

Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering

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Summary

The timing of flowering is important for the reproductive success of plants. Here we describe the identification and characterization of a new MADS-box gene, *FLOWERING LOCUS M* (*FLM*), which is involved in the transition from vegetative to reproductive development. *FLM* is similar in amino-acid sequence to *FLC*, another MADS-box gene involved in flowering-time control. *flm* mutants are early flowering in both inductive and non-inductive photoperiods, and flowering time is sensitive to *FLM* dosage. *FLM* overexpression produces late-flowering plants. Thus *FLM* acts as an inhibitor of flowering. *FLM* is expressed in areas of cell division such as root and shoot apical regions and leaf primordia.

Keywords: MADS-box gene, *FLM*, *AGL27*, *FLC*, *AGL31*, flowering repressor.

Introduction

The transition from vegetative to reproductive growth is a critical transition in the life cycle of flowering plants. In many species the timing of this transition is determined by an interaction between developmental programs and pathways that respond to environmental cues such as daylength and temperature (e.g. Bernier, 1988; Koornneef *et al.*, 1998). These developmental programs and environmental pathways affect both the production of signals that promote or repress flowering, and the competence of shoot apical meristem to undergo the flowering transition (e.g. Bernier, 1988; Levy and Dean, 1998).

Arabidopsis thaliana is a facultative long-day plant, that is, it flowers earlier in long days (LD) than in short days (SD) (Koornneef *et al.*, 1998). Genetic analyses have revealed several genes that, when mutated, cause delayed flowering. These mutants can be grouped according to their flowering response to photoperiod and to exposure to low temperature (the promotion of flowering by low temperature is known as vernalization.) One group of delayed-flowering mutants retains a response to photoperiod and to a vernalizing cold treatment. This group includes *FCA*, *FLOWERING LOCUS D*, *FPA*, *FVE* and *LUMINIDEPENDENS* (*LD*); these genes are thought to promote flowering through a photoperiod-independent pathway known as the autonomous pathway (Levy and

Dean, 1998). Another group of delayed-flowering mutants has little or no response to photoperiod or vernalization. This group, which includes *CONSTANS* (*CO*), *FD*, *FE*, *FHA*, *FT*, *FWA* and *GIGANTEA* (*GI*), comprises the photoperiod pathway (Levy and Dean, 1998). A third pathway is represented by the gibberellin biosynthesis mutant *ga1*. These mutants are only slightly delayed under LD but do not flower in SD (Wilson *et al.*, 1992). Thus *ga1* appears to be required by a pathway that promotes flowering in SD (Levy and Dean, 1998).

In *Arabidopsis*, naturally occurring late-flowering accessions containing the *FRIGIDA* (*FRI*) gene and plants containing mutations in the autonomous floral-promotion pathway are relatively late flowering unless vernalized (Johanson *et al.*, 2000; Michaels and Amasino, 2001). The late-flowering phenotype of these backgrounds is caused by up-regulation of the floral inhibitor *FLOWERING LOCUS C* (*FLC*). After vernalization, however, *FLC* transcript levels are down-regulated and remain low for the remainder of the plant's life (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Thus *FLC* acts as a central regulator of flowering time that is up-regulated by *FRI* and down-regulated by vernalization and the autonomous pathway.

MADS-box genes are a family of transcription factors found in animals, yeast and plants. In *Arabidopsis*, this

family is comprised of at least 46 different genes (Alvarez-Buylla *et al.*, 2000). MADS-box genes regulate many developmental processes (Riechmann and Meyerowitz, 1997; Shore and Sharrocks, 1995). For example, there are MADS-box genes associated with flower meristem identity and the development of floral organs, ovules, fruits, leaves and roots (Riechmann and Meyerowitz, 1997). Recently, four MADS-box genes that regulate flowering time have been identified in *Arabidopsis*: *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999); *SHORT VEGETATIVE PHASE* (*SVP*) (Hartmann *et al.*, 2000); *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000); and *FRUITFULL* (*FUL*) (Ferrandiz *et al.*, 2000). *FLC* and *SVP* are floral inhibitors whereas *SOC1* and *FUL* act to promote flowering (Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000).

Phylogenetic analysis places *FLC* in a subfamily with two other MADS-box genes, *AGL27* and *ALG31* (Alvarez-Buylla *et al.*, 2000). Here we report that *AGL27* is a regulator of flowering time that acts as a repressor of the transition from vegetative to reproductive development, and we designate this gene *FLOWERING LOCUS M* (*FLM*).

Results

Identification and description of flm mutants

FLC was first identified genetically as an inhibitor of flowering that plays a central role in the timing of the transition to flowering in *Arabidopsis* (Koornneef *et al.*, 1994; Lee *et al.*, 1994). *FLC* is a MADS-box gene that is up-regulated by *FRI* and down-regulated by the autonomous pathway or by vernalization (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Phylogenetic analysis reveals that the two closest relatives of *FLC* in the *Arabidopsis* genome are *AGL27* and *AGL31* (Alvarez-Buylla *et al.*, 2000). *AGL31* is organized in tandem repeats forming a cluster of four nearly identical genes at the bottom of chromosome V (GenBank accession numbers AB019236, AB026633 and AB013395), and *AGL27* is located at the bottom of chromosome I (GenBank accession number AC002291). Like *FLC*, *AGL27* is a negative regulator of flowering in *Arabidopsis* (see below). Thus we have designated this gene as *FLOWERING LOCUS M* (*FLM*); the M in this designation reflects that this gene encodes a MADS-domain protein. The MADS domain of *FLM* is 70% identical to *FLC* and 74% identical to *AGL31* (Figure 1a).

Screening of a cDNA library identified two forms of *FLM* resulting from differential splicing at two sites. If differential splicing occurs randomly at these two sites, four mRNA splice variants would be formed. To determine if all splicing variants are produced, *FLM* cDNAs were amplified by RT-PCR, cloned and subjected to diagnostic restriction

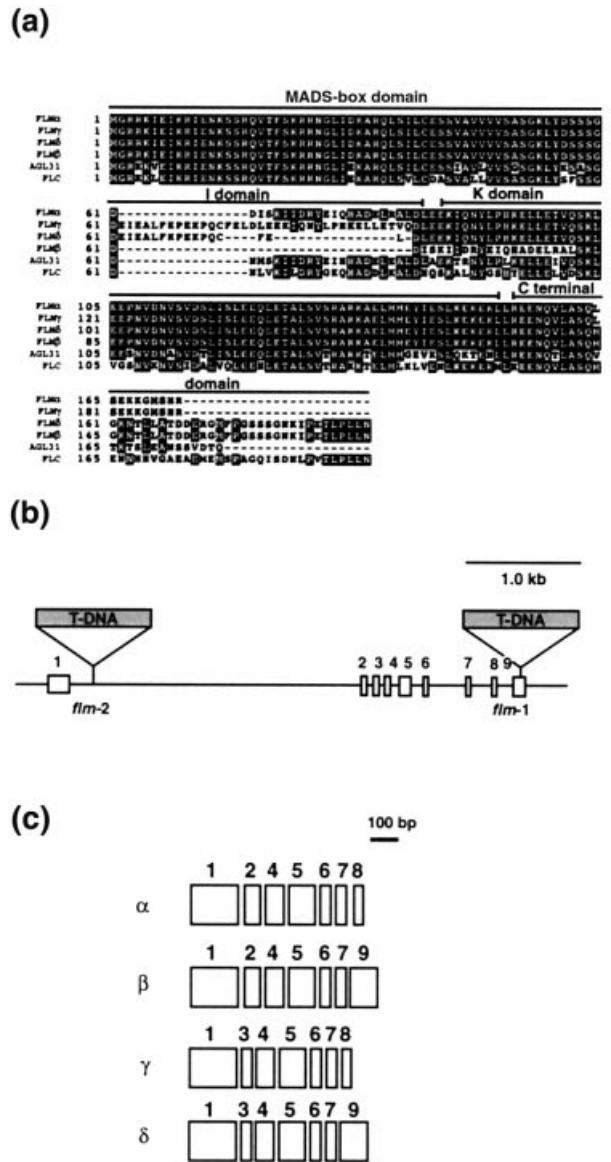


Figure 1. Schematic representation of the *FLM* genomic region, splicing pattern and amino-acid sequence comparison to related proteins. (a) An alignment of amino-acid sequences from *FLC*, *AGL31* and the splice variants of *FLM* using the CLUSTAL W program (Thompson *et al.*, 1994). The MADS, I, K and C-terminal domains are indicated (Riechmann and Meyerowitz, 1997). Identical amino acids are indicated by black boxes, and similar residues by gray boxes. Gaps introduced to optimize the alignment are indicated by dashes. (b) The *FLM* coding region with all nine possible exons shown. Exons are represented by boxes; introns by lines. The positions of T-DNA insertions are also shown. (c) Schematic representation of the four splice variants of *FLM*: *FLM*α, *FLM*β, *FLM*γ and *FLM*δ.

enzyme digestions that would distinguish the four forms. All four predicted transcripts were present, and their identity was confirmed by sequencing (see Experimental procedures). The four forms were designated: *FLM*α; *FLM*β; *FLM*γ; and *FLM*δ (Figure 1a,c). All of these splice

Table 1. Leaf number of *flm* mutants in LD and SD conditions

| Day length | Genotype ^a | Rosette leaf number | Cauline leaf number | Total leaf number ^b |
|------------|-----------------------|---------------------|---------------------|--------------------------------|
| Long | <i>flm-1/flm-1</i> | 5.2 ± 0.5 | 2.1 ± 0.5 | 7.1 ± 0.9 (27) |
| | <i>flm-2/flm-2</i> | 5.2 ± 0.7 | 2.0 ± 0.6 | 7.2 ± 0.9 (34) |
| | <i>flm-1/FLM-1</i> | 6.2 ± 0.6 | 2.1 ± 0.4 | 8.3 ± 0.8 (42) |
| | <i>flm-2/FLM-2</i> | 5.9 ± 0.7 | 2.2 ± 0.5 | 8.2 ± 0.8 (38) |
| | <i>FLM/FLM</i> | 7.1 ± 0.9 | 2.5 ± 0.7 | 9.4 ± 0.8 (44) |
| Short | <i>flm-1/flm-1</i> | 10.0 ± 2.2 | 4.3 ± 0.8 | 14.3 ± 2.4 (15) |
| | <i>flm-2/flm-2</i> | 10.2 ± 2.3 | 4.1 ± 0.9 | 14.2 ± 2.9 (32) |
| | <i>flm-1/FLM-1</i> | 17.1 ± 4.6 | 5.6 ± 1.3 | 21.5 ± 5.6 (24) |
| | <i>flm-2/FLM-2</i> | 16.1 ± 4.6 | 5.4 ± 1.2 | 21.0 ± 5.9 (48) |
| | <i>FLM/FLM</i> | 22.6 ± 4.7 | 7.5 ± 1.2 | 30.6 ± 5.1 (28) |

^aAll genotypes are in the *Ws* genetic background.

^bTotal leaf number was counted as rosette leaves plus cauline leaves. The number of plants analyzed is given in parentheses.

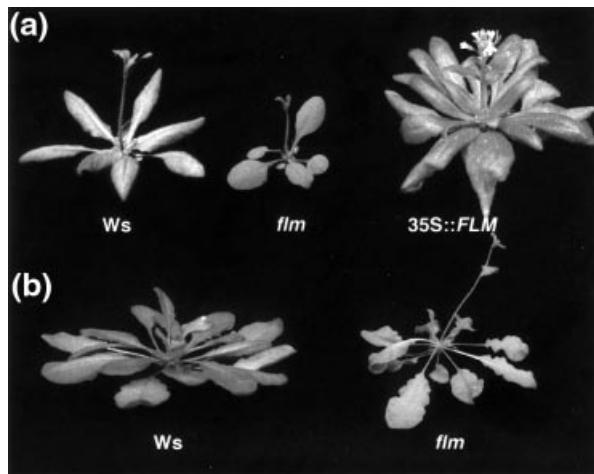


Figure 2. Flowering phenotype of wild type, an *flm* mutant and an *FLM* overexpression line.

(a) Homozygous *flm-1* mutant in *Ws* (middle) compared to overexpression in *Col* background (*35S::FLM*, right) and wild-type *Ws* (left). All plants were grown in LD conditions.

(b) Homozygous *flm-1* mutant in *Ws* (right) compared to wild-type *Ws* (left). Both plants were grown in SD conditions.

variants contain seven of nine possible exons (Figure 1b). *FLM* α is comprised of exons 1, 2, 4, 5, 6, 7 and 8; *FLM* β , exons 1, 2, 4, 5, 6, 7 and 9; *FLM* γ , exons 1, 3, 4, 5, 6, 7 and 8; and *FLM* δ , exons 1, 3, 4, 5, 6, 7 and 9 (Figure 1c). In wild-type Wassilewskija (*Ws*) seedlings, all splicing variants are approximately equally represented (in 87 clones we found 22 clones for the α variant, 19 for β , 28 for γ , 18 for δ).

To determine whether *FLM*, like *FLC*, is a repressor of flowering, we identified two T-DNA-insertion mutations in *FLM* in the *Ws* accession using a reverse genetic approach (Krysan *et al.*, 1999). Each line contained a single T-DNA locus based on DNA blot analysis of a segregating population (data not shown). The *flm-1* allele has a T-

DNA insertion in exon 9, and the *flm-2* allele has a T-DNA insertion in intron 1 (Figure 1b). Plants homozygous for either mutant allele are early flowering (Figure 2). To confirm that the disruption of *FLM* affected flowering behavior, both mutant alleles were back-crossed to wild-type *Ws* and the flowering time and *FLM* genotype of plants in segregating *F*₂ populations were analyzed. Plants from the segregating *F*₂ population for each allele were genotyped by PCR and then analyzed for leaf number. For both alleles, under both LD and SD conditions, the *F*₂ population contained an early, an intermediate and a wild-type class segregating in a 1 : 2 : 1 ratio. The earliest-flowering class was comprised of the homozygous mutants. The *flm-1* and *flm-2* homozygous mutants flowered with an average of two leaves earlier than wild type under LD, and 12 leaves earlier than wild type under SD (Table 1). Plants heterozygous for either allele exhibited the intermediate phenotype, indicating that flowering is sensitive to *FLM* dosage (Table 1). The intermediate phenotype was most apparent in SD (Table 1). Although the *flm-1* allele has a T-DNA insertion in the last exon of the β and δ forms, both mutant alleles produced a similar early flowering phenotype and transcript was not detected in either mutants by RT-PCR, thus both are likely to represent loss-of-function mutations (data not shown).

Overexpression of *FLM* delays flowering

The early flowering phenotype of *flm* mutants indicates that *FLM* is an inhibitor of flowering. To further investigate the role of *FLM*, a genomic clone of *FLM* was fused to the constitutive 35SCaMV promoter. In the first transformed generation (*T*₁), 55 of 76 plants were late flowering compared to the parental line; the remaining lines showed no alteration in flowering time. Photoperiod and vernalization responsiveness was examined in the progeny of

Table 2. Leaf number of *FLM* overexpressors in LD and SD conditions

| Day length | Transgenic line | Leaf number ^a |
|--|-----------------|--------------------------|
| Short | # 29 T2 | 65.0 ± 6.2 (5) |
| | # 42 T2 | 61.6 ± 5.0 (13) |
| | # 71 T2 | na ^c |
| | # 74 T2 | 58.4 ± 2.9 (9) |
| | Col | 41.0 ± 0.8 (5) |
| Long | # 29 T2 | 23.5 ± 5.5 (32) |
| | # 42 T2 | 26.3 ± 3.2 (44) |
| | # 71 T2 | 25.4 ± 2.9 (21) |
| | # 74 T2 | 23.4 ± 2.5 (14) |
| | Col | 10.4 ± 1.1 (5) |
| Long, following vernalization ^b | # 29 T2 | 22.6 ± 2.0 (8) |
| | # 42 T2 | 22.8 ± 3.1 (8) |
| | # 71 T2 | 23.8 ± 2.9 (9) |
| | # 74 T2 | na ^c |

^aLeaf number was counted as rosette leaves; numbers in parentheses correspond to number of plants analyzed.

^bPlants were vernalized at 4°C during 40 days and then transferred to LD conditions.

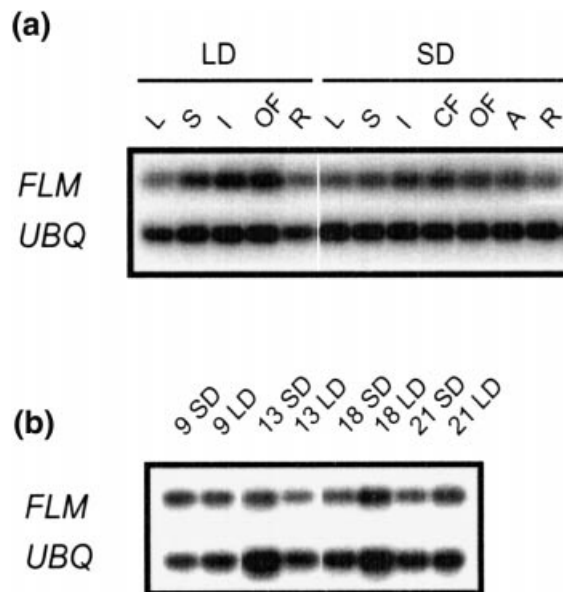
^cna, not analyzed.

several of these transformants (*T*₂ lines). Overexpression of *FLM* delayed flowering under both LD and SD conditions (Table 2). Late-flowering *T*₂ plants flowered with an average of 25 leaves in LD and 60 leaves in SD (compared to 10 and 41 leaves in LD and SD, respectively, for wild type) and thus *FLM* overexpression lines retain a photoperiod response. Also, the delay in flowering resulting from *FLM* overexpression was not significantly affected by vernalizing cold treatments (Table 2), thus *FLM* does not appear to act upstream of vernalization-sensitive components of flowering.

Temporal and spatial expression pattern of *FLM*

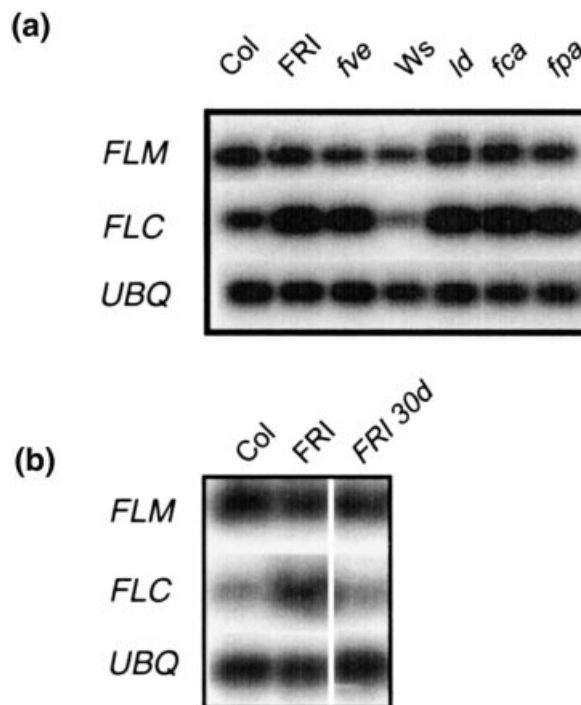
The levels of *FLM* were difficult to detect by RNA blot analysis. Therefore quantitative RT-PCR was used to analyze *FLM* mRNA expression. To avoid cross-hybridization with other MADS-box genes such as *FLC* and *AGL31*, a pair of primers were designed to sequences that were unique to *FLM* and which amplify all splice variants.

FLM expression was detected in all vegetative tissues as well as inflorescences and flowers (Figure 3a). These results are consistent with previous studies of *AGL27* expression (Alvarez-Buylla *et al.*, 2000). Transcript levels did not differ between plants growing in LD and SD, indicating that *FLM* expression was not affected by photoperiod (Figure 3). Transcript levels were also unaffected by plant age during vegetative development (Figure 3b).

**Figure 3.** Expression analysis of *FLM* by RT-PCR.

(a) Expression of *FLM* in leaves (L), stem (S), inflorescence (I), open flowers (OF), closed flowers (CF) and roots (R) of wild-type *Ws* under LD and SD.

(b) Time course of *FLM* expression under LD and SD in whole seedlings.

**Figure 4.** *FLM* expression in various genetic backgrounds and after cold treatment.

(a) RT-PCR analysis of *FLM* mRNA levels in 13-day-old seedlings of (left to right) wild-type Col (which is a *fri* null mutant); *FRI*-SF2 in Col (*FRI*); *fve* mutant in Col; wild-type *Ws*; *ld*, *fca* and *fpa* mutants in *Ws*. *FLC* and *UBQ* are shown as controls.

(b) RT-PCR analysis of *FLM* mRNA levels in (left to right) wild-type Col without cold treatment; *FRI*-SF2 in Col background without cold treatment (*FRI*); and *FRI*-SF2 in Col background after 30 days of cold treatment (*FRI* + 30d). *FLC* and *UBQ* are shown as controls.

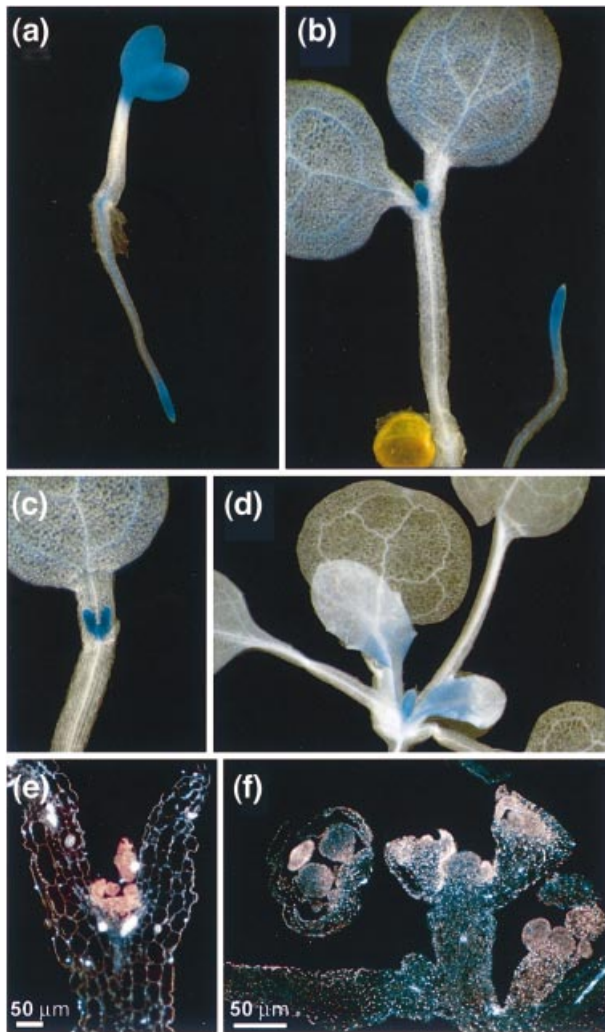


Figure 5. *FLM*-GUS expression pattern. GUS activity is indicated by blue or pink staining.
 (a) At 3 days after germination (DAG), staining is seen in cotyledons, shoot apex and root apex.
 (b) At 5 DAG, expression is restricted to the shoot and root apical regions.
 (c) Enlargement of the shoot apex from a seedling at 5 DAG.
 (d) Shoot expression pattern at 15 DAG.
 (e) Longitudinal section showing the GUS pattern in leaves and in the shoot apex at 5 DAG.
 (f) Longitudinal section showing the GUS pattern in the inflorescence after the transition to flowering at 18 DAG.

FLC is up-regulated by *FRI* and down-regulated by vernalization and the autonomous pathway. Because of the sequence similarity between *FLC* and *FLM*, and their similar roles as inhibitors of flowering, we wished to determine whether *FLM* is regulated similarly to *FLC*. *FLM* transcript levels were unchanged by *FRI*, autonomous-pathway mutants or a 40-day vernalization period (Figure 4a,b). *FLM* mRNA levels were also not affected by the presence of the photoperiod pathway mutants *co* and *gi* (data not shown). Thus the regulation of *FLM* is distinct from that of *FLC*.

To examine more carefully the pattern of *FLM* expression, a construct was created that contained the β -glucuronidase (GUS) gene (Jefferson, 1987) fused in frame to an *FLM* genomic clone at the *NheI* restriction site in exon 7. Analysis of transgenic plants containing this GUS fusion revealed a pattern of fusion protein accumulation that changed during development (Figure 5). In seedlings up to 3 days after germination (DAG; germination refers to radicle emergence), GUS activity is present throughout the plant (Figure 5a). As the seedling grows, the pattern of GUS staining becomes restricted. In plants 5 DAG and older, staining is only seen in the root and shoot apex and in young leaves (Figure 5b–e). After the transition to flowering, staining in the inflorescence meristem is reduced and strong expression is restricted to floral primordia (Figure 5f). A similar expression pattern is also observed for *SVP* (Hartmann *et al.*, 2000).

Discussion

In this study we report the identification of a gene encoding a novel MADS-domain protein from *Arabidopsis* as a flowering repressor. We designated this gene *FLOWERING LOCUS M* (*FLM*) because the amino-acid sequence of the encoded protein is related to the MADS-box gene *FLOWERING LOCUS C* (*FLC*), which has previously been described as an inhibitor of flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Sequence comparison reveals that *FLM*, *FLC* and the tandem repeat of four *AGL31* genes comprise a subfamily of MADS-box genes (Alvarez-Buylla *et al.*, 2000; these authors refer to *FLM* as *AGL27*). Subfamily members often exhibit similar expression patterns and related functions (Theissen *et al.*, 1996). The expression pattern and function of the *AGL31* genes has not been determined, but it would not be surprising if one or more members of the *AGL31* genes also act as flowering repressors. However, determining the role of these genes may be difficult because if two or more of these genes are redundant, classical genetic approaches may require lesions in all active genes to reveal a phenotype. Thus approaches such as overexpression and gene silencing may be required to identify the role of these *AGL31* genes.

FLM has two sites at which differential splicing occurs. The differential splicing at each site is independent, and thus there are four forms of *FLM* mRNA. Comparison of the predicted amino-acid sequence from the four different forms of *FLM* with *FLC* and *AGL31* reveals that the β and δ forms are more similar to *FLC*, whereas the α and γ forms are more similar to *AGL31*. It is not yet known whether one or several of the *FLM* splice variants encode a protein that is active in flowering-time control. The splice variants are present in approximately equal proportions in RNA prepared from seedlings. However, it is possible that regula-

tion of splicing may occur in specific cell types. Future work will address the activities of the individual splice variants and whether regulation of splicing may occur.

The mutant and overexpression phenotypes indicate that *FLM* acts as an inhibitor of flowering. Two independent *flm* mutants are early flowering in both LD and SD conditions. Also, overexpression of *FLM* from the constitutive CaMV 35S promoter delays flowering.

Two mutations that affect flowering time, *fe* and *efs*, are located near *FLM* on the bottom of chromosome I (Koornneef *et al.*, 1998). Because these genes have not yet been molecularly identified, it is possible that they correspond to lesions in *FLM*. The *fe* mutant, however, delays flowering whereas *flm* mutants are early flowering. Although the *efs* mutant flowers early like *flm*, it has pleiotropic effects not seen in *flm* mutants. Furthermore, *efs* is located 3.4 cm from *ADH* (Soppe *et al.*, 1999), but *ADH* is located very close to *FLM* (≈ 20 kb). Thus it seems unlikely that either *fe* or *efs* is an allele of *FLM*. In addition to *FE* and *EFS*, several quantitative trait loci have also been mapped to this region (Koornneef *et al.*, 1998), but whether *FLM* corresponds to any of these loci has not been established.

As discussed above, *FLM* is a flowering repressor that, by sequence comparison, is grouped with two other MADS-box genes: *FLC*, which is also a flowering repressor, and *AGL31*. One additional MADS-box gene that acts as a flowering repressor, *SHORT VEGETATIVE PHASE (SVP)*, has recently been identified in *Arabidopsis* (Hartmann *et al.*, 2000). *SVP* is classified into a distinct subfamily of MADS-box genes that is separate from the *FLC*, *FLM* and *AGL31* subfamily (Alvarez-Buylla *et al.*, 2000). However, *FLC*, *FLM* and *SVP* are similar in that they act as dosage-dependent repressors of flowering (Hartmann *et al.*, 2000; Michaels and Amasino, 1999; Michaels and Amasino, 2000). Also, *FLC*, *FLM* and *SVP* are highly expressed in the shoot apex and young leaves, and are also expressed in roots (Hartmann *et al.*, 2000; Michaels and Amasino, 1999; Michaels and Amasino, 2001).

Despite the close sequence similarity between *FLC* and *FLM*, the function of *FLM* may be most similar to that of *SVP*. For example, *flm* and *svp* mutants have quite similar phenotypes; both mutants flower early in non-inductive SD photoperiods as well as inductive LD photoperiods, and this early flowering phenotype is observed in rapidly flowering summer-annual accessions such as *Ws* and *Col*. In contrast, the early flowering phenotype of *flc* mutations in inductive LD photoperiods is observed only in genetic backgrounds that are otherwise late-flowering, such as *FRI*-containing winter annuals or in autonomous-pathway mutants (Michaels and Amasino, 1999; Michaels and Amasino, 2001). Furthermore, in summer-annual accessions such as *Col*, *flc* mutations have only a slight effect on flowering in SD (Michaels and Amasino, 2001). Another

distinction between *FLM* and *FLC* is the regulation of mRNA levels. *FLC* expression is positively regulated by *FRI* and negatively regulated by autonomous-pathway genes such as *LD*, *FCA*, *FPA* and *FVE*. *FLM*, in contrast, does not exhibit any regulation by *FRI* or the autonomous pathway and, as in *SVP*, expression is also not affected by photoperiod. Given the similarities between *FLM* and *SVP*, it is likely that they act in the same flowering pathway, and it is possible that they act as a heterodimer to repress flowering in *Arabidopsis*.

Experimental procedures

Plant growth conditions

Imbibed seeds were incubated on agar-solidified medium containing 0.65 g l⁻¹ Peters Excel 15-5-15 fertilizer (Grace Sierra, Milpitas, CA) for 2 days at 4°C for stratification before being transferred to soil. Growth conditions were 22°C and 60% relative humidity under approximately 100 $\mu\text{E m}^{-2} \text{sec}^{-1}$ cool-white fluorescent light. Long-day conditions consisted of 16 h light and 8 h dark; short days were 8 h light and 16 h dark. Plants used to obtain roots for RNA extraction were cultured in flasks with liquid Murashige-Skoog medium with 1% sucrose under continuous light with gentle shaking. For vernalization treatment, seeds were incubated on the agar medium mentioned above for 24 h at 21°C and then shifted to 4°C for 40 days under SD conditions.

Identification of *flm* mutants

flm mutant alleles were obtained through a PCR-based screen of T-DNA insertional lines as described (*Arabidopsis* Knockout Facility, Biotechnology Center UW Madison, <http://www.biotech.wisc.edu/Arabidopsis>) (Krysan *et al.*, 1999).

Mutant genotyping

The *flm-1* allele was detected using the primers (5'-GGATAG-AAGCGCTGTTC AAGCCGGA-3') and (5'-TGTCTCCGAAGGAGGT-ACAACACTG-3') which gives a fragment of 1550 bp for *FLM* in wild-type and heterozygous plants, and no amplification in homozygous mutant plants. To verify the presence of the T-DNA in *flm-1* mutants, a PCR reaction was done using the primers (5'-GGATAG AAGCGCTGTTC AAGCCGGA-3') and (5'-CATTTATA-ATAACGCTGCGGACATCTAC-3'), which gives a fragment in the homozygous mutant and heterozygous plants. The *flm-2* allele was detected using the primers (5'-TCCTTTCTGGGTCTCACTCGA-3') and (5'-TGTGTGGCGAGTATCAATGT GG-3') which gives a fragment of 700 bp for *FLM* in wild-type and heterozygous plants, and no amplification in homozygous plants. To verify the presence of the T-DNA in *flm-2*, a PCR reaction was done using the primers (5'-TGTGTGGCGAGTATCAATGTGG-3') and (5'-AGC-ACGGGAAC TGGGATGAC-3'), which gives a fragment in both homozygous mutant and heterozygous plants.

RNA extraction

Total RNA was extracted using the TRI reagent (Sigma, St Louis, MO) as indicated by the manufacturer.

RT-PCR

The total RNA was first treated with DNaseI as recommended by the manufacturer (Promega, Madison, WI). cDNA synthesis was performed using 5 µg of total RNA with superscript reverse transcriptase (Life Technologies, Grand Island, NY). The *FLM* gene was amplified using the primers 5'-GTGAGCTAGGAA-GGCAGAACTGA-3' and 5'-CCGAAGGAGGTACAACACTGATCC-3'; for *FLC* analysis the primers 5'-CCGAAGGAGGTACAACACTGATCC-3' and 5'-AAACGCTCGCCCTTATCAGCGG-3' were used. PCR conditions were as follows: 3 min at 95°C, then 25 cycles of 95°C 30 sec, 65°C 20 sec, 72°C 30 sec, and then 72°C for 2 min. Polyubiquitin was used as a control (primers 5'-GATCTTTGC-CGGAAAACAATTGGAGGATGGT-3' and 5'-CGACTTGTCTTAG-AAAGAAAGAGATAACAGG-3') using the same PCR conditions but with 20 cycles. ExTaq polymerase (Pan Vera/Takara, Madison, WI) was used in these reactions. The amplified fragments were separated on a 1.2% agarose gel, blotted onto a nylon membrane (Biotrace HP, Gelman, Ann Arbor, MI), and hybridized with radiolabeled *FLM*, *FLC* and *UBQ* probes. Signals were visualized using the Molecular Dynamics phosphorimager (Sunnyvale, CA). The RT-PCR analysis was repeated at least three times with independent RNA preparations.

GUS staining and sectioning

Histochemical analyses for GUS activity were conducted according to Jefferson (1987), and sectioning was done according to Patterson (1998) and Fernandez *et al.* (2000). The GUS expression in the sections was analyzed using dark field in a Zeiss Microscope with a 20× magnification lens.

Analysis of *FLM* splice variants

FLM transcripts were amplified using the same conditions as described above using the primers 5'-GGCCATGGGAAGAAG-AAAATCGAGATC-3' and 5'-CCGAAGGAGGTACAACACTGATCC-3'. The RT-PCR product was cloned into pGEMT-Easy (Promega, Madison, WI). Plasmids were digested with *EcoRI* to release the insert from the vector and with *BglII* which cleaves two of the four splice variants. Thus the four forms can be distinguished as follows: α will be cleaved by *BglII* producing fragments of 243 bp and 577 bp; β is not cleaved by *BglII* and gives a fragment of 710 bp; γ will be cleaved by *BglII* producing fragments of 290 bp and 580 bp; δ is not cleaved by *BglII* and gives a fragment of 757 bp. These digestions were analyzed in 2% metaphor agarose gels (ISCBioexpress, Kayville, UT). These plasmids were also sequenced by Perkin Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) using the T7 primer.

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