5 where *FLC* is located (Koornneef et al., 1994; Lee et al., 1994b). Because both *FLF* and *FLC* are semidominant repressors of flowering, it is possible that they are the same gene. However, the alleles of *FLC* present in C24 and L*er* ecotypes behave as nonfunctional, early-flowering alleles (Michaels and Amasino, 1995; Sanda and Amasino, 1995), whereas the alleles of *FLF* present in C24 and L*er* are functional alleles. The *FLF* transcript is present in L*er* at a very low level. This is not a property of the L*er* allele per se, because when the L*er* genotype is modified by mutations in a number of loci (*FCA*, *FVE*, *FPA*, and *VRN2*), *FLF* is transcribed at a higher level and the transcript level correlates with the delay in flowering. These data are consistent with the L*er FLF* gene product having a specific late-flowering effect. The question of *FLF*/*FLC* identity requires molecular data to supplement the existing genetic data on *FLC.*

METHODS

Plant Material and Growth Conditions

Plants (*Arabidopsis thaliana*) were grown either in pots containing a mixture of 50% sand and 50% loam or aseptically in Petri dishes or tubes containing a modified Murashige and Skoog medium (Langridge, 1957). All plants were grown in artificially lit cabinets at 23°C under long-day (16 hr of light and 8 hr of dark) conditions using cool-white fluorescent lights at an intensity of ~200 μM m^{-2} sec^{-1}. The plants

grown for the experiment shown in Figure 5C were grown in lower light and consequently took longer than normal to flower.

Plants were vernalized by germinating seed in the dark for either 3, 4, or 8 weeks at 4°C. After this cold treatment, seedlings were transferred to long-day photoperiods at 23°C, and times to flowering (measured as the time until stem elongation [bolting] was observed) were determined beginning from the first day at the higher temperature.

Mutant seed stocks were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) or from C. Dean (John Innes Centre, Norwich, UK) (*vrn1*, *fca vrn1*, and *fca vrn2*). E.J. Finnegan (Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, Canberra, Australia) provided the methyl transferase antisense line 10.5. R. Amasino (University of Wisconsin, Madison) provided the Landsberg *erecta* (L*er*) lines containing *FRI*–Sf2, *FLC*–Sf2, or *FLC*–Col.

T-DNA Mutagenesis

The late-flowering mutant (*flf-1*) arose from root transformation (Valvekens et al., 1988) of ecotype C24, with a modified binary vector pBinDAc (Finnegan et al., 1993). This vector contains the neomycin phosphotransferase II (*NPTII*) gene under the control of the nopaline synthase promoter together with a deleted maize *Activator* (*Ac*) element inserted within the untranslated leader of the β-glucuronidase gene in the reverse orientation to the direction of transcription.

Construction and Screening of Genomic Libraries

A genomic library of the *flf-1* mutant was constructed by partial digest of total plant DNA with the restriction enzyme Sau3AI and ligation into the phage vector λEMBL4. The resulting library was screened using a ^{32}P-dCTP–labeled probe of the *NPTII* gene (Feinberg and Vogelstein, 1983). Four positive phages were purified and restriction mapped and together spanned 27 kb of plant DNA flanking the T-DNA site of insertion. A 2.3-kb BamHI-EcoRI and a 2.7-kb EcoRI fragment (probes 1 and 2, respectively; Figure 2C) isolated from this flanking plant DNA were subsequently used to probe a genomic library of wild-type Arabidopsis C24 made from BamHI-digested DNA cloned into λEMBL4. Probe 1 isolated a genomic clone containing 6.5 kb of plant DNA spanning the T-DNA insertion site, and probe 2 isolated a genomic clone containing 7.3 kb of adjacent sequence. Each clone was purified and sequenced using the Applied Biosystems (Foster City, CA) dRhodamine dye terminator sequencing mix according to manufacturer's instructions and analyzed using an Applied Biosystems 377 sequencing machine. The Genetics Computer Group (Madison, WI) software package (version 9.1) was employed for sequence analysis, and BLAST searches were performed by using the AtDB WU-BLASTZ server (http://genome-wwwZ.stanford.edu/cgi-bin/AtDB/nph-blastZatdb).

Screening of cDNA Libraries

Arabidopsis cDNA libraries were screened using probes from the plant DNA flanking the T-DNA insertion site. Gene A cDNAs were isolated with a 4.6-kb EcoRI-BamHI fragment (probe 3; Figure 2C) from a L*er* flower cDNA library (Weigel et al., 1992). Gene B cDNAs were isolated from a Columbia (Col) cDNA library (Newman et al., 1994) with a 2.7-kb EcoRI fragment (probe 2; Figure 2C). The cDNAs were sequenced and analyzed as given above.

DNA Gel Blot Analysis

Total genomic DNA was isolated either by following the cetyltrimethylammonium bromide procedure (Dean et al., 1993) or as described by McNellis et al. (1998). Two to three micrograms of DNA was digested with the appropriate restriction enzyme, run on an 0.8% agarose gel, and blotted onto Hybond N+ (Amersham) membranes (Southern, 1975). Probes were labeled with ^{32}P-dCTP, using the random primer method (Feinberg and Vogelstein, 1983).

The 3′ *Ac* probe was a 0.8-kb SphI fragment (Lawrence et al., 1993), and probe 4 was generated by amplification of the wild-type genomic clone with primers 5′-GTATAGGGCACATGCCC-3′ and 5′-CACTCGGAGCTGTGCC-3′. The amplified product contains a 570-bp subset of the probe 2 sequence (Figure 2C), lacking the MADS box, to eliminate cross-hybridization. The filters were exposed to PhosphorImager screens for image detection (Molecular Dynamics, Sunnyvale, CA).

Restriction Fragment Length Polymorphism Mapping

A restriction fragment length polymorphism (RFLP) was detected between the parental lines L*er* and Col by digestion of genomic DNA with BamHI and probing with the gene A cDNA. Sixty-four recombinant inbred lines (Lister and Dean, 1993) were digested with BamHI, and DNA gel blots were probed with gene A. The Mapmaker program (Lander et al., 1987) was employed to compare the data with RFLP data for 68 mapped markers. Fine mapping of the region was performed using DNA from F_2 plants generated from a cross between L*er* and the *flf-1* mutant. An HpaI digest of 62 F_2 DNA was probed with the chromosome 5 RFLP marker 447 (Chang et al., 1988).

RNA Extractions and RNA Gel Blot Analysis

Total RNA was extracted from ~1 g of plant tissue, following the method of Longemann et al. (1987). Total RNA (10 to 20 μg) was run on 2.2 M formaldehyde–agarose gels and blotted onto Hybond N nylon filters. T7 or SP6 polymerase transcription of the linearized gene A or gene B (*FLF*) plasmid template (containing the complete cDNA for gene A; linearized to remove MADS box for gene B) was used to generate antisense ^{32}P-dUTP–labeled riboprobes. Filters were hybridized, as described by Dolferus et al. (1994), and washed with a final solution of 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65°C. For gene A–probed filters, it was necessary to treat filters with RNase A, as described by Dolferus et al. (1994), to remove ribosomal trapping. The filters were exposed to PhosphorImager screens for quantification of signal intensity by using a PhosphorImager (Molecular Dynamics). RNA size markers were used to determine the size of the gene A and gene B transcripts.

Generation of Transgenic Plants

A binary construct containing the *FLF* cDNA under the control of a cauliflower mosaic virus 35S promoter was generated by cloning an XhoI-SpeI–digested polymerase chain reaction (PCR) product into XhoI-XbaI–digested pART7 (Gleave, 1992) containing a 35S promoter. The PCR product was amplified using 20 ng of the *FLF* cDNA clone as template with primers 5′-CCG**CTCGAG**CTTAGTATCTCC-GGCG-3′ and 5′-GG**ACTAGT**CGCCCTTATCAGCGGA-3′ (restric-

tion sites are shown in boldface; sequences hybridizing to gene B cDNA are underlined). The amplification reaction was conducted in a 20-μL final volume that contained 1 μM each oligonucleotide primer, 0.2 units of Ampitaq polymerase (Perkin-Elmer), and 125 μM each of the four deoxynucleotides. Conditions for the amplification were as follows: 94°C for 3 min, 30 cycles consisting of 30-sec denaturation at 94°C, annealing at 50°C for 30 sec, and polymerization at 72°C for 1 min, and a final extension at 72°C for 5 min before the temperature was decreased to 25°C. The PCR product was sequenced to ensure that no mutations had been introduced during the amplification procedure. The 35S::*FLF* cassette was then subcloned using NotI into pART27 (Gleave, 1992) and introduced into *Agrobacterium tumefaciens* GV3101 by triparental mating, employing pRK2013 as the helper plasmid. Transgenic plants were generated by in-plant transformation (Bechthold et al., 1993), using the *NPTII* gene as a selectable marker to identify transgenic plants.

The 35S::*FLF* antisense binary construct was generated by cloning an EcoRI-SpeI–digested 630-bp PCR product containing the *FLF* cDNA sequence lacking the MADS box and amplified as described above with primers 5′-CG**GAATTC**TCACACGAATAAGGTAC-3′ and 5′-GG**ACTAGT**GGTCAAGATCCTTGATC-3′ (restriction sites are shown in boldface; sequences hybridizing to gene B cDNA are underlined). The PCR product was cloned into pART7 and a derivative of pART27, and transgenic plants were generated as given above, except that the *Bar* gene was used as the selectable marker.

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