Regulation of Flowering in Arabidopsis by an *FLC* Homologue

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The Arabidopsis *FLC* gene encodes a MADS domain protein that acts as a repressor of flowering. Late-flowering vernalization-responsive ecotypes and mutants have high steady-state levels of *FLC* transcript, which decrease during the promotion of flowering by vernalization. Therefore, FLC has a central role in regulating the response to vernalization. We have isolated an Arabidopsis gene, *MAF1*, which encodes a protein that is closely related to FLC. Overexpression studies demonstrate that *MAF1* produces comparable effects to *FLC*, and likely has a similar function in the regulation of flowering. In contrast to *FLC*, however, *MAF1* expression shows a less clear correlation with the vernalization response. In addition, *MAF1* overexpression does not influence *FLC* transcript levels. Thus, *MAF1* likely acts downstream or independently of *FLC* transcription. We further report identification of a cluster of four additional *FLC*-like genes in the Arabidopsis genome.

To maximize reproductive success, plants have evolved complex mechanisms to ensure that flowering occurs under favorable conditions. Analysis of late-flowering mutants and ecotypes in Arabidopsis has revealed that such mechanisms depend upon several genetic pathways that might contain 80 or more genes (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Martinez-Zapater et al., 1994; Koornneef et al., 1998a, 1998b; Levy and Dean, 1998). Together these loci coordinate flowering time with environmental variables (e.g. day length, temperature, light quality, and nutrient availability) and with the developmental state of the plant (Martinez-Zapater et al., 1994).

Arabidopsis flowers rapidly when grown under long-day conditions of 16 h or continuous light, but flowers much later under short-day conditions of 8 to 10 h of light. Genes regulating this response constitute the photoperiod pathway and were revealed by mutations that cause late flowering under long days but do not alter flowering in short-day conditions (Fig. 1). Examples of loci from this group, which promote flowering in response to long days, include *CONSTANS* (CO), *GIGANTEA* (*GI*), *FT*, *FWA*, *FE*, *FD*, and *FHA*. A second group of genes, which includes *LUMINIDEPENDENS* (*LD*), *FCA, FVE, FY,* and *FPA*, forms an autonomous pathway that is active under all day length conditions (Fig. 1). Mutants for this second class of genes flower later than wild-type controls irrespective of the day length conditions (Koornneef et al., 1991, 1998a, 1998b; Martinez-Zapater et al., 1994).

In addition to differing in their response to day length, mutants from the photoperiod and autonomous pathways show a differential response to prolonged cold (vernalization) treatments (Vince-Prue, 1975). Through a vernalization response, Arabidopsis ecotypes from northern latitudes, such as Stockholm, are adapted to flower in the spring following exposure to cold winter conditions (Napp-Zinn, 1957). This avoids flowering in the late summer when seed maturation might be curtailed by the onset of winter conditions (Reeves and Coupland, 2000). When these ecotypes are grown in the laboratory they flower late, but will flower much earlier if subjected to a cold period of 4 to 8 weeks while the seed is germinating. In a comparable manner, mutants from the autonomous pathway exhibit a very marked reduction in flowering time when subjected to vernalization. In contrast, mutants from the photoperiod pathway only show a minor response to cold treatments (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Bagnall, 1992; Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994; Chandler et al., 1996; Koornneef et al., 1998b). Thus, vernalization can overcome the requirement for the autonomous pathway (Martinez-Zapater and Somerville, 1990; Reeves and Coupland, 2000).

Genetic analysis of natural ecotypes has demonstrated that the vernalization requirement results from synergistic interactions between dominant alleles of two loci: *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*; Napp-Zinn, 1957, Burn et al., 1993; Clarke and Dean, 1994; Koornneef et al., 1994; Lee et al., 1994; Sanda and Amasino, 1996; Johanson et al., 2000). Late-flowering ecotypes such as Pitztal and Stockholm contain active alleles of *FLC* and *FRI*, whereas early-flowering ecotypes contain a recessive allele of either one or both genes. Therefore, the effects of *FRI* and *FLC* are suppressed by vernalization (Lee and Amasino, 1995).

FLC has recently been cloned and found to encode a MADS domain transcription factor (Michaels and Amasino, 1999; Sheldon et al., 1999). Molecular analysis has now allowed the position of *FLC* within the

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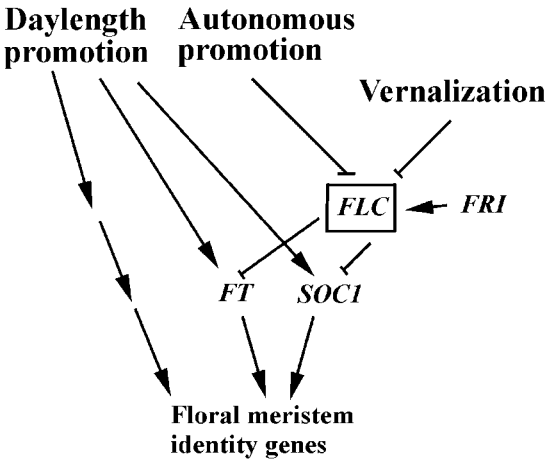

Figure 1. Schematic diagram (after Samach et al., 2000) showing the network of genes controlling flowering time in Arabidopsis. The day length pathway (also known as the photoperiod pathway) and the autonomous pathway of floral promotion are thought to converge prior to up-regulation of the floral meristem identity genes.

flowering control pathways to be determined. Plants containing a dominant allele of the *FRI* gene and mutants from the autonomous pathway all contain high steady-state levels of *FLC* transcript, which declines in response to a cold treatment. Thus, *FLC* expression appears to be supported by *FRI* and repressed by the products of genes from the autonomous pathway. Mutants from the photoperiod pathway, on the other hand, exhibit relatively low levels of *FLC* expression. Furthermore, it has been shown that high levels of *FLC* transcript are sufficient to produce very late flowering in Landsberg *erecta* (L*er*), which lacks a functional *FRI* allele (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000; Johanson et al., 2000). Hence, *FLC* has a central function in the maintenance of a vernalization requirement.

A key question now is to identify the components of the downstream pathway by which FLC exerts repression of flowering. It has been shown recently that the photoperiod and autonomous pathways likely converge via at least two genes, *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), prior to the activation of floral meristem identity genes (Borner et al., 2000; Lee et al., 2000; Onouchi et al., 2000; Samach et al., 2000). It has been suggested that the levels of these genes might be determined through a balance of *CONSTANS* and *FLC* activity (Fig. 1). Although *SOC1* and *FT* are probably direct targets of CO, their interactions with *FLC* may be indirect (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). Therefore, it is probable that additional genes act in the pathway alongside or downstream of *FLC*.

We have isolated and commenced characterization of a novel Arabidopsis gene, *MAF1* (MADS Affecting Flowering 1), which encodes a protein highly related to FLC. Mutant alleles of this gene are not yet available, but overexpression analyses indicate that it functions in the regulation of flowering time in a similar manner to *FLC*. Moreover, analysis of the Arabidopsis genome sequence reveals four additional genes that are very similar to *FLC* and *MAF1*.

RESULTS

Identification of an *FLC* Homolog

We identified a MADS box gene, *F22K20.15*, within BAC F22K20 (GenBank accession no. AC002291) from chromosome 1 that was predicted to encode a protein closely related to FLC (this gene was also noted by Michaels and Amasino, 1999). An 872-bp cDNA clone for this *FLC* homolog was identified among clones isolated from a library derived from leaf mRNA. The encoded protein was 196 amino acids in length, and shared 62% overall amino acid sequence identity with FLC, and 82% identity within the MADS DNA binding domain (Fig. 2). Based on the results to be described below, we named this novel gene *MAF1*. *MAF1/F22K20.15* also corresponds to the recently described gene *AGL27*, of which the similarity to *FLC* was noted in the phylogenetic analysis of 45 Arabidopsis MADS box genes (Alvarez-Buylla et al., 2000a).

Reverse transcriptase (RT)-PCR studies detected *MAF1* transcript in a variety of different tissues (Fig. 3A). A recent paper, published during the preparation of this article, has also shown the ubiquitous expression of *MAF1/AGL27* by RNA-blot analyses (Alvarez-Buylla et al., 2000b). Therefore, as for *FLC*, expression of *MAF1* is not restricted to a specific region of the Arabidopsis plant (Michaels and Amasino, 1999; Sheldon et al., 1999).

The genetic map position of *MAF1* could be precisely defined because BAC F22K20 contains the *ALCOHOL DEHYDROGENASE* (*ADH1*) gene. However, no known flowering time regulator was located in this region of the genetic map (Koornneef et al.,

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MAF1     1 MGRRKIEIKRIENKSSRQVTFSKRRNGLIDKARQLSILCE  40
FLC      1 MGRKKLEIKRIENKSSRQVTFSKRRNGLIEKARQLSVLCD  40
           ***.*.**********************.******.**.

MAF1    41 SSVAVVVVSASGKLYDSSSGDDISKIIDRYEIQHADELRA  80
FLC     41 ASVALLVVSASGKLYSFSSGDNLVKILDRYGKQHADDLKA  80
           .***..*********  **** . **.***  ****.*.*

MAF1    81 LDLEEKIQNYLPHKELLETVQSKLEEPNVDNVSVDSLISL 120
FLC     81 LDHQSKALNYGSHYELLELVDSKLVGSNVKNVSIDALVQL 120
           ** . *  **  * **** * ***   ** ***.*.*. *

MAF1   121 EEQLETALSVSRARKAELMMEYIESLKEKEKLLREENQVL 160
FLC    121 EEHLETALSVTRAKKTELMLKLVENLKEKEKMLKEENQVL 160
           **.*******.**.*.***.  .* ******.*.******

MAF1   161 ASQMGKNTLLATDDERGMFPGSSSGNKIPETLPLLN 196
FLC    161 ASQMENNHHVGAEAEMEMSPAGQISDNLPVTLPLLN 196
           ****  *  . .. *  * *       .* ******
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Figure 2. Sequence comparison of the MAF1 and FLC proteins. Asterisks indicate identical amino acids, and similar residues are depicted by dots. The *MAF1* cDNA sequence has been deposited in GenBank (accession no. AF342808).

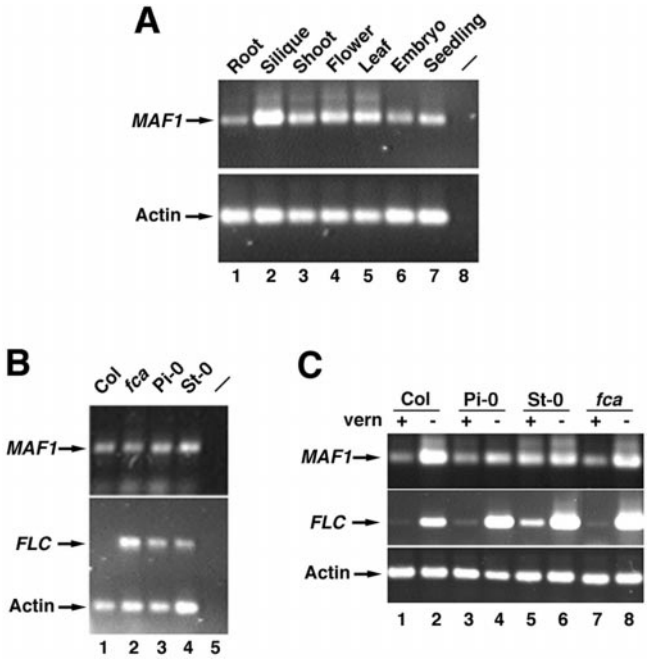

Figure 3. *MAF1* is ubiquitously expressed and shows a less consistent response to vernalization than *FLC*. A, *MAF1* expression was determined by RT-PCR in a variety of tissues (top: root, silique, shoot, flower, leaf, embryo, and whole seedling tissue samples). RT-PCR was also performed with actin primers as control (bottom). B, Comparable levels of *MAF1* expression are found in vegetative seedlings of different genetic backgrounds (top), whereas *FLC* is expressed at higher levels in vernalization responsive backgrounds (middle row). C, *MAF1* levels of expression are reduced by vernalization in *fca-9* and wild-type Columbia, but are altered less markedly in Stockholm and Pitztal (top). *FLC* transcript levels are substantially reduced by vernalization in all four backgrounds (middle). Col, Columbia; Pi-0, Pitztal; St-0, Stockholm; vern, vernalization. −, Blank RT-PCR control lane.

μ1998a; Levy and Dean, 1998; Arabidopsis genetic map available at The Arabidopsis Information Resource, http://www.arabidopsis.org/).

Overexpression of *MAF1* Modifies Flowering Time in a Comparable Manner to *FLC* Overexpression

Dominant alleles of *FLC*, and overexpression of the gene in the L*er* background, have been reported to delay flowering, whereas null *flc* mutants are early flowering (Koornneef et al., 1994; Lee et al., 1994; Michaels and Amasino, 1999; Sheldon et al., 1999). Thus, *FLC* acts to prevent premature flowering. Because *MAF1* mutant alleles were unavailable, we used an overexpression strategy to investigate whether this gene has a similar function to that of *FLC*. Transgenic Arabidopsis plants, of ecotype Columbia, were produced in which the *MAF1* cDNA was constitutively expressed from a cauliflower mosaic virus 35S promoter. Out of 40 T_1 lines, 31 individuals flowered earlier than control plants transformed with an empty vector (Fig. 4A). These transformants produced visible inflorescences approximately 7 to 14 d earlier than the control plants. Their mean rosette leaf number at the time of flowering was 12.4 $\pm$ 0.8, whereas the control plants flowered with 27.4 $\pm$ 1.2 rosette leaves (12-h photoperiod). While two T_1 plants flowered at the same time as controls, seven lines produced visible inflorescences 2 to 3 weeks after wild type and were clearly late flowering (Fig. 4B). In most instances, therefore, *MAF1* promoted flowering, but in a minority of cases delayed flowering. These results appeared to indicate that *MAF1* could have an opposing role to *FLC*. However, whereas overexpression of *FLC* in L*er* causes a delay in flowering (Michaels and Amasino, 1999; Sheldon et al., 1999), it has also been reported that 35S::*FLC* can cause early flowering. When a 35S::*FLC* construct was introduced into the C24 background, only two of 23 T_1 plants were late flowering, whereas 17 of 23 flowered early (Sheldon et al., 1999). Thus, our results for 35S::*MAF1* in Columbia were similar to those obtained for 35S::*FLC* in the C24 ecotype.

To further explore these discrepancies between ecotypes, we transformed a 35S::*FLC* construct into both Columbia and L*er*, and 35S::*MAF1* into L*er*. Under continuous light conditions, nine of 17 T_1

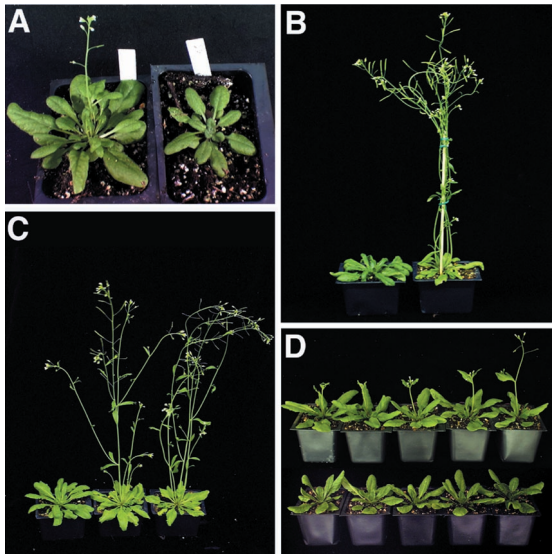

Figure 4. Effects of *MAF1* overexpression in Arabidopsis plants of Columbia, Stockholm, and Pitztal ecotypes. A, Early-flowering 35S::*MAF1* Columbia T_1 transformant (left plant) compared with wild-type Columbia (right plant) at 5 weeks after sowing. Plants were grown under 12-h light. B, Late-flowering 35S::*MAF1* Columbia T_2 plant of line 4 (left plant), compared with wild type (right plant) at 36 d after sowing. Plants were grown under continuous light. C, Effects of 35S::*MAF1* in Stockholm are comparable to the effects of vernalization in that ecotype. Wild-type non-vernalized plant (left), 35S::*MAF1* non-vernalized T_1 plant (middle), and wild-type vernalized plant (right) are shown at 34 d after sowing. Plants were grown in continuous light. D, Selection of early-flowering 35S::*MAF1* Pitztal T_1 lines (top row) compared with Pitztal wild-type plants at 34 d after sowing, grown under continuous light.

35S::*FLC* Columbia plants flowered approximately 1 week earlier than controls (mean rosette leaf no. of 5.1 ± 0.6 compared with 11.6 ± 0.5 for wild type). Although four of 17 plants had a wild-type phenotype, the remaining four of 17 T_1 plants were markedly late flowering. In fact, three of the late-flowering 35S::*FLC* Columbia T_1 plants had still not flowered after 5 months of growth. In addition, the plants developed multiple axillary shoots from among the primary rosette leaves, which formed a mass of secondary rosettes (not shown). It is noteworthy that this phenotype is comparable to that of a dominant mutant of *FLC* obtained by Sheldon et al. (1999) in the C24 background.

In contrast to Columbia transformants, none of our 35S::*FLC* L*er* T_1 plants were noted to flower earlier than wild-type controls (mean rosette leaf no. of 6.3 ± 0.3). However, ten of 19 35S::*FLC* L*er* T_1 plants were clearly late flowering and typically bolted more than 1 week later than wild type. Moreover, four of these plants were extremely late flowering and had still not flowered after 3 months. Similar results were obtained for 35S::*MAF1* in L*er*: of 12 T_1 plants examined: none flowered early, nine of 12 were similar to wild type, and three of 12 were distinctly late flowering. These individuals made approximately 20 rosette leaves and flowered around 2 weeks later than wild type.

In summary, overexpression of *MAF1* and *FLC* produced equivalent effects. In the Columbia background, both genes yielded a majority of early-flowering lines and a few late-flowering lines. By contrast, neither gene was noted to cause early flowering in L*er* plants, but each generated a number of late flowering lines. It should be noted, however, that *FLC* appeared to be a more potent repressor of flowering than *MAF1*. None of the 35S::*MAF1* plants were as late flowering as the most extreme 35S::*FLC* plants.

Effects of Genetic Background and Vernalization on *MAF1* Expression

A key observation with regard to *FLC* activity is that certain late-flowering ecotypes (with an active *FRI* allele or defects in autonomous promotion) have high *FLC* transcript levels, which fall when flowering has been induced by vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000). To determine whether *MAF1* expression parallels that of *FLC* with respect to the influence of genetic background, we studied its expression in a mutant for the *FCA* gene (a component of the autonomous pathway) and in two late-flowering ecotypes, Stockholm and Pitztal. Plants were grown under continuous light conditions and RNA was prepared from whole vegetative seedlings. RT-PCR using gene-specific primers revealed no obvious differential expression of *MAF1* between wild-type Columbia and *fca* mutant, Stockholm, or Pitztal seedlings (Fig. 3B). *FLC* expression, however, was markedly higher in those samples than in wild-type Columbia, confirming previous observations (Fig. 3B; Michaels and Amasino, 1999; Sheldon et al., 1999, 2000).

To examine how *MAF1* transcript levels are affected by vernalization, germinating seedlings were cold treated for approximately 8 weeks. Then they were transferred to a growth cabinet under continuous light conditions along with freshly sown non-vernalized seedlings, and the levels of *MAF1* and *FLC* expression evaluated by RT-PCR after 8 d. In wild-type Columbia and *fca* mutant backgrounds, *MAF1* transcript levels were distinctly higher in samples from non-vernalized than vernalized plants (Fig. 3C). In the Pitztal and Stockholm backgrounds, however, the difference in *MAF1* levels caused by the vernalization treatment was less apparent. To confirm the effectiveness of the vernalization, RT-PCR was performed over 30 cycles with *FLC* primers: *FLC* transcript was present in untreated seedlings at much higher levels than in the vernalized samples for all four genetic backgrounds (Fig. 3C). These results for *MAF1* and *FLC* were replicated in three independent experiments for which different batches of vernalized and non-vernalized seedlings were used. Furthermore, batches of plants were grown to maturity. As expected, *fca*, Stockholm, and Pitztal all showed a strong vernalization response and flowered several weeks earlier than non-treated controls. Wild-type Columbia displayed a clear but much weaker response, with vernalized plants producing visible flower buds about 5 d earlier than non-treated plants.

In summary, expression of *MAF1* does not completely parallel that of *FLC*. *MAF1* levels are decreased by vernalization, but the effects are less consistent between different genetic backgrounds than for *FLC*. In contrast to *FLC*, *MAF1* transcript is present at similar moderate levels in the Columbia background (which lacks an active allele of the *FRI* gene) and in Stockholm and Pitztal (which both possess an active *FRI* allele). *FLC*, the expression of which is supported by *FRI*, is expressed at relatively low levels in the Columbia background, unless the *FCA* gene is inactive.

MAF1 Overexpression Can Promote Flowering in the Late Ecotypes Stockholm and Pitztal

To test whether an increase in *MAF1* expression could influence the repression of flowering imposed by high *FLC* levels, we overexpressed *MAF1* in the late-flowering ecotypes Stockholm and Pitztal. In a first experiment, 32 primary transformants of Pitztal and 32 of Stockholm were grown interspersed with wild-type control plants under continuous light conditions. In both cases, around 50% of the transformants flowered markedly earlier than any wild-type plant, and in some transformants, time to flowering (based on total leaf no. or days to open flower) was

approximately halved (Fig. 4, C and D; Table I). However, just as was observed with overexpression of *MAF1* in Columbia, a minority of the Pitztal and Stockholm transformants were clearly late flowering compared with the wild-type plants. In fact, one Stockholm T_1 line was extremely late flowering, generated masses of secondary rosettes, and did not produce flower buds for more than 3 months under continuous light. Nevertheless, in these ecotypes the most common effect of *MAF1* overexpression was early flowering.

To explore whether overexpression of *MAF1* produces comparable effects to vernalization, batches of wild-type Pitztal and Stockholm seedlings were cold treated for 6 weeks at 4°C, then grown among a second set of 35S::*MAF1* T_1 Pitztal, 35S::*MAF1* T_1 Stockholm, and non-vernalized wild-type plants (Table I). As expected, vernalization markedly and uniformly reduced flowering time in both Pitztal and Stockholm wild-type plants (Table I). Among the 35S::*MAF1* Stockholm lines, the earliest flowering T_1 group (eight of 23 lines) was indistinguishable from vernalized plants. For Pitztal, however, the early-flowering T_1 plants were on average marginally later than the vernalized plants.

Late Flowering of 35S::*MAF1* Plants Correlates with the Highest Levels of Overexpression

Because both late- and early-flowering plants could be identified among the 35S::*MAF1* T_1 lines, we speculated that one of the two phenotypes could correspond to cosuppressed individuals in which the transgene and endogenous genes had become silenced to reveal a mutant phenotype for *MAF1*. To investigate this, we examined the T_2 progeny of three late-flowering (lines 4, 5, and 11) and three early-

Table I. *Flowering time phenotypes of 35S::MAF1 Stockholm and Pitztal T_1 lines*[a]

ND, Not determined.

Experiment	Phenotype[b,c]	Penetrance	Days to Visible Flower Bud	Days to First Open Flower	Rosette Leaf No.	Cauline Leaf No.	Total Leaf No.
Experiment 1							
Pitztal							
Wild type	Wild type	16/16	31–34 (ND)	33–36 (34.6 ± 0.5)	22–27 (25.4 ± 0.9)	6–9 (7.3 ± 0.5)	28–36 (32.7 ± 1.2)
35S::*MAF1*	Early	18/32	15–30 (ND)	18–32 (26.9 ± 2.3)	7–18 (12.8 ± 1.6)	1–6 (2.8 ± 0.6)	8–24 (15.1 ± 1.9)
	Slightly early	2/32	31–32 (ND)	33–34 (33.5 ± ND)	18–18 (18.0 ± ND)	4–5 (4.5 ± ND)	22–23 (22.5 ± ND)
	Wild type	3/32	31–34 (ND)	33–36 (34.7 ± ND)	23–26 (24.3 ± ND)	4–10 (7.0 ± ND)	28–36 (31.4 ± ND)
	Late	9/32	36–48 (ND)	39–50 (42.9 ± 3.2)	34–47 (38.8 ± 3.8)	9–12 (10.7 ± 0.9)	43–59 (49.4 ± 4.5)
Stockholm							
Wild type	Wild type	16/16	33–40 (ND)	37–43 (39.6 ± 1.0)	28–43 (36.4 ± 2.7)	6–8 (7.2 ± 0.5)	36–50 (43.6 ± 2.4)
35S::*MAF1*	Early	12/32	23–32 (ND)	26–35 (32.5 ± 2.3)	11–25 (17.5 ± 3.2)	1–6 (4.1 ± 1.4)	12–31 (21.6 ± 4.3)
	Slightly early	5/32	31–35 (ND)	35–40 (38.2 ± 2.4)	25–28 (26.4 ± 1.7)	5–7 (6.2 ± 1.0)	30–34 (32.6 ± 2.4)
	Wild type	11/32	35–40 (ND)	39–43 (40.3 ± 1.1)	28–38 (32.8 ± 2.4)	5–8 (7.2 ± 0.7)	35–46 (40.0 ± 2.7)
	Late	4/32	45–49 (ND)	50–52 (51.0 ± 1.8)	47–62 (ND)	7–12 (9.0 ± 3.4)	56–70 (ND)
Experiment 2[d]							
Pitztal							
Wild type	Wild type	22/22	26–33 (27.5 ± 0.9)	29–37 (31.8 ± 0.8)	14–28 (18.7 ± 1.5)	1–9 (6.1 ± 0.9)	16–34 (24.6 ± 2.0)
Vernalized wild type	Early	24/24	17–19 (18.0 ± 0.5)	19–25 (22.3 ± 0.4)	8–13 (10.6 ± 0.6)	2–3 (2.7 ± 0.2)	10–16 (13.2 ± 0.7)
35S::*MAF1*	Early	4/23	22–22 (22.0 ± ND)	24–27 (25.0 ± 2.3)	10–11 (10.5 ± 0.9)	1–4 (2.3 ± ND)	12–14 (12.8 ± 1.5)
	Slightly early	9/23	22–26 (23.4 ± ND)	25–30 (27.2 ± 1.4)	12–16 (13.9 ± 1.2)	3–4 (3.2 ± 0.3)	16–20 (17.1 ± 1.3)
	Wild type	7/23	25–33 (28.6 ± 2.6)	28–36 (32.3 ± 2.8)	16–27 (21.0 ± 4.0)	5–8 (6.1 ± 1.1)	21–34 (27.1 ± 4.8)
	Slightly late	2/23	30–33 (31.5 ± ND)	34–36 (35.0 ± ND)	25–30 (27.5 ± ND)	9–10 (9.5 ± ND)	35–39 (37.0 ± ND)
	Late	1/23	36–36 (36.0 ± ND)	40–40 (40.0 ± ND)	33–33 (33.0 ± ND)	8–8 (8.0 ± ND)	41–41 (41.0 ± ND)
Stockholm							
Wild type	Wild type	23/23	27–35 (30.9 ± 0.9)	31–39 (34.4 ± 0.9)	22–34 (28.0 ± 1.5)	4–10 (7.3 ± 0.6)	26–44 (35.3 ± 1.8)
Vernalized wild type	Early	24/24	17–22 (20.3 ± 0.7)	21–28 (23.8 ± 1.0)	9–13 (11.1 ± 0.5)	1–5 (2.1 ± 0.3)	11–18 (13.3 ± 0.7)
35S::*MAF1*	Early	8/23	16–26 (20.5 ± 2.5)	18–29 (24.6 ± 3.3)	8–12 (10.6 ± 1.5)	1–4 (2.4 ± 0.8)	9–18 (13.0 ± 2.3)
	Slightly early	5/23	26–27 (26.6 ± 0.7)	31–31 (31.0 ± ND)	12–19 (16.2 ± 3.4)	2–5 (4.0 ± 1.5)	14–24 (20.2 ± 4.8)
	Wild type	7/23	27–35 (32.7 ± 2.9)	31–39 (36.6 ± 2.8)	23–35 (28.1 ± 4.2)	7–10 (8.3 ± 1.4)	31–44 (36.4 ± 5.2)
	Slightly late	1/23	36–36 (36.0 ± ND)	40–40 (40.0 ± ND)	25–25 (25.0 ± ND)	8–8 (8.0 ± ND)	33–33 (33.0 ± ND)
	Late	2/23	41–>90[e] (ND)	45–>90[e] (ND)	41–ND (ND)	7–ND (ND)	48–ND (ND)

[a] The range of values obtained followed by mean ± SE with 95% confidence limits attached (parentheses) are shown for each class. [b] Transformants classified as early or late, flowered outside the wild-type range in terms of both days to first open flower and total leaf no. [c] Transformants classified as slightly early or slightly late flowered outside wild-type range in terms of days to first open flower or total leaf no. [d] For unknown reasons, in the second sowing, the non-vernalized wild-type plants exhibited greater variation in flowering time than in the first sowing. [e] One plant produced no flowers within the duration of the experiment (90 d).

flowering (lines 1, 3, and 6) 35S::*MAF1* Columbia plants (Table II).

In this experiment, plants were grown under continuous light. All T_2 plants from line 4 were uniformly late flowering and had a total leaf number more than double that of wild type. Late flowering was also evident in the T_2 populations from lines 5 and 11, but in these cases, a minor proportion of the plants lost the phenotype.

For the early-flowering lines, under the extremely inductive conditions of continuous light, a very marginal reduction in flowering time was observed that only slightly reduced the total leaf number (Table II). It is interesting that in the T_2 progeny from line 3, although 18 of 20 individuals flowered a little earlier than wild type, two individuals flowered markedly late, with 32 and 35 leaves, respectively.

When T_2 populations for early-flowering 35S::*MAF1* lines were regrown in 12-h-light conditions, a clear early-flowering phenotype was noted (Table II). Overall, then, it appeared that the early-flowering effects of *MAF1* overexpression were most evident under less inductive circumstances such as when Columbia plants were grown under a 12-h photoperiod, or in late-flowering ecotypes.

It is unclear why lines 5 and 11 produced some non-late-flowering individuals, and why the early-flowering T_1 line 3 yielded occasional late-flowering plants in the T_2 generation. We speculate that this occurred due to quantitative changes in expression level of the transgene through gene silencing type phenomena. To examine how expression levels correlated with flowering time, RNA was extracted from pooled T_2 vegetative seedlings of each line and from leaves of individual adult T_2 plants that were flowering. RT-PCR was performed using *MAF1*-specific primers at a low number (25) of cycles. The highest levels of *MAF1* expression were detected in late-flowering individual plants (Fig. 5A, lanes 2, 4, 5, and 7) or in samples from pooled seedlings that contained late-flowering individuals (Fig. 5A, lanes 12–14 and 16). Plants that showed only moderate or low levels of overexpression compared with wild type were slightly early flowering or normal (Fig. 5A, lanes 3, 6, 8, 9, 11, and 15). The trend was also observed in 35S::*MAF1* Stockholm (Fig. 5B) and Pitztal T_1 plants (not shown). RT-PCR was performed with two early- and two late-flowering lines in each background: Again, the late-flowering lines contained the higher levels of *MAF1* expression (Fig. 5B, lanes 2 and 3). Thus, late flowering does not arise from cosuppression of *MAF1*. Rather, the factor appears to affect flowering time in a quantitative manner; a modest level of overexpression triggers early flowering, whereas a larger increase delays flowering.

Late Flowering of 35S::*MAF1* Plants Is Independent of *FLC* Expression and Does Not Respond to Vernalization

Because *FLC* acts as a repressor of flowering, we explored whether late-flowering 35S::*MAF1* Columbia plants contained elevated *FLC* transcript levels. RT-PCR was repeated using *FLC* specific primers over 30 cycles (Fig. 5A). No correlation was noted between lateness of flowering and the level of *FLC* transcript. Hence, it appears that the phenotype of late-flowering lines is not dependent on *FLC* expression. In addition, early- and late-flowering 35S::*MAF1* Stockholm plants were both found to contain comparable levels of *FLC* transcript to non-vernalized Stockholm controls, verifying that *MAF1* does not affect *FLC* transcription (Fig. 5B). Thus, in late-flowering 35S::*MAF1* lines, overexpression of *MAF1* activated a repression pathway independent of *FLC* or it influenced the autonomous pathway downstream of *FLC*

Table II. *Flowering time phenotypes of 35S::MAF1 Columbia lines*

ND, Not determined.

Line	T_1 Phenotype (12-H Light)	No. of Insertion Points[a]	*MAF1* Expression Level[b]	T_2 Phenotype (24-H Light)[c,d]	T_2 Phenotype (12-H Light)[c,d]
4	Late	1	***	20/20 Late (34.2 ± 1.0)	20/20 Late (ND)
5	Late	1	***	16/20 Late (32.3 ± 1.3)	14/19 Late (ND)
			**	4/20 Normal (12.4 ± 2.1)	5/19 Normal (ND)
11	Late	1	***	12/20 Late (31.9 ± 1.0)	10/20 Late (ND)
			ND	8/20 Normal (12.1 ± 1.8)	10/20 Normal (ND)
1	Early	1	**	20/20 Normal (13.1 ± 0.9)	ND
3	Early	Multiple	**	18/20 Slightly early (11.1 ± 0.8)	17/20 Early (18.9 ± 2.5)
			***	2/20 Late (33.5 ± ND)	3/20 Late (63.7 ± 6.2)
6	Early	Multiple	**	20/20 Slightly early (11.7 ± 0.7)	19/20 Early (17.2 ± 2.2)
					1/20 Normal (30 ± ND)
2	Early	ND	ND	ND	17/19 Early (13.2 ± 3.0)
					2/19 Late (54.5 ± ND)
Wildtype	Normal	0	*	20/20 Normal (13.2 ± 0.5)	20/20 Normal (33.2 ± 2.0)

[a] No. of independent insertion points was assessed via kanamycin resistance segregation in the T_2 populations. [b] *, Low; **, moderate; ***, high, indicate level of *MAF1* expression detected by RT-PCR analysis. [c] Nos. in brackets indicate mean total leaf no. ± SE with 95% confidence limits. [d] All T_2 populations, except line 1, were selected on kanamycin plates and transferred to soil 7 d after sowing.

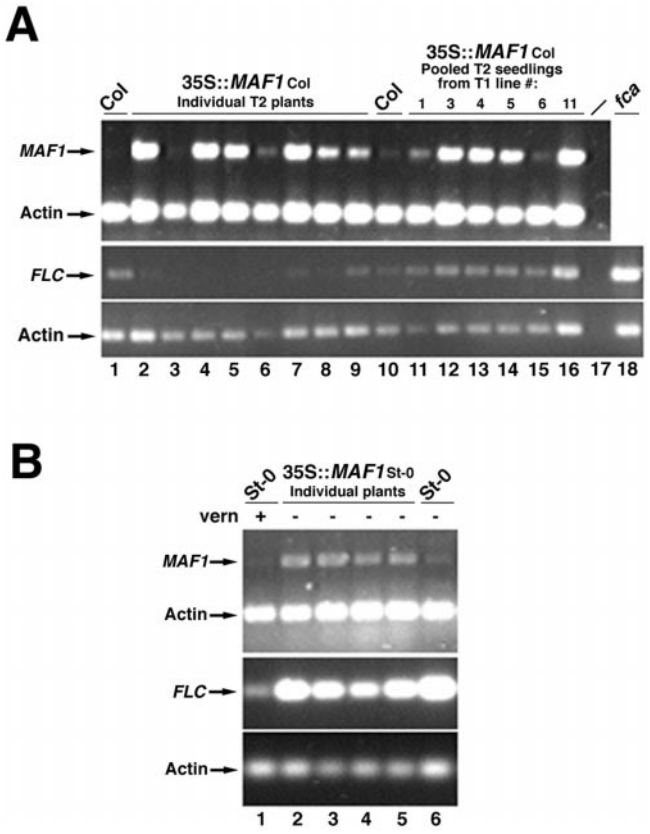

Figure 5. Late flowering of 35S::*MAF1* plants correlates with the highest levels of overexpression and is independent of *FLC* expression. RT-PCR expression profiles of *MAF1* (upper) and *FLC* (middle) in 35S::*MAF1* plants of Columbia (A) and Stockholm (B) ecotypes are shown. All plants were grown under continuous light conditions. A, Samples in lanes 2 through 9 are derived from rosette leaves of individual adult T_2 plants. Lanes 2, 4, 5, and 7, showing the highest levels of *MAF1* expression, correspond to late flowering plants. Plants that showed only moderate or low levels of overexpression compared with wild type were slightly early flowering or normal (lanes 3, 6, 8, and 9). Lane 1 corresponds to a wild-type control plant. Samples in lanes 11 through 16 are derived from pooled vegetative T_2 seedlings of 35S::*MAF1* lines 1, 3, 4, 5, 6, and 11, as indicated. Samples from pooled seedlings that contained late-flowering individuals (lanes 12, 13, and 16) showed the highest levels of *MAF1* expression. Lane 10 corresponds to wild-type control pooled seedlings. For both individual plants and pooled seedlings, there is no clear association between *FLC* levels and lateness of flowering. High levels of *FLC* are detected in the *fca* control. B, Samples in lanes 2 through 5 are from rosette leaf tissue of individual 35S::*MAF1* Stockholm T_1 adult plants. Lanes 2 and 3 correspond to late-flowering plants; lanes 4 and 5 correspond to early-flowering plants. All lines contain high levels of *FLC* transcript compared with the vernalized wild-type control (lane 1), and the early- and late-flowering 35S::*MAF1* lines both contain high levels of *FLC* transcript comparable to those in the non-vernalized wild type (lane 6). Col, Columbia; St-0, Stockholm; vern, vernalization. −, Blank RT-PCR control lane.

transcription (Fig. 1). We have observed, however, that late-flowering 35S::*MAF1* plants are responsive to photoperiod. In an experiment conducted under short day conditions of 8 h of light, we obtained a number of 35S::*MAF1* Columbia T_1 plants that flowered up to a month later than wild-type controls (data not shown). This response shows that late-flowering 35S::*MAF1* plants possess a functional photoperiod promotion pathway, and therefore are more likely defective in the autonomous (or some other) pathway.

To confirm that the late flowering effects caused by *MAF1* overexpression were independent of *FLC* transcription, we tested whether late-flowering 35S::*MAF1* Columbia plants were responsive to vernalization. No significant change in flowering time was noted: In continuous light conditions, vernalized T_2 plants of line 4 had a total of 31.3 ± 1.8 leaves compared with 30.1 ± 1.3 when non-vernalized. Control *fca* plants verified that the treatment was effective: Vernalized plants flowered after only 10.3 ± 0.9 leaves compared with more than 40 leaves for the non-vernalized controls. Thus, the late-flowering phenotype caused by MAF1 could not be overcome by vernalization, a result that would be expected if the delay occurred independently of changes in *FLC* expression.

Arabidopsis Contains a Family of Six *FLC*-Like Genes

The complex and quantitative effects of *MAF1* on flowering time suggested that its overexpression might have influenced the targets of other transcription factors, such as FLC. A search of the Arabidopsis genomic sequence for additional *FLC* and *MAF1* homologs identified four other highly related genes, which form a tight cluster at the bottom of chromosome 5. The gene cluster occupies approximately 22 kb and consists of genes *MXK3.30* (which corresponds to AGL31; Alvarez-Buylla et al., 2000a), *F15O5.2*, *F15O5.3*, and *F15O5.4* (GenBank accession nos. BAB10332, BAA97510, BAA97511, and BAB11644, respectively). The MADS domains of the proteins encoded by these four genes are highly conserved with those of FLC and MAF1: 76% to 91% of amino acid sequence identity, depending on the pair-wise comparison (Fig. 6). It has been previously shown that *FLC*, *MAF1/AGL27*, and *MXK3.30/AGL31* form a monophyletic group within the Arabidopsis MADS gene family (Alvarez-Buylla et al., 2000a). Phylogenetic analysis shows that *F15O5.2*, *F15O5.3*, and *F15O5.4* also form part of the *FLC* clade (Fig. 7). The close evolutionary relationship among these six genes suggests that they all might be involved in the regulation of flowering time.

DISCUSSION

We have begun characterization of a MADS box gene, *MAF1*, that encodes a protein with a large degree of identity to the floral repressor, FLC. Mutant alleles for *MAF1* are currently unavailable, but overexpression of this gene in Columbia, L*er*, and two late-flowering ecotypes indicates that MAF1 activity affects flowering time.

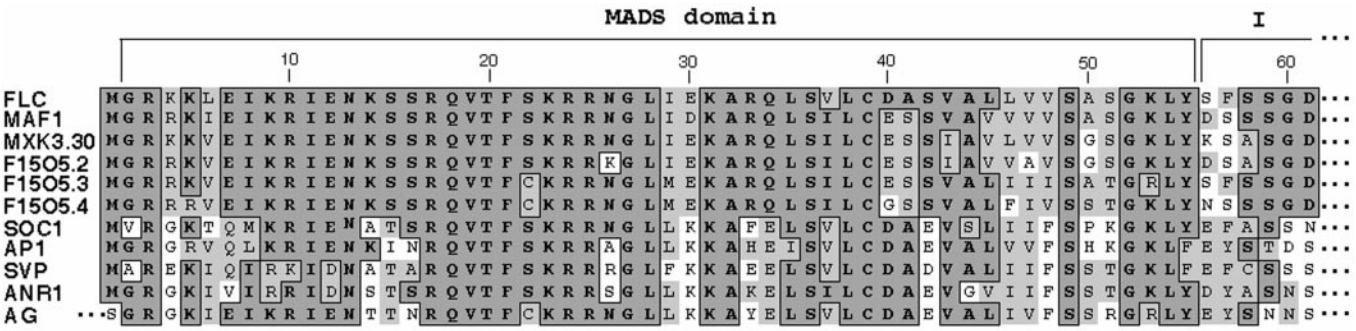

Figure 6. Sequence comparison of the predicted MADS domains (along with the first six amino acids from the adjacent I region) of MAF1, MXK3.30, F15O5.2, F15O5.3, F15O5.4, FLC, and other Arabidopsis MADS domain proteins. FLC, MAF1, MXK3.30, F15O5.2, F15O5.3, and F15O5.4 are almost identical within the MADS domain, and they all possess an acidic residue (D or E) at position 30. In other MADS domain proteins, a K residue, which is known to contact the phosphate backbone of DNA, invariably occupies this position (Pellegrini et al., 1995).

Overexpression of *MAF1* caused early flowering in the majority of Columbia, Stockholm, and Pitztal lines examined. However, in a smaller number of instances, 35S::*MAF1* plants showed delayed flowering. The reason for this discrepancy is unknown, but we found that the highest levels of overexpression correlate with late flowering and that lower levels of overexpression are associated with early flowering. In addition, preliminary results indicate that late-flowering 35S::*FLC* Columbia lines also possess higher levels of overexpression than early-flowering 35S::*FLC* Columbia lines (data not shown).

Genetic and expression analysis provide convincing evidence that *FLC* is a floral repressor (Koornneef et al., 1994; Lee et al., 1994; Michaels and Amasino, 1999; Sheldon et al., 1999). That *FLC* overexpression causes late flowering in L*er* supports this conclusion. However, no clear explanation has been offered for the fact that in the C24 ecotype, most 35S::*FLC* lines flower early (Sheldon et al., 1999). We have found that 35S::*FLC* Columbia lines are mainly early flowering and behave in a similar manner to 35S::*MAF1* Columbia lines. In addition, both 35S::*FLC* and 35S::*MAF1* were noted to cause only late flowering in L*er*. Thus, the effects of *FLC* and *MAF1* overexpression are comparable, and clearly dependent upon ecotype.

Under the conditions of these studies, L*er* plants flower very rapidly and make only five to six rosette leaves before bolting. It is possible that there are additional floral repressors, active in the other backgrounds (but not present in L*er*), with which *FLC* or *MAF1* overexpression can interfere. Lower levels of *FLC* or *MAF1* overexpression could "titrate out" or silence such factors, to produce early flowering. On the other hand, excessively high *FLC* or *MAF1* levels might still repress flowering even though those factors were inactivated.

The level of *FLC* transcript in an Arabidopsis plant has been put forward as the molecular basis for whether it will show a vernalization response (Sheldon et al., 2000). The presence of an active *FRIGIDA* allele, or defects in components of the autonomous pathway of floral promotion, result in high levels of *FLC* expression and late flowering. Hence, these genes are thought to have opposite effects on *FLC*, with *FRI* supporting *FLC* levels and the autonomous pathway having a negative effect (Fig. 1). When a cold treatment is supplied, *FLC* levels fall and flowering is derepressed (Michaels and Amasino, 1999; Sheldon et al., 1999, 2000). In contrast to *FLC*, we did not detect any marked effect of genetic background on the level of *MAF1* transcript, suggesting that there is a less critical requirement for *FRI* in maintaining *MAF1* levels. In addition, *MAF1* transcript levels showed a less consistent decrease than *FLC* levels upon vernalization in the Stockholm and Pitztal ecotypes. Nevertheless, like *FLC*, *MAF1* transcript significantly declined when wild-type Columbia or *fca-9* plants (that mutant was obtained in the Columbia background; Page et al., 1999) were vernalized. Hence, a decline in *MAF1* levels due to vernalization could be dependent upon ecotype, or changes in its expression might be too subtle to be consistently detected.

Given the equivalent effects of *MAF1* and *FLC* overexpression, it seems probable that *MAF1* has a similar function to *FLC*. If MAF1 does act as repressor, it is possible that it acts in combination with FLC. In such a scenario, the switch from the vegetative to the flowering state might mainly be effected by a change in *FLC* levels, with *MAF1* levels remaining relatively constant. MAF1 alternatively could be regulated posttranslationally, in which case the interactions between MAF1 and FLC would likely include additional unidentified factors.

35S::*MAF1* plants were unresponsive to vernalization and did not contain altered levels of *FLC* transcript, indicating that *MAF1* can influence events downstream of *FLC* transcription. Moreover, late-flowering 35S::*MAF1* lines were responsive to photoperiod and showed delayed flowering in both long-day and short-day conditions. Thus, these plants were more likely altered in the autonomous

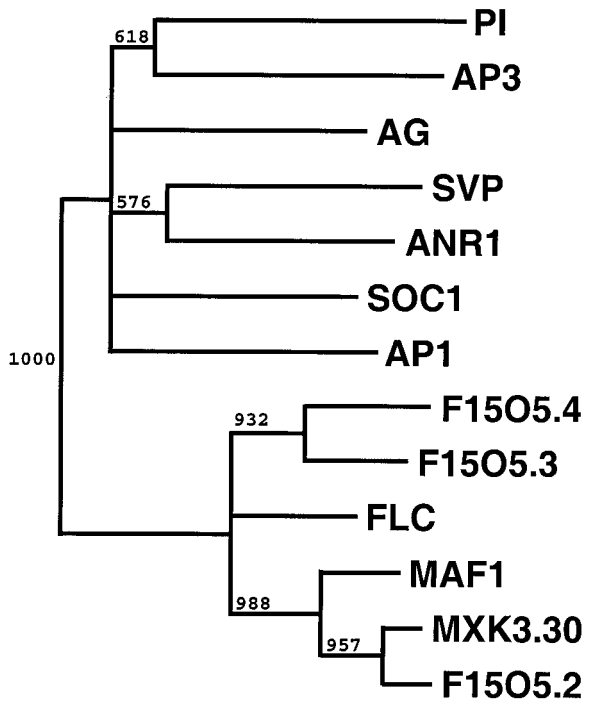

Figure 7. Phylogenetic analysis showing the relationships between MAF1, MXK3.30, F15O5.2, F15O5.3, F15O5.4, FLC, and other Arabidopsis MADS domain proteins. The neighbor-joining tree was based on an alignment of sequences that comprised the MADS domain and the first 20 amino acids of the adjacent I region. Bootstrap values are shown on branches. Branches with less than 50% of bootstrap support are collapsed. The same FLC clade is obtained irrespective of whether the tree is constructed with only the genes represented here, or whether all the MADS box genes so far identified in the Arabidopsis genome are included in the comparison (not shown).

(or some other) pathway rather than in the photoperiod pathway of floral promotion (Fig. 1). It has been proposed recently that both the autonomous and photoperiod-responsive pathways of floral promotion act via common downstream components that include *FT* and *SOC1* (Fig. 1; Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). FT is a putative lipid-binding protein, the mode of action of which is not yet clear (Kardailsky et al., 1999; Kobayashi et al., 1999). However, it is interesting that SOC1 is also a MADS domain transcription factor (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). It is an emerging possibility, therefore, that a whole group of MADS transcription factors function to control flowering via the same regulatory network as FLC, and that some of them, such as MAF1 (along with other, as yet uncharacterized, components), might link FLC to downstream components of the pathway. It is worth noting that amino acid residue 30 is acidic (E or D) in FLC, MAF1, MXK3.30, F15O5.2, F15O5.3, and F15O5.4, whereas in all other Arabidopsis MADS domain proteins so far identified, that position is occupied by a positively charged Lys residue (Fig. 6 and data not shown). The crystal structure of the human SRF MADS domain bound to DNA has shown that this Lys residue (which is also conserved in yeast (*Saccharomyces cerevisiae*) MCM1 and human MEF2A proteins) contacts the phosphate backbone of the DNA target site (Pellegrini et al., 1995). Therefore, that specific amino acid difference could confer DNA-binding properties to FLC and the FLC-like proteins distinct from those of the other Arabidopsis MADS domain factors.

The analysis of *MAF1* activity is further complicated by the presence of alternative splicing. Two alternative cDNA sequences for *MAF1*, different from the one reported here, have been recently deposited in GenBank (accession nos. AGL27-I and AGL27-II; AF312665 and AF312666, respectively). Both AGL27-I and AGL27-II mRNAs result from omitting an exon and using in its place sequence segments that correspond to intronic sequences for the *MAF1* cDNA reported here. AGL27-II mRNA translation would result in a protein with an altered sequence in its I region. The I region of the plant MADS domain proteins has been shown to play a role in dimerization (Riechmann and Meyerowitz, 1997), and its sequence is highly conserved among FLC, MAF1, MXK3.30, F15O5.2, F15O5.3, and F15O5.4 (not shown). Therefore, AGL27-II protein might have altered properties with respect of those of MAF1. Translation of AGL27-I mRNA would result in a truncated MAF1 protein in which the last 30 amino acids are replaced by a smaller segment of seven residues. The contribution that the different splice variants could make to *MAF1* function remains to be investigated.

Analysis of the Arabidopsis genome sequence indicates that it contains at least 82 MADS box genes, of which approximately 50% might have partially redundant functions (Riechmann and Ratcliffe, 2000; Riechmann et al., 2000). Numerous MADS box genes have been found to participate in the regulation of flower development, often in a functionally overlapping manner for those genes that belong to the same clade (Bowman et al., 1991; Coen and Meyerowitz, 1991; Kempin et al., 1995; Riechmann and Meyerowitz, 1997; Ferrandiz et al., 2000; Liljegren et al., 2000; Pelaz et al., 2000). The high degree of sequence similarity among *FLC*, *MAF1*, *MXK3.30*, *F15O5.2*, *F15O5.3*, and *F15O5.4* also raises the possibility of (partial) functional redundancy among them. Because *SOC1* and a further flowering time gene, *SHORT VEGETATIVE PHASE* (Hartmann et al., 2000), also encode MADS proteins, it appears that the gene family might have an equally critical role in the regulation of flowering time.

MATERIALS AND METHODS

All experiments were performed using Arabidopsis of ecotype Columbia except where otherwise indicated. The Stockholm (CS6863) and Pitztal (CS6832) lines were sup-

plied by the Arabidopsis Biological Resource Center at Ohio State University (Columbus). The *fca-9* allele was in a Columbia background (Page et al., 1999; kindly provided as a gift to O. Ratcliffe by Dr. Caroline Dean [John Innes Centre, Norwich, UK]). In all experiments, seeds were sterilized by a 2-min ethanol treatment followed by 20 min in 30% (v/v) bleach/0.01% (v/v) Tween and five washes in distilled water. Seeds were sown to Murashige and Skoog (MS) agar in 0.1% (w/v) agarose and stratified for 3 to 5 d at 4°C, before transfer to growth rooms with a temperature of 20°C to 25°C. MS medium was supplemented with 50 mg L^{-1} kanamycin for selection of transformed plants. Plants were transplanted to soil after 7 d of growth on plates. For vernalization treatments, seeds were sown to MS agar plates, sealed with micropore tape, and placed in a 4°C cold room with low-light levels for 6 to 8 weeks. The plates were then transferred to the growth rooms alongside plates containing freshly sown non-vernalized controls. Rosette leaves were counted when a visible inflorescence of approximately 3 cm was apparent.

The *MAF1* cDNA was identified among clones isolated from a library derived from Arabidopsis leaf mRNA. The *FLC* cDNA was isolated (based on the published sequence, Michaels and Amasino, 1999) by RT-PCR from whole vegetative Columbia seedlings. Arabidopsis plants were transformed by the floral dip method (Bechtold and Pelletier, 1998; Clough and Bent, 1998) using *Agrobacterium tumefaciens* carrying a standard transformation vector, which contained a kanamycin resistance selectable marker and either the *MAF1* or *FLC* cDNA downstream from the cauliflower mosaic virus 35S promoter. For RT-PCR expression studies, RNA was extracted from plant tissue using a cetyltrimethylammonium bromide-based protocol (Jones et al., 1995), poly(A^+) RNA RNA was purified using oligo(dT) cellulose (Gibco BRL, Rockville, MD), and first stand cDNA synthesis was performed using a SuperScript kit (Gibco BRL). Primers used in *MAF1* RT-PCR experiments were: primer 1, 5′-GGCATAACCCTTATCGGAGATTTGAAGC; primer 2, 5′-ACACAAACTCTGATCTTGTCTCCGAAGG; primer 3, 5′-GCATAACCCTTATCGGAGATTTGAAGCCAT; and primer 4, 5′-AACATTCCTCTCTCATCATCTGTTGCCAGC.

Experiments were performed as follows: for tissue distribution, primers 1 and 2, 35 PCR cycles (Fig. 3A); for *MAF1* expression in different genetic backgrounds, primers 1 and 2, 30 cycles (Fig. 3B); for *MAF1* expression in vernalization studies, primers 3 and 4, 25 to 30 cycles (results of 30 PCR cycles shown, Fig. 3C); and for *MAF1* expression in 35S::*MAF1* plants, primers 1 and 2, 25 cycles (Fig. 5).

Primers used in *FLC* RT-PCR experiments were: primer 5, 5′-AACGCTTAGTATCTCCGGCGACTTGAAC; primer 6, 5′-CTCACACGAATAAGGTACAAAGTTCATC; primer 7, 5′-TTAGTATCTCCGGCGACTTGAACCCAAACC; and primer 8, 5′-AGATTCTCAACAAGCTTCAACATGAGTTCG.

Experiments were performed as follows: for *FLC* expression in different genetic backgrounds, primers 5 and 6, 35 PCR cycles (Fig. 3B); and for *FLC* expression in vernalization studies and in 35S::*MAF1* plants, primers 7 and 8, 30 PCR cycles (Figs. 3C and 5). Primer specificity was verified by sequencing RT-PCR products. Samples were standardized via 20 to 25 cycles of PCR with actin primers 5′-AGAGATTCAGATGCCCAGAAGTCTTGTTCC and 5′-A-ACGATTCCTGGACCTGCCTCATCATACTC.

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