The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*

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The very late-flowering behavior of *Arabidopsis* winter-annual ecotypes is conferred mainly by two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). A MADS-domain gene, *AGAMOUS-LIKE 20* (*AGL20*), was identified as a dominant *FRI* suppressor in activation tagging mutagenesis. Overexpression of *AGL20* suppresses not only the late flowering of plants that have functional *FRI* and *FLC* alleles but also the delayed phase transitions during the vegetative stages of plant development. Interestingly, *AGL20* expression is positively regulated not only by the redundant vernalization and autonomous pathways of flowering but also by the photoperiod pathway. Our results indicate that *AGL20* is an important integrator of three pathways controlling flowering in *Arabidopsis*.

[*Key Words*: Flowering; MADS domain protein; AGL20; phase transition; activation tagging]

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Plants undergo several distinct phase transitions during their life cycle (Poethig 1990; Schultz and Haughn 1993; Telfer et al. 1997). The most dramatic phase change is the transition from vegetative growth to reproductive growth, or flowering. In addition, juvenile and adult phases can be distinguished during vegetative development, discernible by the distribution of trichomes on the leaf surface in *Arabidopsis* (Telfer et al. 1997). The transition to flowering is regulated by a complex genetic network that monitors the developmental state of the plant as well as environmental conditions. The genetic control of flowering has been extensively studied in *Arabidopsis* (Weigel 1995; Koornneef et al. 1998; Levy and Dean 1998). *Arabidopsis* is a facultative long-day plant that flowers faster under long days than short days. Genetic analyses of late-flowering mutants have identified more than 20 genes, which have been placed in at least three parallel genetic pathways based on the effect of each mutation on the response to environmental conditions. Genes such as *CONSTANS* (*CO*), *GIGANTEA* (*GI*), *FT*, *FWA*, *FHA*, *FE*, and *FD* have been placed in the long day-dependent pathway because mutations in them cause lateness in flowering under long days but have little effect under short days. Genes such as *FCA*, *FPA*, *FVE*, *FY*, and *LUMINIDEPENDENS* (*LD*) have been placed in the autonomous pathway because mutations in them cause delay in flowering under both long days and short days. A third pathway, which acts redundantly with the autonomous pathway, accelerates flowering upon vernalization (an extended cold treatment).

The genes defective in six late-flowering mutants, *LD*, *CO*, *FCA*, *FHA*, *GI*, and *FT*, have been isolated. *LD* and *CO* seem to encode transcription factors, whereas *FCA* encodes a putative RNA-binding protein that may have a role in posttranscriptional regulation (Lee et al. 1994a; Putterill et al. 1995; Macknight et al. 1997). *FHA* encodes a blue-light photoreceptor, CRYPTOCHROME 2, and *GI* encodes a protein that is regulated by a circadian clock (Guo et al. 1998; Fowler et al. 1999; Park et al. 1999), suggesting that the two genes are involved in photoperiod perception. *FT*, whose biochemical function is unclear, acts downstream from *CO* (Kardailsky et al. 1999; Kobayashi et al. 1999).

Flowering-time genes modulate the activity of floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA 1* (*AP1*). The induction of *CO* activity causes the rapid expression of *LFY* followed by later expression of *AP1* (Simon et al. 1996). Conversely, *FT* and *FWA* function to activate *AP1* in parallel with *LFY* or downstream from *LFY* transcription (Ruiz-García et al. 1997). Genetic analyses have also shown that some genes such as *CO*, *GI*, *FCA*, and *FVE* affect the transcriptional induction of *LFY*, whereas other genes such as *FWA*, *FE*, and *FT* affect the response to *LFY* activity (Nilsson et al. 1998). Therefore, it has been suggested that flowering-

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time genes regulate flowering by either activating *LFY* expression or modulating the response to *LFY*. Consistently, *LFY* expression is activated by the transition to flowering, and constitutive expression of *LFY* or *AP1* causes early flowering (Mandel and Yanofsky 1995; Weigel and Nilsson 1995; Blázquez et al. 1997).

Genetic analyses of naturally-occurring variations of flowering time among *Arabidopsis* wild-type strains or ecotypes have identified additional loci that control flowering time. Among them, two loci, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), account for most of the difference in flowering time between early- and late-flowering ecotypes (Napp-Zinn 1985; Burn et al. 1993; Lee et al. 1993; Clarke and Dean 1994; Koornneef et al. 1994). Late-flowering ecotypes have dominant alleles of *FRI* and *FLC* that act synergistically to suppress flowering, whereas early-flowering ecotypes have a recessive allele of *FRI* and/or weak allele of *FLC* (Koornneef et al. 1994; Lee et al. 1994b; Sanda and Amasino 1995; Sheldon et al. 2000). *FRI* and *FLC* confer winter annual behavior to late-flowering ecotypes such that the effect of *FRI* and *FLC* is suppressed by vernalization. *FRI*- and *FLC*-containing lines show delayed flowering under both long and short days, indicating that these two genes act in the autonomous pathway (Lee and Amasino 1995). *FLC* has been recently isolated and found to encode a MADS-domain protein (Michaels and Amasino 1999; Sheldon et al. 1999). Dominant, late-flowering alleles of *FRI* increase *FLC* transcript levels whereas vernalization decreases *FLC* levels. In addition, *LD*, a gene involved in the autonomous pathway, represses *FLC* expression. Therefore, it has been proposed that the modulation of *FLC* expression by the autonomous and vernalization pathways is critical to the control of flowering (Michaels and Amasino 1999; Sheldon et al. 2000).

To further elucidate the genetic control of flowering by *FRI* and *FLC*, we have screened for *FRI* suppressor mutants by activation-tagging mutagenesis. We have isolated a dominant *FRI* suppressor in which *AGAMOUS-LIKE 20* (*AGL20*), a gene encoding a MADS-domain protein, is overexpressed. Our studies of *AGL20* expression in various flowering-time mutants show that it is regulated by all three flowering-time pathways. Therefore, we propose that the level of AGL20 activity is critical to the control of flowering time and that *AGL20* integrates signals from the photoperiod, vernalization, and autonomous floral induction pathways.

Results

Isolation of an FRI *suppressor by activation tagging*

To isolate *FRI* suppressor mutants, we performed activation-tagging mutagenesis (Hayashi et al. 1992; Weigel et al. 2000) in a line in which the *FRI* allele of the ecotype San Feliu-2 (SF2) was introgressed into Columbia (Col) through eight backcrosses (*FRI-SF2; FLC-Col*, referred to as *FRI FLC* below; Michaels and Amasino 1999). One of the primary transformants showed a very early-flowering phenotype and was designated as *fsu1-1D/+ FRI FLC* (*fsu1* stands for *FRI suppressor 1*; Fig. 1A,B). T2 populations of *fsu1-1D/+ FRI FLC* showed an approximately 3:1 segregation ratio for the transgene marker (57 basta-resistant:20 basta-sensitive in basta selection; $\chi^2 = 0.039$, $P < 0.5$), as well as for early to late flowering (56 early-flowering:24 late-flowering; $\chi^2 = 1.6$, $P < 0.1$). The late-flowering progeny produced more than 55 rosette leaves before bolting, which was similar to the *FRI FLC* parent. Early-flowering T2 plants fell into two classes. The very early class produced on average five rosettes, whereas the other class produced on average 11 rosette leaves. The ratio of the two classes was roughly 1:2 (16 very early plants:40 intermediately early plants, $\chi^2 = 0.57$, $P < 0.1$). In addition, in the T3 population obtained from self-pollination of T2 progeny, all of the progeny from late-flowering plants were basta-sensitive (no T-DNA) and the progeny of the intermediately early class segregated 3:1 for basta resistance (hemizygous for T-DNA). The progeny of the very early class was uniformly basta-resistant (homozygous for T-DNA). The cosegregation of the T-DNA insert with the early-flowering phenotype and the simple 1:2:1 segregation ratio strongly indicated that a single T-DNA insertion was closely linked to the locus causing early flowering in *fsu1-1D FRI FLC* plants.

Overexpression of AGL20 *causes early flowering in* FRI FLC

DNA gel blot analysis confirmed that there was only a single T-DNA insert in *fsu1-1D FRI FLC* mutants (data

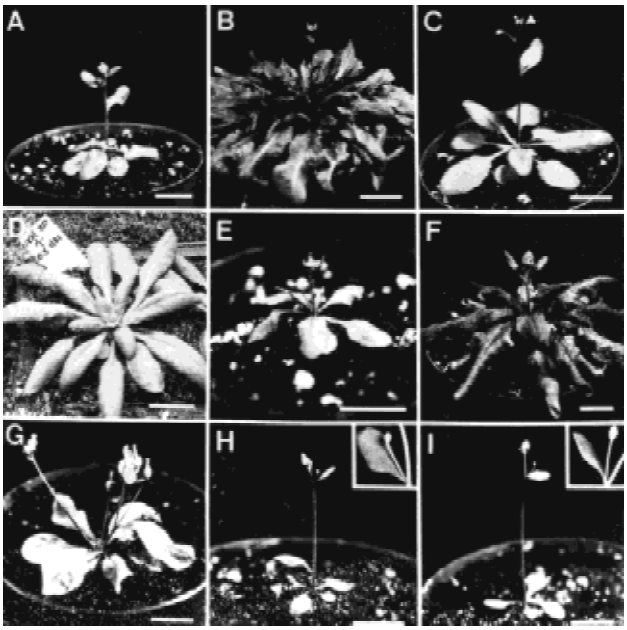

Figure 1. Flowering phenotype of lines described in this study. (*A*) Homozygous *fsu1-1D FRI FLC*; (*B*) *FRI FLC*; (*C*) Columbia; (*D*) *agl20*; (*E*) *35S::LFY/–*; (*F*) *35S::LFY/– FRI/–*; (*G*) *fsu1-1D/–35S::LFY/– FRI/–*; (*H*) *fsu1-1D FRI flc-3*; (*I*) *fsu1-1D fri-Col FLC*. The *insets* in (*H*) and (*I*) show ectopic flowers subtended by cauline leaves. All plants were grown under long days (16 h light/ 8 h dark). Bars, 1 cm.

not shown). Plant DNA flanking the right border of the T-DNA insertion site was isolated by plasmid rescue (Fig. 2A)(Lee et al. 1994a). Sequence analysis of rescued plant DNA revealed that the insertion was in a part of the genome represented by bacterial artificial chromosome (BAC) clone F17K2 (GenBank accession no. AC003680). The rescued plant sequences spanned nucleotides 63,367 to 64,214 of BAC F17K2 and included sequences of the first exon of *AGL20* (Fig. 2B). The four repeats of the 35S enhancer were inserted 677 base pairs upstream of the *AGL20* start codon in the *fsu1-1D FRI FLC* mutant.

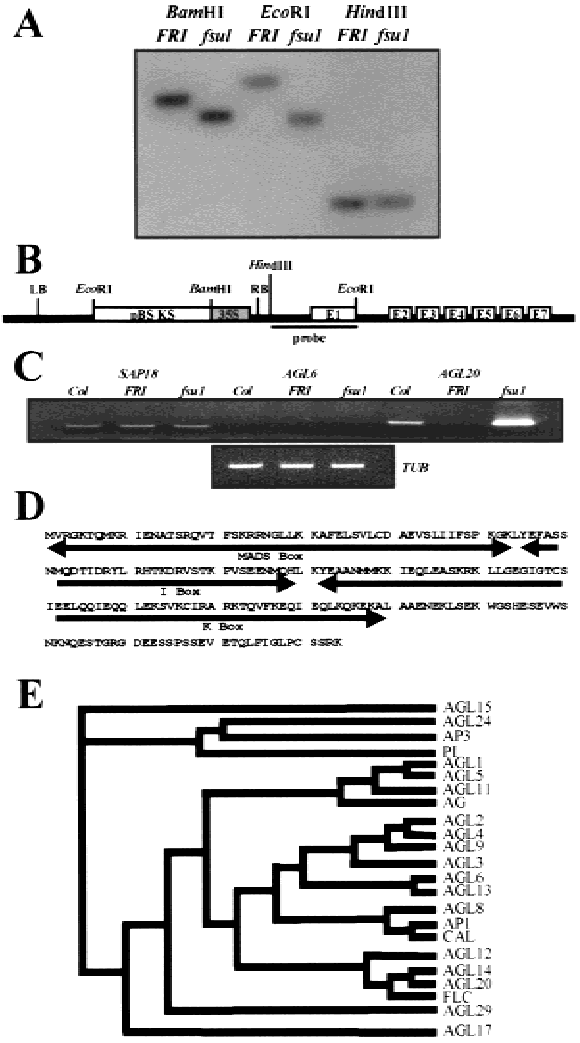

Figure 2. Insertion of 35S enhancer in the promoter region of *AGL20* causes overexpression. (*A*) DNA gel blot analysis showing the polymorphism between *fsu1-1D FRI FLC* (*fsu1*) and *FRI FLC* (*FRI*). (*B*) Diagram of genomic organization showing the insertion of 35S enhancer into the *AGL20* region in *fsu1-1D FRI FLC*. LB and RB are left border and right border of T-DNA, respectively. A bar represents the DNA fragment obtained by plasmid rescue and used for DNA gel blot analyses. (*C*) RT-PCR result for *SAP18*, *AGL6*, and *AGL20* in the leaves of 3-week-old Columbia (*Col*), *FRI FLC* (*FRI*), and *fsu1-1D FRI FLC* (*fsu1*). The β-*tubulin* gene (*TUB*) was amplified as a quantitative control. (*D*) The amino acid sequence of *AGL20*. MADS, I, and K domains are indicated. (*E*) Neighbor-joining phylogenetic tree for *Arabidopsis* MADS-domain proteins.

Reverse transcription-polymerase chain reaction (RT-PCR) of RNA isolated from the leaves of 3-week-old Columbia, *FRI FLC*, and the *fsu1-1D FRI FLC* mutant showed that *AGL20* is overexpressed in *fsu1-1D FRI FLC* (Fig. 2C). Conversely, two putative genes near the T-DNA insertion site, *AGL6* (F17K2.18) and *sin3 associated polypeptide* (*SAP18*, F17K2.17) were not overexpressed in the *fsu1-1D FRI FLC* mutant (Fig. 2C). This result indicates that the acceleration of flowering in *fsu1-1D FRI FLC* results from overexpression of *AGL20*. Consistent with this, when the *AGL20* coding sequence under control of the CaMV 35S promoter was introduced into *FRI FLC*, all transgenic lines flowered earlier than *FRI FLC* (Table 1). Henceforth, we renamed *fsu1-1D* to *agl20-101D*.

AGL20 consists of seven exons and six introns (Fig. 2B). It encodes a typical MADS-domain protein that contains MADS, I (intervening), K, and C-terminal domains (Fig. 2D). Phylogenetic analysis using the M-I-K region showed that *AGL20* is most similar to the MADS-domain genes *AGL14* and *FLC* in *Arabidopsis* (Fig. 2E). The amino acid sequence identity in the M-I-K region is 65.4% with *AGL14* and 43.6% with *FLC*, whereas overall amino acid sequence identity is 55.9% with *AGL14* and 37.7% with *FLC*.

The normal role of *AGL20* in the regulation of flowering was confirmed with a T-DNA insertional mutant, which flowered late in both long and short days (Fig. 1D). The *agl20* mutants flowered later under short days than under long days, which indicates that the *agl20* mutant is responsive to photoperiod (Table 1).

agl20-101D FRI FLC *responds to photoperiod and vernalization*

The effect of photoperiod and vernalization on the flowering time of *agl20-101D FRI FLC* is shown in Figure 3. Similar to Columbia, *agl20-101D FRI* flowered more rapidly under long days than under short days. Vernalization reduced flowering time in *agl20-101D FRI FLC*, but the reduction in *agl20-101D FRI FLC* was higher than in Columbia. Therefore, *agl20-101D FRI FLC* responds to both photoperiod and vernalization, indicating that *agl20-101D* does not abolish the sensitivity to either photoperiod or vernalization in the *FRI FLC* background.

agl20-101D *accelerates phase transition in* FRI FLC

In *Arabidopsis*, a marker of phase change is the distribution of trichomes on the leaf surface (Telfer et al. 1997). The leaves produced during the juvenile phase have trichomes only on their adaxial side, whereas the leaves produced during adult phase possess trichomes on both adaxial and abaxial sides. The cauline leaves produced during the reproductive phase lack trichomes on

Table 1. *Comparison of phase transitions and flowering time in the plants with different combinations of transgenics and mutations*

Genotype	Juvenile leaves[a]	Total rosette leaves	Cauline leaves[b]	*n*
		Experiment 1[c]		
Columbia[d]	4.8 ± 0.4	9.2 ± 0.4	2.5 ± 0.5	20
FRI FLC	9.3 ± 0.5	61.0 ± 1.4	12.5 ± 0.7	10
agl20-101D FRI FLC[e]	3.3 ± 0.5	5.2 ± 0.4	5.3 ± 0.9	25
agl20-101D/– FRI FLC[f]	ND	10.6 ± 1.4	5.4 ± 0.5	27
35S::LFY/ – FRI/– FLC	ND	16.7 ± 1.9	7.4 ± 1.7*	7
agl20-101D/– 35S::LFY/ – FRI/ – FLC	ND	7.0 ± 1.1	4.5 ± 1.2*	10
agl20-101D fri FLC	ND	2.0 ± 0.4	3.5 ± 0.7*	17
agl20-101D FRI flc-3	ND	2.0 ± 0.3	3.3 ± 0.6*	21
35S::AGL20 FRI FLC S1	ND	14	6	1
35S::AGL20 FRI FLC S2	ND	12	7	1
35S::AGL20 FRI FLC S3	ND	17	8	1
		Experiment 2[g]		
Columbia, LD	ND	11.9 ± 0.3	3.3 ± 0.2	30
agl20, LD	ND	26.2 ± 1.0	5.0 ± 0.6	5
Columbia, SD	ND	30.0 ± 1.0	9.0 ± 1.0	30
agl20, SD	ND	68.8 ± 4.9	15.2 ± 0.8	5

[a]Number of juvenile leaves determined by identifying the last leaf that had trichomes only on the adaxial side. (ND) not determined.
[b]The cauline leaves produced in plants marked with an asterisk subtend ectopic flowers instead of secondary shoots.
[c]In experiment 1, plants were grown under long days (16 h light/8 h dark).
[d]The genotype of Columbia is *fri FLC.*
[e]Homozygous *agl20-101D FRI FLC.*
[f]Heterozygous *agl20-101D/+ FRI FLC.*
[g]In experiment 2, plants were grown under long days (LD) and short days (SD; 10 h light/14 h dark cycle).

their adaxial surface. To test whether *agl20-101D* also affected the transition from the juvenile to adult phase, we compared the trichome distribution in different lines (Table 1). *FRI FLC* produced approximately twice as many juvenile leaves as Columbia, indicating that *FRI FLC* delays the transition from juvenile to adult phase as well as flowering. *FRI FLC* also produced more cauline leaves with associated secondary shoots than Columbia. The juvenile phase was found to be dramatically shortened in *agl20-101D FRI FLC*. However, *agl20-101D FRI FLC* produced significantly more cauline leaves than Columbia, although homozygous *agl20-101D FRI FLC* produced fewer rosette leaves than Columbia. This result suggests that *AGL20* overexpression can partially rescue the delay in the transition from secondary shoots to flowers in *FRI FLC*.

Interestingly, the flowering phenotype of *agl20-101D FRI FLC* is semidominant, if flowering time is measured by rosette leaf number (Table 1). That is, homozygous *agl20-101D FRI FLC* plants produce fewer rosette leaves than hemizygotes, indicating that the promotion of flowering by *agl20-101D* is dosage-dependent and that the effects of *AGL20* overexpression are not saturated in *agl20-101D FRI FLC*. However, the number of cauline leaves with associated secondary shoots is not distinguishable between hemizygous and homozygous *agl20-101D FRI FLC* (Table 1). This result indicates differential effects of *agl20-101D* on bolting and flowering.

FRI FLC *represses* LFY *expression*

An important regulator of the transition from secondary shoots to flowers is the *LFY* transcription factor (Weigel and Nilsson 1995). Therefore, we monitored *LFY* expression levels in *agl20-101D FRI FLC* using a *LFY::GUS* fusion gene (Blázquez et al. 1997). We compared *LFY* promoter activity in *agl20-101D/+; FRI/fri; FLC/FLC*, which flowered at the same time as *agl20-101D/+ FRI FLC*, with *fri/fri; FLC/FLC* (Columbia wild type). Hemizygous *agl20-101D/+ FRI FLC* produced five to six nodes of cauline leaves with secondary shoots above rosette leaves, but none of these showed GUS staining (Fig. 4A). Conversely, Columbia produced two to three cauline leaves with secondary shoots above rosette leaves, followed by flowers. In the Columbia background, GUS staining was detected in the cauline leaf primordia as well as floral meristems that are produced at the same nodes where secondary shoots are produced in hemizygous *agl20-101D FRI FLC* plants (Fig. 4B). This result indicates that *LFY* expression is repressed by *FRI FLC* and that *AGL20* overexpression cannot overcome such repression at least until after five to six secondary shoots are formed.

It has been reported that *35S::LFY* can partially rescue the late-flowering phenotype of *FRI FLC* (Nilsson et al. 1998). Reexamination of the effect of *35S::LFY* on *FRI FLC* confirmed the previous report such that the number of rosette leaves was reduced dramatically by *35S::LFY*. But the number of cauline leaves in *FRI FLC* was not

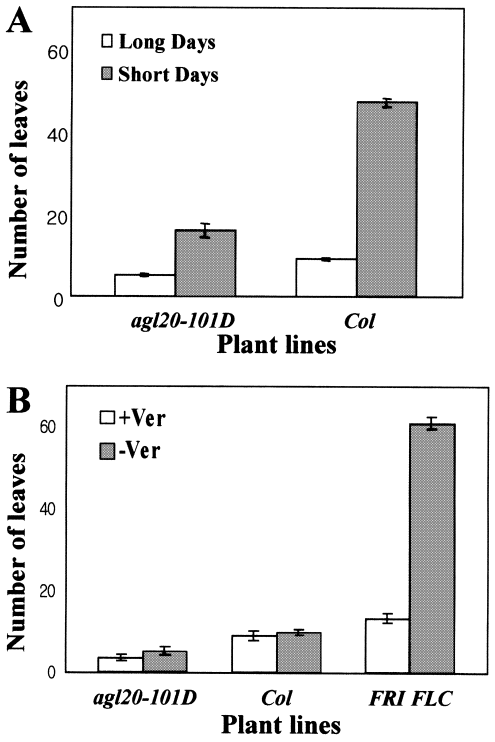

Figure 3. Effect of photoperiod and vernalization on the flowering time of *agl20-101D FRI FLC*. (*A*) The effect of photoperiod; (*B*) the effect of vernalization. *agl20-101D FRI FLC* is designated as *agl20-101D*. Flowering time was measured as the number of rosette leaves. *agl20-101D* was originally designated *fsu1-1D*.

correspondingly reduced by *35S::LFY* (Fig. 1F; Table 1). However, all of the secondary shoots in the axils of cauline leaves were converted to solitary flowers by *35S::LFY*. This result is in contrast to the effect of *agl20-101D* on *FRI FLC* because secondary shoot-to-flower conversions were not observed in *agl20-101D FRI*, even though *agl20-101D FRI FLC* bolted earlier than *35S::LFY* in the *FRI FLC* background (Table 1). This result may suggest that *AGL20* and *LFY* affect flowering through at least partially independent pathways. Consistent with this hypothesis, the effect of *agl20-101D* and *35S::LFY* on flowering was additive such that both rosette leaf and cauline leaf numbers were reduced in *agl20-101D/– 35S::LFY/–* plants when compared with *agl20-101D/–* or *35S::LFY/–* in the *FRI FLC* background (Fig. 1G; Table 1).

Because *FRI FLC* delays both flowering time and the transition from cauline leaves with secondary shoots to flowers, we investigated the phenotype of *agl20-101D* in the absence of *FRI* or *FLC*. We combined the *fri-Col* allele, which is nonfunctional, with *agl20-101D* as well as the *flc-3* null mutant allele (Levy and Dean 1998; Michaels and Amasino 1999). Flowering time was further accelerated by the addition of *fri-Col* or *flc-3* mutations (Fig. 1; Table 1). The rosette leaf number was reduced to two in both *agl20-101D FRI flc-3* and *agl20-101D fri-Col FLC* lines. Interestingly, both lines showed solitary flowers in the axils of cauline leaves (Fig. 1H,I). Such a conversion of secondary shoots to flowers is also observed in *35S::LFY* or *35S::AP1* plants (Mandel and Yanofsky 1995; Weigel and Nilsson 1995). This result may indicate that *AGL20* overexpression can activate flower meristem identity genes such as *LFY* or *AP1* in shoot meristems, once *FRI FLC* is removed.

Expression of AGL20

The expression of *AGL20* RNA was determined by in situ hybridization in Landsberg *erecta* (L*er*) wild type (Fig. 4C,D). The strong expression of *AGL20* in the shoot apical meristem and slightly weaker expression in the leaf primordia were detected during vegetative growth (8 days after germination). Four days later when shoot apical meristem produced flower meristems, the expression domain of *AGL20* was expanded in the shoot apical meristem, and *AGL20* transcript was also detected in developing leaves. To gain further insight into the role of *AGL20* in regulating phase transition and flowering, we determined *AGL20* expression levels in various tissues by RT-PCR (Fig. 5). In Columbia wild type, *AGL20* was expressed most strongly in leaves, but the transcript was also detected in vegetative apices, inflorescence, stems of flowering plants, and roots (Fig. 5A). Temporal changes in *AGL20* expression were also determined in whole plants. In our long-day conditions, flowers in Columbia wild type initiate around 9 days after germination, as determined by first detection of *AP1* expression (Fig. 5B). *AGL20* expression was increased significantly around 9 days after germination (Fig. 5B).

To investigate how *AGL20* expression is regulated by *FRI* and *FLC*, RT-PCR was performed using RNA extracted from whole plants of *FRI FLC*, *FRI flc-3*, and *fri* (FN235) *FLC* mutants at 6, 9, and 12 days after germination (Fig. 5C). *AGL20* expression was very weak in *FRI FLC*, but an increased level of *AGL20* expression was detected in *fri* (FN235) *FLC* or *FRI flc-3* mutants. The expression level in *fri* (FN235) *FLC* or *FRI flc-3* mutants was similar to that in Columbia. This result suggests that *AGL20* expression is negatively regulated by *FRI* and *FLC*. Conversely, *FLC* expression level was increased in *FRI FLC* but decreased in the *fri* (FN235) *FLC* mutant, confirming that *FRI* positively regulates *FLC*, as previously reported (Fig. 5C)(Michaels and Amasino 1999).

The effect of photoperiod on *AGL20* expression was also determined. For this experiment, Columbia was grown under long and short days, and plants at a similar developmental stage (when the plants showed four rosette leaves; 9 days old under long days and 14 days old under short days) were compared for *AGL20* expression level. Plants grown under short days showed approximately 40% less *AGL20* expression than plants grown under long days, in contrast to *FLC* expression, which showed no difference between long days and short days (Fig. 5D).

To determine the effect of vernalization on *AGL20* expression, the *FRI FLC* line was used, because *FRI FLC*

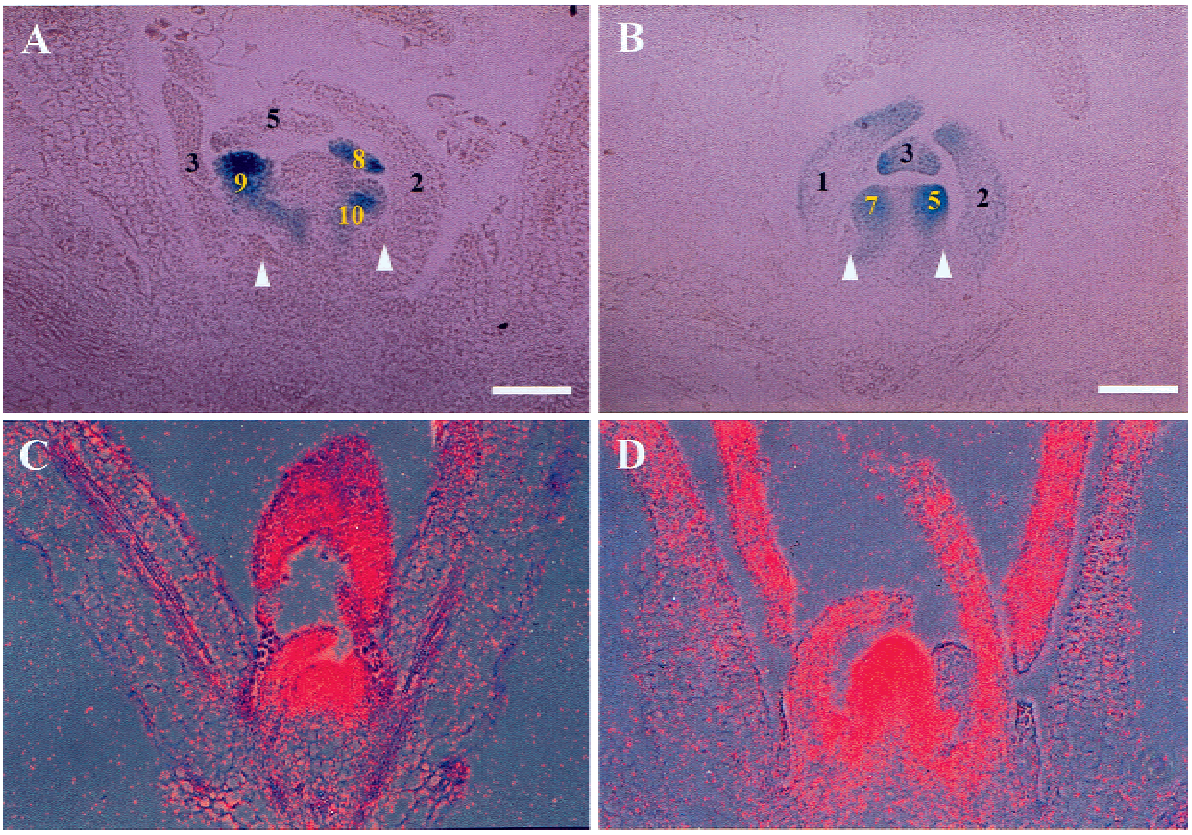

Figure 4. Analysis of *LFY* promoter activity in *agl20-101D FRI FLC* and in situ localization of *AGL20* RNA in L*er*. Expression of a *LFY::GUS* transgene introduced into *agl20-101D FRI FLC* (*A*) and Columbia (*B*). The numbers on leaf primordia and floral meristems indicate node numbers above rosette leaves. Arrowheads indicate the position of secondary shoot primordia. In situ localization of *AGL20* in the shoot apices of 8-day-old (*C*) and 12-day-old (*D*) L*er* plants. *agl20-101D* was originally designated *fsu1-1D*. Bars, 50 µm.

shows strong acceleration of flowering by vernalization. The *FRI FLC* line was subjected to 4 weeks of cold treatment and was further grown under long days until it produced four rosette leaves. As a control, the *FRI FLC* line was grown under long days for 9 days, at which time it produced four rosette leaves, without vernalization. *AGL20* was barely detectable by RT-PCR in the *FRI FLC* line without vernalization. However, 4 weeks of vernalization dramatically increased the *AGL20* expression level (Fig. 5D). Conversely, vernalization reduced the expression level of *FLC*, consistent with previous reports (Fig. 5D)(Michaels and Amasino 1999). Thus, vernalization of the *FRI FLC* line causes acceleration of flowering and a concomitant decrease of *FLC* and increase of *AGL20* expression. We also determined the *AGL20* expression level in vernalized *fca-1*, which is in the L*er* background. Although L*er* has only a weakly active *FLC* allele, flowering of *fca-1* is strongly accelerated by vernalization. When vernalized, *AGL20* expression increased and *FLC* expression decreased in *fca-1* (Fig. 5D). This suggests that vernalization, which represses *FLC* expression, activates *AGL20*. In conclusion, we found that *AGL20* expression levels are regulated by both photoperiod and vernalization.

AGL20 *expression is regulated by other flowering-time genes*

We next investigated whether genes in the autonomous pathway regulate *AGL20* expression. *AGL20* expression was strongly reduced in the *fve-3* mutant (70% reduction compared with Columbia wild type), whereas *FLC* was increased (Fig. 5F). Reduced *AGL20* expression and increased *FLC* expression were also observed in the *ld-1* mutant (Fig. 5F). A simple model to account for this and the results presented above is that *FLC* is an upstream negative regulator of *AGL20*, and that genes such as *LD* or *FVE* activate *AGL20* expression through repression of *FLC*. Alternatively, *AGL20* and *FLC* may repress each other's expression, and the increased *FLC* expression in *ld-1* and *fve-3* leads to the repression of *AGL20* expression. To distinguish the two possibilities, we compared *FLC* expression level in *agl20-101D FRI FLC*, *35S::AGL20 FRI FLC*, and *FRI FLC* (Fig. 5E). *FLC* expression levels in all of the lines were very similar, although higher expression of *AGL20* was detected in *agl20-101D* and *35S::AGL20* in *FRI FLC* background. In addition, *FLC* expression levels in *agl20* loss-of-function mutants was similar to that of Columbia (Fig. 5E). These results show that *AGL20* does not repress *FLC* expression and

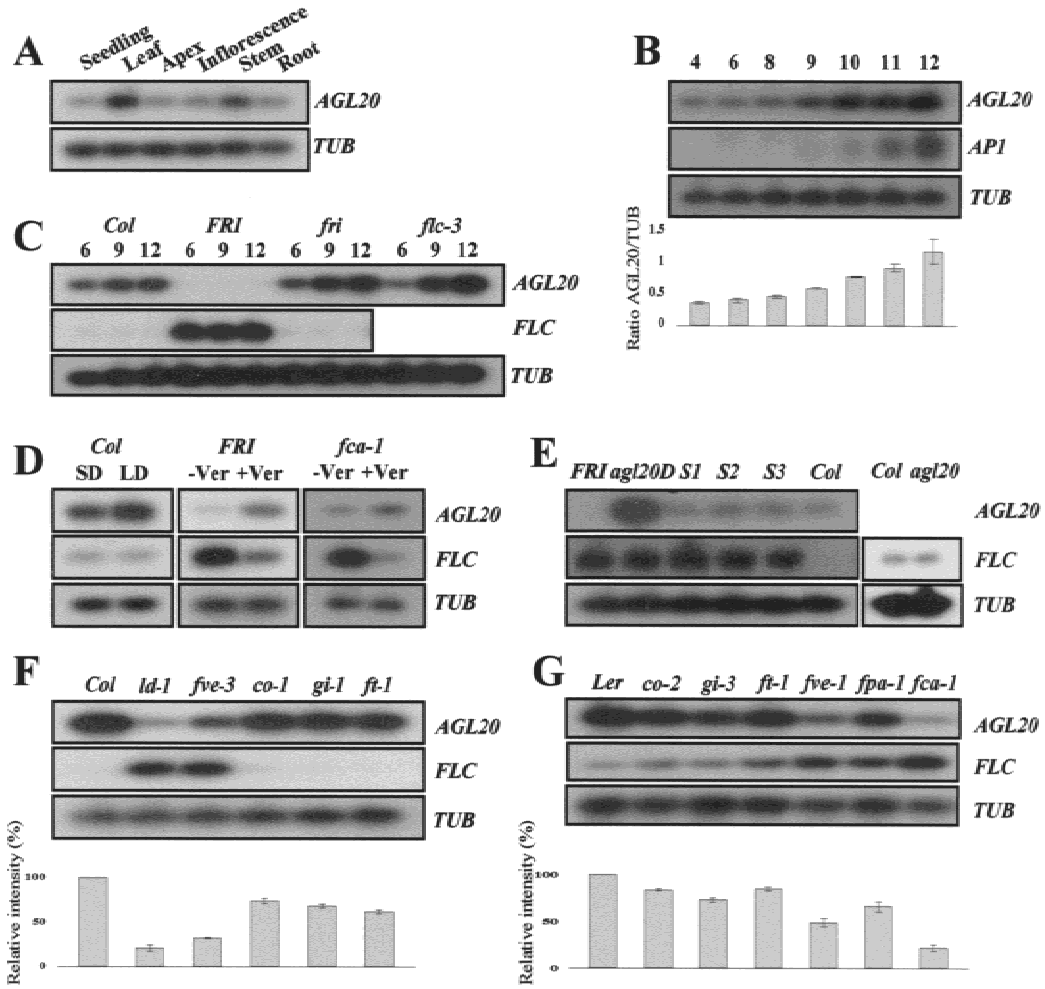

Figure 5. Analysis of *AGL20* expression by RT-PCR. (*A*) Expression of *AGL20* in various organs. (*B*) A time course of expression of *AGL20*, *FLC*, and *AP1*. Lane numbers indicate days after germination. (*C*) Comparison of expression levels of *AGL20* and *FLC* in Columbia (*Col*), *FRI FLC* (*FRI*), *fri* (FN235) *FLC* (*fri*), and *FRI flc-3* (*flc-3*). (*D*) Effect of photoperiod and vernalization on the expression of *AGL20* and *FLC*. To detect *FLC* expression in Columbia grown under short days and long days, the filter was overexposed. (*E*) Expression of *AGL20* and *FLC* in *agl20-101D FRI FLC* (*agl20D*), *35S::AGL20 FRI FLC* (*S1*, *S2*, *S3*), and *agl20* knockout mutant. For *agl20*, the filter was overexposed to detect *FLC*. (*F*) Expression of *AGL20* in late-flowering mutants in Columbia background. (*G*) Expression of *AGL20* in late-flowering mutants in L*er* background. The bar graphs below panels *B*, *F*, and *G* represent the relative expression level of *AGL20*. Standard deviation obtained from three independent experiments is indicated. The β-*tubulin* gene (*TUB*) was amplified as a quantitative control.

support the idea that *FLC* is an upstream negative regulator of *AGL20*.

We also compared how other late-flowering mutants affect the expression levels of *AGL20* and *FLC* (Fig. 5F,G). In the Columbia background, which has a dominant *FLC* allele, mutants of the photoperiod pathway, such as *co-1*, *gi-1*, and *ft-1* (introgressed into Columbia), showed approximately 30%–40% reduction of *AGL20* expression, but *FLC* expression was similar to Columbia wild type (Fig. 5F). In summary, the results show that *AGL20* expression is positively regulated by flowering-time genes, although it is more strongly dependent on genes in the autonomous pathway.

Similar results were obtained from late-flowering mutants in L*er*, which has a weak *FLC* allele (Fig. 5G). All of the late-flowering mutants in L*er* showed decreased levels of *AGL20* expression. However, the photoperiod pathway mutants, *co-2*, *gi-3*, and *ft-1*, showed less reduction in *AGL20* expression than the autonomous pathway mutants, *fca-1*, *fve-1*, and *fpa-1*, confirming that *AGL20* expression is more strongly dependent on the autonomous pathway than the photoperiod pathway also in the L*er* background (Fig. 5G). Interestingly, autonomous pathway mutant *fpa-1* consistently showed higher expression of *AGL20* than *fca-1* and *fve-1*. It may indicate that *FPA* acts more or less differently with the other genes involved in the autonomous pathway. In conclusion, *AGL20* expression is regulated by three floral inductive pathways, including the photoperiod, vernalization, and autonomous pathways.

Discussion

AGL20 *is a floral activator*

Winter-annual *Arabidopsis* strains such as Stockholm and SF2 flower very late in the absence of vernalization treatment. Previous genetic analyses showed that such lateness of flowering is mainly caused by the synergistic interaction of two genes, *FRI* and *FLC* (Napp-Zinn 1985; Burn et al. 1993; Lee et al. 1993; Clarke and Dean 1994; Koornneef et al. 1994; Lee et al. 1994b). To define the regulatory mechanism of *FRI* and *FLC*, a screen for downstream factor(s) repressed by *FRI* and *FLC* was pursued by the random overexpression strategy of activation tagging mutagenesis. The rationale is that if the late-flowering phenotype of the *FRI FLC* line is suppressed by overexpression of a certain gene, that gene may be a downstream target gene of *FRI FLC* repression. One of the suppressor mutants, *agl20-101D FRI FLC*, shows overexpression of a MADS-domain gene, *AGL20*. The results presented in this study show that *AGL20* acts as a floral activator and is repressed by the interaction of *FRI* and *FLC*. A positive role for *AGL20* is confirmed by *agl20* loss-of-function mutants, which flower late.

The expression level of *AGL20* correlates very well with flowering time. In the *FRI FLC* lines, which flower very late, *AGL20* expression is very weak. The homozygous *agl20-101D FRI FLC* mutant overexpresses *AGL20* in the *FRI FLC* background and flowers even earlier than Columbia. Consistently, *35S::AGL20* transformants in the *FRI FLC* background show accelerated flowering with increased level of *AGL20* expression (Fig. 5E). Finally, *fri* (FN235) *FLC* or *FRI flc-3* strains, whose flowering times are similar to that of Columbia, show similar levels of *AGL20* expression as Columbia. Such a correlation between *AGL20* expression level and flowering time may explain the responsiveness of *agl20-101D FRI FLC* to both photoperiod and vernalization. The activation of endogenous *AGL20* by long days and vernalization may have an additive effect on flowering of *agl20-101D FRI FLC*.

AGL20 is very similar to the SaMADS A from *Sinapsis alba* (95.3% amino acid sequence identity; Menzel et al. 1996). *SaMADS A* is expressed most highly at the shoot apical meristem of plants after they have been induced to flower. Based on the expression pattern, it has been suggested that *SaMADS A* has an important function during the transition to flowering. Our genetic analysis of *AGL20* in *Arabidopsis* has shown that *AGL20* is indeed an important regulator of flowering. In addition, we have shown that *AGL20* is expressed in vegetative tissues, consistent with a more general role of *AGL20* in regulating phase transitions. *AGL20* expression is gradually increased during vegetative growth. Such a gradual increase before flower initiation has also been observed in *LFY* and *FT*, two regulators of flowering (Blázquez et al. 1997; Kardailsky et al. 1999; Kobayashi et al. 1999).

AGL20 is a member of the MADS-domain family, a large family of transcription factors. Many MADS-domain proteins are involved in flower development. For example, *AP1* and *CAULIFLOWER* function as floral meristem identity genes, and *APETALA3* and *PISTILLATA* are involved in floral organ development (Riechmann and Meyerowitz 1997). However, *AGL15* plays a role during embryogenesis and *AGL12*, *AGL14*, and *AGL17* are expressed only in root tissues, suggesting that MADS-domain family members play diverse roles in plant development (Heck et al. 1995; Rounsley et al. 1995). Interestingly, the floral repressor FLC is also a member of MADS-domain family and shows the second highest amino acid sequence identity with AGL20 among *Arabidopsis* MADS-domain proteins.

AGL20 *activates phase transition*

The interaction of *FRI* and *FLC* causes not only a delay of flowering but also a delay of phase transition during all stages of plant development. Such an overall delay in plant development is observed in most late-flowering mutants (Martínez-Zapater et al. 1995; Telfer et al. 1997). *agl20-101D* accelerates both the transition from the juvenile to adult phase and that from vegetative to reproductive phase in the *FRI FLC* background. A weaker acceleration is also found in the transition from cauline leaves with secondary shoots to flowers. The different acceleration of phase transitions may result from a dual role of *FRI* and *FLC*, which are likely to regulate flowering not only through *AGL20*, but also through meristem identity genes such as *LFY*. We propose that the interaction of *FRI* and *FLC* not only inhibits expression of *AGL20*, but also has an independent effect on *LFY*, as suggested by our analysis of *LFY* promoter activity in *agl20-101D FRI FLC* plants. *LFY* is a key regulator of flower formation, but not of vegetative phase change (Weigel and Nilsson 1995). Thus, the repression of *LFY* by *FRI FLC* may weaken the acceleration of the transition from cauline leaves with secondary shoots to flowers in *agl20-101D FRI FLC*. Consistent with this hypothesis, when *35S::LFY* is introduced into *FRI FLC*, all of the secondary shoots are converted to flowers (Nilsson et al. 1998). Although not apparent in the *FRI FLC* background, *agl20-101D*seems to activate *LFY* expression once *FRI FLC* repression is removed. In the *agl20-101D fri FLC* or *agl20-101D FRI flc* lines, *agl20-101D* causes the replacement of secondary shoots by ectopic flowers as is observed in *35S::LFY*. The phenotype of *agl20-101D FRI FLC* also supports the idea that *Arabidopsis* has a distinct inflorescence phase I, during which cauline leaves with associated secondary shoots are produced (Ratcliffe et al. 1998, 1999). Although homozygous *agl20-101D FRI FLC* lines make the transition to flowering more quickly than Columbia, these lines produce a higher number of cauline leaves than Columbia.

Floral induction pathways merge to activate AGL20

In addition to *FRI* and *FLC*, we have identified several other regulators of *AGL20* expression, and a model of how three floral inductive pathways are integrated by

AGL20 is presented in Figure 6. In this model, *AGL20* plays a pivotal role in the control of flowering time, with the three floral inductive pathways merging to activate *AGL20*. Activation of *AGL20* by the autonomous pathway genes, *LD*, *FCA*, and *FVE*, is likely mediated by repression of *FLC*, as is activation of *AGL20* upon vernalization.

In addition to the autonomous and the vernalization pathways, the photoperiodic pathway activates *AGL20*. However, the reduction of *AGL20* expression in *co*, *gi*, and *ft* mutants is relatively modest, even though at least *co* and *gi* mutants flower much later in long days than does *agl20*. These observations indicate that *AGL20* is not the only factor downstream from the photoperiodic pathway. We have recently found that the photoperiod pathway and the autonomous pathway interact through *AGL20* activity. *agl20-101D* can partly suppress the late-flowering phenotype of the *co* mutant (H. Lee and I. Lee, unpubl.), consistent with a role of *AGL20* downstream from *CO*. Conversely, *35S::CO* can compensate for the delay in flowering in *fca* mutants (Piñeiro and Coupland 1998). Thus, there must be some cross-talk between the autonomous and photoperiod pathway, and we propose that *AGL20* is a component of this cross-talk.

A question remains which other genes integrate flowering signals. Two other candidates for integration of floral inductive signals are *FT* and *LFY*. Similar to *AGL20*, *FT* and *LFY* act partially downstream from *CO*, a gene that promotes flowering in response to long days (Simon et al. 1996; Kardailsky et al. 1999; Kobayashi et al. 1999). In addition, the expression of *FT* and *LFY* is regulated by *FCA*, which is involved in the autonomous pathway (Nilsson et al. 1998; J.H. Ahn and D. Weigel, pers. comm.). The analysis of *agl20-101D* mutants suggests that *LFY* acts at least in part downstream from *AGL20*. On the other hand, our data suggest that *FT* affects *AGL20* expression, indicating that there is substantial cross-regulation among the three genes.

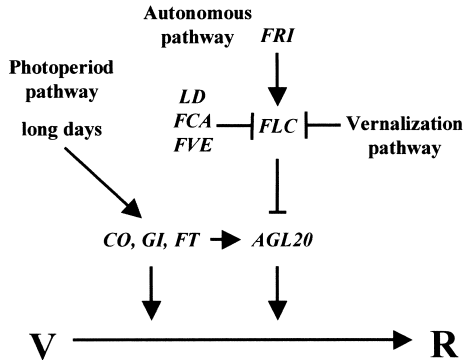

Figure 6. Model for the integrative role of *AGL20* and the interaction of flowering pathways. The horizontal line represents the vegetative (V) to reproductive (R) transition. Arrows indicate promotion, and T-bars indicate repression. In the autonomous pathway, *FRI* activates *FLC*, and *FLC* represses *AGL20*. *AGL20* acts as a floral activator. Other autonomous pathway genes, such as *LD*, *FCA*, and *FVE*, promote flowering by activating *AGL20* through the repression of *FLC*. Vernalization also promotes flowering by activating *AGL20* expression through the repression of *FLC*. Photoperiod pathway genes, *CO* and *GI*, promote flowering by activating *AGL20* but act also through other factor(s).

In addition to the photoperiod, vernalization, and autonomous pathways, it has been suggested that a gibberellin pathway promotes flowering (Levy and Dean 1998). A gibberellin biosynthetic mutant, *ga1*, does not flower under short days, and exogenous treatments of gibberellin accelerate flowering of many late-flowering mutants of both photoperiod and autonomous pathways (Wilson et al. 1992; Chandler and Dean 1994). The effect of gibberellin on *AGL20* expression may lead to further understanding how the pathways controlling flowering in *Arabidopsis* are integrated.

Materials and methods

Plant materials and growth conditions

Plants (*Arabidopsis thaliana*) were grown in long days (16 h light/8 h dark) or short days (8 h light/16 h dark), under cool white fluorescent lights (100 μmole/m^2/sec), at 23 ± 2°C, 60 ± 10% relative humidity. For vernalization studies, seeds were imbibed on 0.8% phytoagar containing half strength of Murashige-Skoog (MS) medium (GIBCO BRL) and incubated for 4 weeks at 4°C under short days. *FRI FLC* line is a Columbia near-isogenic line with *FRI* allele from SF2 obtained by eight backcrosses into Columbia, which was described previously (Michaels and Amasino 1999). The mutants, *fri* (FN235) *FLC* and *FRI flc-3*, are the lines obtained by fast neutron mutagenesis of *FRI FLC*. The late-flowering mutants, *co-1*, *gi-1*, *fve-3*, *ld-1*, and *agl20*, are in the Columbia background, but *co-2*, *gi-3*, *ft-1*, *fd-1*, *fha-1*, *fe-1*, *fwa-2*, *fca-1*, *fve-1*, and *fpa-1* are in the L*er* background. *ft-1* introgressed into Columbia was provided by D. Weigel (Salk Institute).

Screening of activation-tagging mutants

FRI FLC line was transformed with pSKI015 (Weigel et al. 2000) by vacuum infiltration (Bechtold et al. 1993). For screening of activation-tagging mutants, T0 seeds were sown on soil under long days and treated with 0.1% basta (AgrEvo, USA) after 7 d. For basta segregation analysis, seeds were germinated on half-strength MS media with 0.007% basta.

Construction of 35S::AGL20

To generate a *35S::AGL20* construct, an *AGL20* cDNA was amplified by RT-PCR using total RNA extracted from Columbia leaf tissues. *Bam*H1 sites were introduced before the ATG start codon and after the TGA stop codon in the cDNA. The primers used in the PCR were 5′-CCC GGA TCC ATG GTG AGG GGC AAA ACT CAG-3′ and 5′-CCC GGA TCC TCA CTT TCT TGA AGA ACA AGG-3′. The RT-PCR product was subcloned into the pGEM-Teasy vector (Promega) to yield pHR2. The sequence of *AGL20* cDNA in pHR2 was confirmed using Sequenase version 2.0 kit (U.S. Biochemical Corp.). The 645-bp insert of pHR2 was digested with *Bam*H1 and introduced into the *Bam*H1 site of pCGN18 vector (Jack et al. 1994). Constructs were introduced into the *FRI FLC* line by vacuum infiltration.

Phylogenetic analysis

Nucleotide and predicted amino acid sequences of MADS-domain proteins in *Arabidopsis* were obtained from GenBank.

Distance matrices were calculated using the DNADIST program of PHYLIP version 3.5 (Department of Genetics, University of Washington, Seattle, WA), and the numbers of nucleotide substitutions were estimated using the two-parameter method of Kimura (1980). Distance trees were constructed using the neighbor-joining method (Saitou and Nei 1987) and implemented using the NEIGHBOR program in PHYLIP.

RT-PCR

Total RNA was extracted as described previously (Puissant and Houdebine 1990). One µg of total RNA from each tissue was reverse-transcribed with oligo-$dT_{12\text{-}18}$ (GIBCO BRL) in a 20 µL reaction mixture using the Reverse Transcription System (Promega). After heat inactivation of the reaction mixture, PCR was performed using 1 µL of the first-stranded cDNA sample with 25 pmole of the primers in a 50 µL reaction. PCR conditions were as follows: 94°C (3 min); 25 cycles of 94°C (30 sec), 55°C (1 min), 72°C (1 min), and 72°C (10 min). PCR products were electrophoresed on an agarose gel, blotted onto a NYTRAN-PLUS membrane (Schleicher & Schuell), and hybridized with the appropriate probes. RT-PCR was repeated at least three times for the samples harvested separately. The primers used for RT-PCR were as follows: For β-*tubulin* (*TUB2*), two primers, 5′-CTC AAG AGG TTC TCA GCA GTA-3′ and 5′-TCA CCT TCT TCA TCC GCA GTT-3′ were used. For *AGL20*, two primers, 5′-CCC CAT ATG GTG AGG GGC AAA ACT C-3′ and 5′-CCC GGA TCC TCA CTT TCT TGA AGA ACA AGG-3′ were used. For *FLC*, two primers, 5′-CCC CAT ATG GGA AGA AAA AAA CTA G-3′ and 5′-CCC GGA TCC CTA ATT AAG TAG TGG GAG-3′ were used. For *AP1*, two primers, 5′-GCA CCT GAG TCC GAC GTC-3′ and 5′-GCG GCG AAG CAG CCA AGG-3′ were used.

GUS staining

For histochemical analysis of GUS, X-gluc staining and tissue fixation were performed as described by Blázquez et al. (1997). Eight-µm sections were prepared and mounted and, after removal of paraplast with xylenes, photographed under bright field on an Olympus BX50 microscope. *LFY::GUS* used in this study was strain DW150-209 (Blázquez et al. 1997).

Genotyping of FRI

To identify *fsu1-1D* with the *fri-Col* allele, genomic DNA was amplified by PCR using two primers, 5′-CAA CGA CCA AAC ACA ACG AC-3′ and 5′-CGC GAG ACT GAA CCT CAC GG-3′ and digested with *Rsa*1. The *FRI-SF2* allele yielded four fragments (100, 200, 300, and 350 bp), and the 300 bp fragment was replaced by a 330 bp fragment in the *fri-Col* allele.

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