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

Candice C. Sheldon, Joanne E. Burn, Pascual P. Perez, Jim Metzger, Jennifer A. Edwards, W. James Peacock, and Elizabeth S. Dennis

Commonwealth Scientific and Industrial Research Organisation, Division of Plant Industry, GPO Box 1600, Canberra, ACT, 2601, Australia

Biocem, 24 avenue des Landais, 63107, Aubiere, Cezeaux, France

Ohio State University, Department of Horticulture, 2001 Fyffe Court, Columbus, Ohio 43210-1096

Cooperative Research Centre for Plant Science, Research School of Biological Sciences, Australian National University, Canberra, ACT, 2601, Australia

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 (GA3) 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 (L. erecta [Ler]). 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
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 gibberelin (GA) 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 T0 plants were allowed to self, and the T1 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 T1 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 did not have 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 ~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 T1 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 T1 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 polymor-
FLF encodes a repressor of flowering (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 (M1 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 M2 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 M2 plants, resulting in the regeneration of the original-sized band. Individual early-flowering M2 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 1 in gene B (Figure 4C). RNA isolated from rosette leaves of early-flowering M2 plants and from C24 and flf-1 plants, resulting in the regeneration of the original-sized band. Individual early-flowering M2 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 1 in gene B (Figure 4C). RNA isolated from rosette leaves of early-flowering M2 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 T2 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).
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 M1 flf-8, 60 days. In the M2 progeny, 50 days; and flf-7, 85 days; flf-1, 50 days; and flf-9, 60 days. In the M1 progeny of flf-3, 36 plants flowered at the same time as did their M1 parent; however, five plants flowered 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 T1 plants and in the rosette leaves of two nonflowering T1 plants. In a further experiment, we generated transgenic plants containing the same 35S::FLF construct in the Ler ecotype. Of the 24 T1 lines generated, nine bolted earlier than did wild-type Ler (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 T1 plants had a very high level of expression of the transgene (Table 1).

These data show that in Ler, 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 T1 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 T1 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
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 Ler 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.
FLF-1 plants (in C24: B2, B5, B11, B12; or in Ler: B36, B45) were selected on Murashige and Skoog plates containing 50 mg/L kanamycin and grown at 23°C. The other plants were grown under identical conditions but without selection on kanamycin.

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 methyltransferase 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 GA3 by flowering earlier; however, a single treatment of 1 μg of GA3 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 GA3 were required to induce early flowering: 4-week-old flf-1 homozygotes treated with 1 μg of GA3 every second day for a total of 2 weeks flowered 2 weeks after the final GA3 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, GA3 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 allele (Col, Wassilewskija [Ws], and Ler; Lee et al., 1994b). Ler lines containing late alleles of either FRI or FLC are several days late to flower (Lee and Amasino, 1995); FLF is expressed in the Ler 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 Ler containing FLC-Col but not in the Col ecotype suggests that either the FRI–Ler and FRI-Col alleles are not the same, although both have been designated as early, or that the Ler 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 Ler 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 Ler orWs have been classified into three classes based on their responses to vernalization and on the effects on flowering time of double mutant combinations (Koomoef et al., 1991; Lee et al., 1994a). We found that the FLF gene is upregulated in the

<table>
<thead>
<tr>
<th>Plants</th>
<th>Flowering Time (Days)</th>
<th>Relative Expression Level of FLF mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C24</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B5</td>
<td>18</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B11</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B12</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Ler</td>
<td>20</td>
<td>ND§</td>
</tr>
<tr>
<td>B36</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B45</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>flf-1</td>
<td>&gt;100</td>
<td>2</td>
</tr>
</tbody>
</table>

*Transgenic T1 plants (in C24: B2, B5, B11, B12; or in Ler: B36, B45) were selected on Murashige and Skoog plates containing 50 mg/L kanamycin and grown at 23°C. Plants were then transferred to soil at 20 days. The other plants were grown under identical conditions but without selection on kanamycin.

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

The FLF transcript level was determined in early-flowering T2 progeny for B2 and B5, from young leaves of the T1 late-flowering plants (B11, B12, B36, and B45), and from in vitro-grown C24, Ler, and flf-1 plants.

§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.

Table 1. Flowering Time of 35S::FLF T1 Transgenic Plants

<table>
<thead>
<tr>
<th>Plants</th>
<th>Flowering Time (Days)</th>
<th>Relative Expression Level of FLF mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C24</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B5</td>
<td>18</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B11</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B12</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Ler</td>
<td>20</td>
<td>ND§</td>
</tr>
<tr>
<td>B36</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B45</td>
<td>&gt;100</td>
<td>&gt;10</td>
</tr>
<tr>
<td>flf-1</td>
<td>&gt;100</td>
<td>2</td>
</tr>
</tbody>
</table>

* Transgenic T1 plants (in C24: B2, B5, B11, B12; or in Ler: 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.

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

The FLF transcript level was determined in early-flowering T2 progeny for B2 and B5, from young leaves of the T1 late-flowering plants (B11, B12, B36, and B45), and from in vitro-grown C24, Ler, and flf-1 plants.

§ND, not detectable.
vernalization-responsive fca, fve, fpa, and ld mutants but not in the vernalization-nonresponsive late-flowering mutants co, gl, fha, ft, and fwa. However, there was a slight up-regulation in the vernalization-nonresponsive late-flowering mutant fd (Figure 6C). FLF expression was not altered in the early-flowering tf1 mutant when compared with Ler. Two mutants that have a reduced response to vernalization are vrn1 and vrn2. These mutants were isolated in an fca (Ler) mutant background (Chandler et al., 1996); we did not detect any increase in expression of the FLF gene in vrn1 above the Ler 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
then grown at 23°C. Plants were grown in soil (20 plants per 20-cm pot). All plants were grown in test tubes and exposed to a steady state level of light. Data from three different sources indicate that an increase in the steady state level of light results in later flowering, and a decrease in the FLF transcript level results in earlier flowering. These data are consistent with the gene encoding FLF playing a role in post-transcriptional regulation (Macknight et al., 1994a).

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 Id (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.

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

<table>
<thead>
<tr>
<th>Plants</th>
<th>Length of Vernalization(^a)</th>
<th>0 Weeks</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>C24 (FLF/FLF)</td>
<td>25.2 ± 0.2</td>
<td>13.6 ± 0.3</td>
<td>—–b</td>
<td></td>
</tr>
<tr>
<td>FLF/flf-1</td>
<td>71.4 ± 1.2</td>
<td>39.3 ± 3.7</td>
<td>20.2 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>flf-1/flf-1</td>
<td>&gt;150</td>
<td>100.8 ± 10.7</td>
<td>17.6 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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.
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 6. Expression of the FLF Gene in Ecotypes and Late-Flowering Mutants.](image)

(A) FLF expression in 12-day-old in vitro-grown seedlings of five Arabidopsis ecotypes: Pitztal, C24, Col, Ws, and Ler. 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 Ler, 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 Ler seedlings and

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 Ler. 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 Ler, 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 Ler seedlings and
The FLF Gene May Correspond to FLC

FLF maps to the region of chromosome 5 where FLC is located (Koomneef 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 Ler ecotypes behave as nonfunctional, early-flowering alleles (Michaels and Amasino, 1995; Sanda and Amasino, 1995), whereas the alleles of FLF present in C24 and Ler are functional alleles. The FLF transcript is present in Ler at a very low level. This is not a property of the Ler allele per se, because when the Ler 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 Ler 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) (vm1, fca vm1, and fca vm2). 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 (Ler) lines containing FRI-Sf2, FLC-Sf2, or FLC-Col.

T-DNA Mutagenesis

The late-flowering mutant (ffl-1) arose from root transformation (Valwegen 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 ffl-1 mutant was constructed by partial digestion of total plant DNA with the restriction enzyme Sau3AI and ligation into the phage vector λEMBL4. The resulting library was screened using a [32P]-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 377 sequencing machine. The Genetics Computer Group (Madison, WI) software package (version 9.1) was employed to compare the data with RFLP data for 68 mapped markers. Fine mapping of the region was performed using DNA from F2 plants generated from a cross between Ler and the ffl-1 mutant. An Hpal digest of 62 F2 DNA was probed with the chromosome 5 RFLP marker 447 (Chang et al., 1988).

DNA Gel Blot Analysis

Total genomic DNA was isolated either by 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 [32P]-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 S′-GATAGGGGACATGCC-3′ and S′-CACTCGGAGCTGTGCGCC-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 Ler 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 F2 plants generated from a cross between Ler and the ffl-1 mutant. An Hpal digest of 62 F2 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 [32P]-dUTP-labeled riboprobes. Filters were hybridized, as described by Dolfers 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 Dolfers 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 Xhol-SpeI–digested polymerase chain reaction (PCR) product into Xhol-XbaI–digested pART7 (Gleeve, 1992) containing a 35S promoter. The PCR product was amplified using 20 ng of the FLF cDNA clone as template with primers S′-CGCTCGAGCTTAGATCTCCGGCG-3′ and S′-GGACTATCCGCGTATACGCGGA-3′ (restricted by the PstI cloning site).
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 AmpliTaq 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 355::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 planta transformation (Bechthold et al., 1993), using the NPTII gene as a selectable marker to identify transgenic plants.

The 355::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′-CGGAACTTCACACGAATAAGGTAC-3′ and 5′-GGACTAGTGCTCAAGATCCTTGTAC-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 pART7, and transgenic plants were generated as given above, except that the Bar gene was used as the selectable marker.

ACKNOWLEDGMENTS

We thank J anice Norman, Chong-Xin Zhao, and Geoff Ellacott for excellent technical assistance and Dr. E. J. Finnegan for providing an initial sample of RNA from the methyl transferase antisense line 10.5 and the Ac SpHII fragment. C. C. S. was supported in part by the Ken and Yasuko Myer Plant Science Research Fund.

Received December 28, 1998; accepted January 25, 1999.

REFERENCES


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


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

Candice C. Sheldon, Joanne E. Burn, Pascual P. Perez, Jim Metzger, Jennifer A. Edwards, W. James Peacock and Elizabeth S. Dennis

*Plant Cell* 1999;11;445-458

DOI 10.1105/tpc.11.3.445

This information is current as of July 11, 2017

References

This article cites 41 articles, 15 of which can be accessed free at:
/content/11/3/445.full.html#ref-list-1

Permissions


eTOCs

Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain

CiteTrack Alerts

Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain

Subscription Information

Subscription Information for *The Plant Cell* and *Plant Physiology* is available at:
http://www.aspib.org/publications/subscriptions.cfm