

The molecular basis of vernalization: The central role of *FLOWERING LOCUS C* (*FLC*)

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In *Arabidopsis*, the MADS-box protein encoded by *FLOWERING LOCUS C* (*FLC*) is a repressor of flowering. Vernalization, which promotes flowering in the late-flowering ecotypes and many late-flowering mutants, decreases the level of *FLC* transcript and protein in the plant. This vernalization-induced reduction in *FLC* transcript levels is mitotically stable and occurs in all tissues. *FLC* activity is restored in each generation, as is the requirement of a low-temperature exposure for the promotion of flowering. The level of *FLC* determines the extent of the vernalization response in the promotion of flowering, and there is a quantitative relationship between the duration of cold treatment and the extent of down-regulation of *FLC* activity. We conclude that *FLC* is the central regulator of the induction of flowering by vernalization. Other vernalization-responsive late-flowering mutants, which are disrupted in genes that encode regulators of *FLC*, are late-flowering as a consequence of their elevated levels of *FLC*.

Recently, we reported on a gene, *FLOWERING LOCUS F* (*FLF*), from *Arabidopsis* whose activity, as determined by steady-state mRNA levels, is correlated quantitatively with the period taken to initiate flowering (1). The *FLF* gene encodes a putative transcription factor of the MADS-box class, which, we concluded, acts as a repressor of floral induction. Genetic analyses of *Arabidopsis* ecotypes previously identified *FLOWERING LOCUS C* (*FLC*) as a semidominant repressor of floral induction (2–4). Because *FLF* has similar properties to *FLC* and maps to the same chromosomal region, the possibility arose that *FLF* and *FLC* are one and the same gene; this hypothesis has been confirmed by Michaels and Amasino (5). We suggest that the gene be referred to as *FLC* and the mutants *flf-1* to *flf-9* now be referred to as *flc-11* to *flc-19*.

In many plant species, flowering is promoted by a period of exposure to low temperature through a process known as vernalization. A number of the late-flowering mutants and ecotypes of *Arabidopsis* have a vernalization response. We found that the vernalization-responsive late-flowering mutants had a higher level of *FLC* transcript than was present in the wild types and, importantly, that the non-vernalization-responsive late-flowering mutants did not have increased *FLC* transcript levels. The vernalization-responsive ecotype C24, which is somewhat late-flowering, also has a high level of *FLC* transcript that is reduced by the low-temperature treatment (1). These observations suggested to us that *FLC* must be directly involved in the vernalization response.

We have shown that reduction in the level of genomic methylation largely substitutes for the low-temperature treatment in promoting flowering (6–8), implicating a reduction in DNA methylation level as a component in the vernalization response. Early-flowering *METHYLTRANSFERASE1* antisense plants, with a decreased level of genomic methylation, have a reduced level of *FLC* activity as compared with the nontransformed control (1). The correlation between *FLC* transcription and methylation levels implies that *FLC* is regulated by methylation status, but we are not able to say whether this is through an alteration of the methylation status of the *FLC* gene itself, or whether it involves other genes that regulate *FLC* expression.

In this paper, we demonstrate that the *FLC* gene is involved directly in controlling the response to vernalization and more generally in the timing of the transition to the reproductive phase of development. We find that a reduction in *FLC* activity is a key component of the vernalization response in six late-flowering vernalization-responsive mutants and in the C24 and Pitztal ecotypes. The late-flowering mutants are mutated in genes that regulate the activity of the *FLC* gene. We further show that the quantitative responses in the promotion of flowering to various periods of exposure to low temperature are paralleled by the levels of *FLC* transcript, and that the changes observed at the transcriptional level are reflected in the level of the *FLC* protein.

One of the key properties of vernalization, the resetting of the requirement for low-temperature induction in each generation, is mirrored in the resetting of *FLC* transcript levels.

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis* seeds sown aseptically in Petri dishes containing a modified Murashige and Skoog medium (9) were cold-treated in the dark (at 5°C ± 1°C) for the stated period before growth under cool-white fluorescent lights with a 16-h photoperiod at 21°C to 23°C. Plates for flowering-time experiments contained 21 seeds, evenly spaced, in a 90-mm or 140-mm plate, whereas plates for RNA extraction contained ≈100–150 seeds per 90-mm plate. Unless indicated, seedlings were harvested for RNA or protein extraction at 14 days for 1-day cold-treated plates, or at a comparable developmental stage (5–6 rosette leaves visible) for plates that were cold-treated for longer periods. Flowering time was recorded as the number of rosette leaves when the floral bolt was 1 cm high.

Mutant seed stocks were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus) or from C. Dean (John Innes Centre, Norwich, U.K.).

The Pitztal line used by Sheldon *et al.* (CS1454; ref. 1) and another Pitztal line (CS1456), both obtained from the ABRC, flower after ≈30 days, in contrast to the reported late-flowering of ≈100 days (10, 11). The line used in the experiments described in this paper is a late-flowering glabrous Pitztal line from our archive stocks, designated Pitztal-1 (Ptz-1).

The *flc-13* mutant contains an *Ac* element in intron I (1), which excises in ≈1 in 20 plants to give a revertant late-flowering heterozygote. Such revertants were identified in flowering-time experiments by their much later flowering time (more than 20 leaves at flowering) and were excluded from the data presented.

Generation of *FLC* Antisense Transgenic Plants. The 35S::*FLC* antisense binary vector (1) was introduced into the *fca-1* mutant by the floral-dip method (12). T₁ plants were selected by using

Abbreviations: Col, Columbia; Ler, Landsberg *erecta*; Ptz-1, Pitztal-1; Ws-2, Wassilewskija-2.

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glufosinate ammonium (Basta; Riedel-deHaen, Germany), and individuals flowering considerably earlier than *fca-1* control plants were identified. The flowering time of three lines was examined in the T₂ generation.

RNA Extraction and RNA Gel Blot Analysis. Total RNA was extracted from ≈0.5 g of tissue as described (13), or from smaller quantities of tissue by using the RNeasy Plant Mini Kit (Qiagen). RNA gel blot analysis was performed as described in ref. 1.

Reverse Transcription–PCR Amplification and Sequencing of *FLC-Ler*. First-strand cDNA, generated from 5 μg of *fca-1* [in *Landsberg erecta* (*Ler*)] total RNA by using Superscript II according to the manufacturer's instructions (GIBCO/BRL), was used as template in a PCR with primers 5'-CCGCTCGAGCTTAG-TATCTCCGGCG-3' and 5'-GGACTAGTCGCCCTTAT-CAGCGGA-3', as described (1). The product was purified by using the QIAquick PCR purification kit (Qiagen) and sequenced with the two primers above and internal primers as described (1). The Wisconsin Package, Version 10.0 (Genetics Computer Group, Madison, WI), was used for sequence analysis.

Immunodetection of FLC Protein. A rabbit polyclonal antibody was raised against a bacterially expressed histidine-tagged FLC protein lacking the first 80 N-terminal amino acids, which was purified by using Talon metal-affinity resin (CLONTECH). The apex and developing leaves of *Arabidopsis* seedlings were ground in liquid nitrogen and homogenized in extraction buffer (0.1 M sodium phosphate, pH 7.2/1 mM EDTA), and the insoluble material was pelleted by centrifugation. The supernatant was boiled immediately in one-third volume of 6× SDS sample buffer (0.5 M Tris, pH 6.8/10% SDS/0.6 M DTT/0.012% bromophenol blue) for 5 min before being separated on a denaturing 12% polyacrylamide gel and blotted onto Protran nitrocellulose (Schleicher & Schuell). Blots were amido black-stained (Sigma) and incubated with either preimmune serum diluted 1:1000 (vol/vol) or FLC polyclonal antibody serum diluted 1:3000 (vol/vol). The immunoreactive proteins were visualized by using the enhanced chemiluminescence Western blotting analysis system (Amersham), with the secondary antibody diluted 1:2000 (vol/vol), and by exposure to x-ray film (Fuji) for 1 to 10 min.

Results and Discussion

***FLC* Is Central to the Vernalization Response.** *Ler* is an early-flowering ecotype with little response to vernalization under long-day growth conditions. However, mutants at a number of loci (e.g., *fca-1*, *fve-1*, and *fpa-1*) in the *Ler* background are late-flowering and respond strongly to vernalization (ref. 14; Fig. 1A). This finding suggests that either the loci that are mutated in these mutants all independently control flowering time and independently confer an ability to respond to vernalization, or, more likely, that these loci act through a common target gene, which is responsible for the vernalization-responsive late-flowering phenotype. All three mutants have an increased level of *FLC* mRNA (ref. 1; Fig. 1B) relative to *Ler*, implicating *FLC* as the common target. This conclusion is supported by observations of late-flowering vernalization-responsive mutants in two other ecotypes, *fld-2* in Columbia (*Col*) (15) and *ld-3* in Wassilewskija-2 (*Ws-2*) (16). As with *Ler*, *Col* and *Ws-2* display only a small vernalization response and have a low level of *FLC* transcript, whereas *fld-2* and *ld-3* have a higher level of *FLC* transcript (Fig. 1) and respond to vernalization with a decreased time to flower.

In the *C24* ecotype, which has a high level of *FLC* transcript, the level of the transcript is reduced by vernalization (1), as is the time to flower. If the late-flowering phenotype of mutants of these five different genes is caused by the elevated level of *FLC*

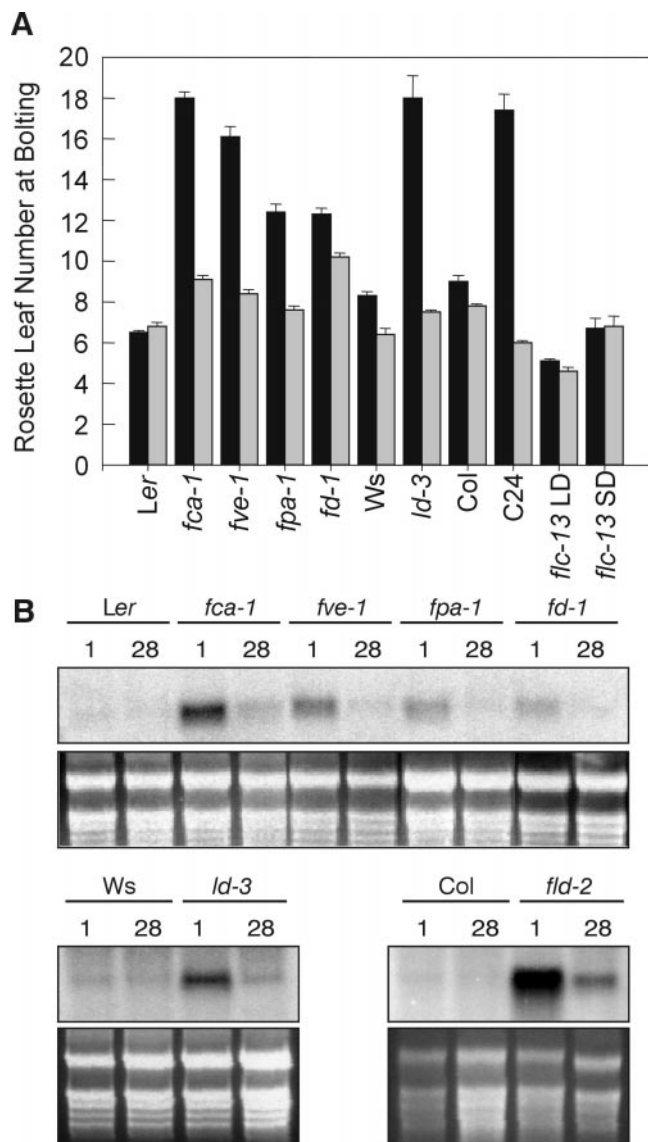


Fig. 1. Vernalization decreases both flowering time and *FLC* transcript level in vernalization-responsive late-flowering mutants. (A) Flowering time expressed as rosette leaf number for mutants and their corresponding wild types, *fca-1*, *fve-1*, *fpa-1*, and *fd-1* in *Ler*; *ld-3* in *Ws-2*; and *flic-13* in *C24*, cold-treated for either 1 (black bars) or 28 (gray bars) days. The *flic-13* mutant was grown under both a 16-h photoperiod (LD) and an 8-h photoperiod (SD). One-day cold-treated *fld-2* plants remained vegetative throughout the experiment (terminated on day 90); *fld-2* plants that had been cold-treated for 28 days flowered with an average of 14.7 ± 0.4 rosette leaves. These data are not included, but the data for the wild-type *Col* are. The error bars represent the SE. (B) *FLC* expression in seedlings of *Ler*, *fca-1*, *fve-1*, *fpa-1*, *fd-1*, *Ws-2*, *ld-3*, *Col*, and *fld-2*, cold-treated for either 1 or 28 days, as indicated above the figure. The ethidium bromide-stained ribosomal bands are shown below the blots as loading controls.

transcript, then we might expect that, after vernalization, each of the mutants would have substantially reduced levels of *FLC* transcript. This is the case (Fig. 1B). Thus, cold treatment is able to overcome the up-regulation of the *FLC* gene caused by mutations of these loci and to overcome the delay in flowering.

The late-flowering mutant *fd-1* has been reported to lack a vernalization response (14). Under our growing conditions, however, it shows a modest decrease in flowering time in response to cold treatment (Fig. 1A), paralleled in the nonvernalized plant by a slightly elevated *FLC* transcript, which is reduced by the cold

treatment (Fig. 1B). Other late-flowering mutants that do not respond to vernalization and that do not have elevated *FLC* levels (*co*, *gi*, *pha*, *fe*, *ft*, and *fwa*) (1) presumably contain lesions in the flowering pathway that are downstream of *FLC*, or the lesions may be in alternative flowering pathways, such as the photoperiod-responsive pathway.

In every ecotype or mutant that we have examined, there is a correlation between the level of *FLC* transcript and the response to vernalization. The ecotypes *Ler*, *Col*, and *Ws-2*, which have only slight vernalization responses under the conditions used, have a low level of *FLC*, even in the absence of vernalization (Fig. 1). The *flc-13* mutant, which has an undetectable level of *FLC* transcript (1), has a minimal response to vernalization, in contrast to the strong vernalization response of the C24 wild type (Fig. 1A).

Our data indicate that in order for a plant to flower earlier in response to vernalization, *FLC* transcript in the nonvernalized plant is required, and that the magnitude of this response depends on the level of *FLC* transcript in the nonvernalized plant.

The *fca-1* Mutant Is Late-Flowering Because of an Elevated Level of *FLC*. The correlation between high *FLC* transcript in the late-flowering nonvernalized mutants and low *FLC* transcript in the vernalized mutants that flower earlier (Fig. 1A) suggests that the cause of the late-flowering in the nonvernalized mutants is the elevated level of *FLC*. However, because the expression of other genes may also be altered by vernalization, we have tested whether a change in *FLC* activity is sufficient for the response. A construct containing an *FLC* antisense gene under the control of the cauliflower mosaic virus 35S promoter, which reduced *FLC* transcript levels and promoted flowering in T₂ C24-transgenic lines (Fig. 2), was introduced into one of the mutants, *fca-1*. Fig. 2 shows the analysis of three T₂ families, segregating for glufosinate ammonium (Basta) resistance, the selectable marker in the transgene construct. Resistant T₂ plants from all three lines showed marked reductions in the levels of *FLC* transcript and flowered earlier than the nontransformed *fca-1* mutant. The flowering time of the T₂ plants was comparable to the flowering time of 28-day cold-treated *fca-1* (Fig. 1A), indicating that much of the late-flowering phenotype of the *fca-1* mutant can be overcome by reducing *FLC* transcript levels. When the same lines were sown in the absence of herbicide selection and scored for flowering time, the segregation was 3:1 for early flowering (as in Fig. 2A) and late flowering (equivalent to *fca-1*), indicating that the transgenic lines were in the *fca-1* background. We conclude that a large proportion of the delay in flowering in the *fca-1* mutant, and presumably in the other late-flowering vernalization-responsive mutants, is caused by their elevated level of *FLC* transcript.

The Difference in Activities of *FLC* Alleles in Ecotypes Is Not Caused by Differences in Their Coding Sequences. Genetic studies with ecotypes of *Arabidopsis* have led to the conclusion that C24 and *Ler* contain alleles of the *FLC* locus that suppress the late-flowering phenotype caused by dominant alleles of the *FRIGIDA* (*FRI*) locus, whereas *Col* contains an allele of *FLC* that does not suppress the late-flowering caused by dominant *FRI* alleles (2–4). The nucleotide sequence of the coding region of the C24 *FLC* locus is identical to the allele present in the *Col* ecotype (1). We have isolated the *FLC* cDNA from the *Ler* ecotype (by using the *fca-1* mutant), and this allele has the same predicted amino acid sequence as the C24 and *Col* alleles. Clearly, the difference in *FLC* activity between ecotypes is not caused by changes in the coding sequence, because all three are identical. It is likely, therefore, that the *FLC* alleles in these ecotypes differ in some aspect of their regulation, perhaps as a result of sequence differences in the promoter region of the *FLC* gene. We have

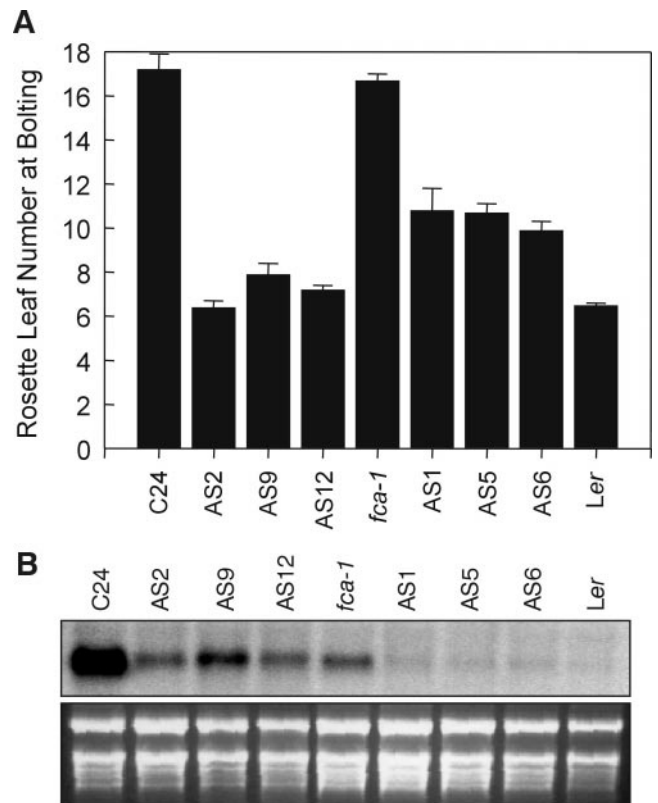


Fig. 2. Reduction in *FLC* transcript level caused by a 35S::*FLC* antisense construct results in early-flowering in C24 and in the *fca-1* mutant. (A) Flowering time of untransformed C24 and three C24 T₂ lines (AS2, AS9, and AS12) containing the 35S::*FLC* antisense construct, and of untransformed *fca-1*, three *fca-1* T₂ lines (AS1, AS5, and AS6), and *Ler*, expressed as rosette leaf number. Error bars represent the SE. (B) *FLC* expression in bolting, early-flowering T₂ segregants and from bolting C24, *fca-1*, and *Ler* plants. The ethidium bromide-stained ribosomal bands are shown below the blots as loading controls.

shown that a number of genes regulate *FLC* expression, and this regulation may depend on the allele of *FLC* present in any given line. For example, the expression of *FLC* in a *Ler* line containing the functional *FRI* allele from the San-Feliu-2 ecotype is markedly lower (1) than is *FLC* expression in the Ptz-1 ecotype, which also has a functional *FRI* allele (ref. 11; see Fig. 4A and B), suggesting that the *FLC-Ler* allele may have a reduced capacity to be up-regulated. Similarly, the difference in the *FLC* expression levels between C24 and Ptz-1, both possessing functional *FRI* alleles (4, 11), may be because of their different *FLC* alleles. The low level of *FLC* expression in the *Ler* ecotype is likely to be because of a deletion in the *FRI-Ler* allele (17).

***vrn1* and *vrn2* Block the Vernalization-Induced Down-Regulation of *FLC*.** *vrn1* and *vrn2*, two mutants with reduced responses to vernalization, have been isolated in the *fca-1* mutant background (in *Ler*) (18). *fca-1 vrn2* is later-flowering than *fca-1* (ref. 18; Fig. 3A) and has a higher *FLC* transcript level than *fca-1* (ref. 1; Fig. 3B), suggesting that apart from any role in the vernalization response, the wild-type *VRN2* gene acts to repress *FLC* expression and thereby to promote flowering. *fca-1 vrn1* has a similar flowering time to *fca-1* (ref. 18; Fig. 3A) and a level of *FLC* transcript similar to *fca-1* (Fig. 3B). Both *fca-1 vrn1* and *fca-1 vrn2* have less reduction in flowering time in response to vernalization than does *fca-1* (ref. 18; Fig. 3A), which is matched by a smaller vernalization-induced reduction in *FLC* transcript level (Fig. 3B). This result indicates that the wild-type *VRN1* and

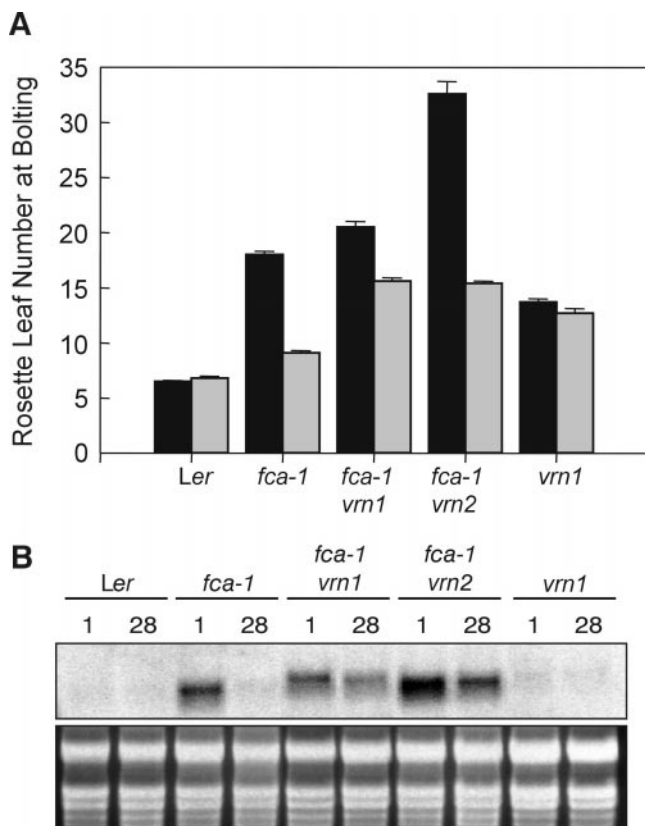


Fig. 3. Mutants with reduced responses to vernalization maintain a high *FLC* transcript level after cold treatment. (A) Flowering time expressed as rosette leaf number for *Ler*, *fca-1*, *fca-1 vrn1-1*, *fca-1 vrn2-1*, and *vrn1-1*, cold-treated for either 1 (black bars) or 28 (gray bars) days. Error bars represent the SE. (B) *FLC* expression in seedlings of *Ler* and the mutants in A, cold-treated for either 1 or 28 days, as indicated above the figure. The ethidium bromide-stained ribosomal bands are shown below the blots as loading controls.

VRN2 genes are involved in mediating the vernalization-induced down-regulation of the *FLC* gene.

vrn1, segregated away from the *fca* mutation and placed in an *FCA* background (18), is late-flowering in our growth conditions, although it does not have an up-regulated *FLC* transcript level (Fig. 3). This finding suggests that the *VRN1* gene may be active in an *FLC*-independent flowering pathway as well as in the *FLC*-dependent pathway. *vrn1* shows little vernalization response, presumably because of the low level of *FLC* transcript level.

The Down-Regulation of *FLC* Is Proportional to the Duration of Cold Treatment.

In a number of plant species, including *Arabidopsis thaliana* the magnitude of the promotion of flowering by cold treatment is proportional to the duration of the cold treatment (10, 19). In the C24 ecotype, we found correlations between the duration of the cold treatment, the promotion of flowering, and the decrease in *FLC* transcript levels (Fig. 4A). In Ptz-1, a late-flowering ecotype, the same correlation exists between the length of the vernalization period and the decrease in *FLC* transcript level and time to flower (Fig. 4B). However, the period of cold treatment required to saturate the vernalization response of Ptz-1 is greater than that required for C24.

The late-flowering overexpression mutant *flc-11* requires a 56-day cold treatment to effect a substantial reduction in flowering time, whereas C24 has a saturated vernalization response with a 28-day cold treatment (1). Consistent with this finding, the

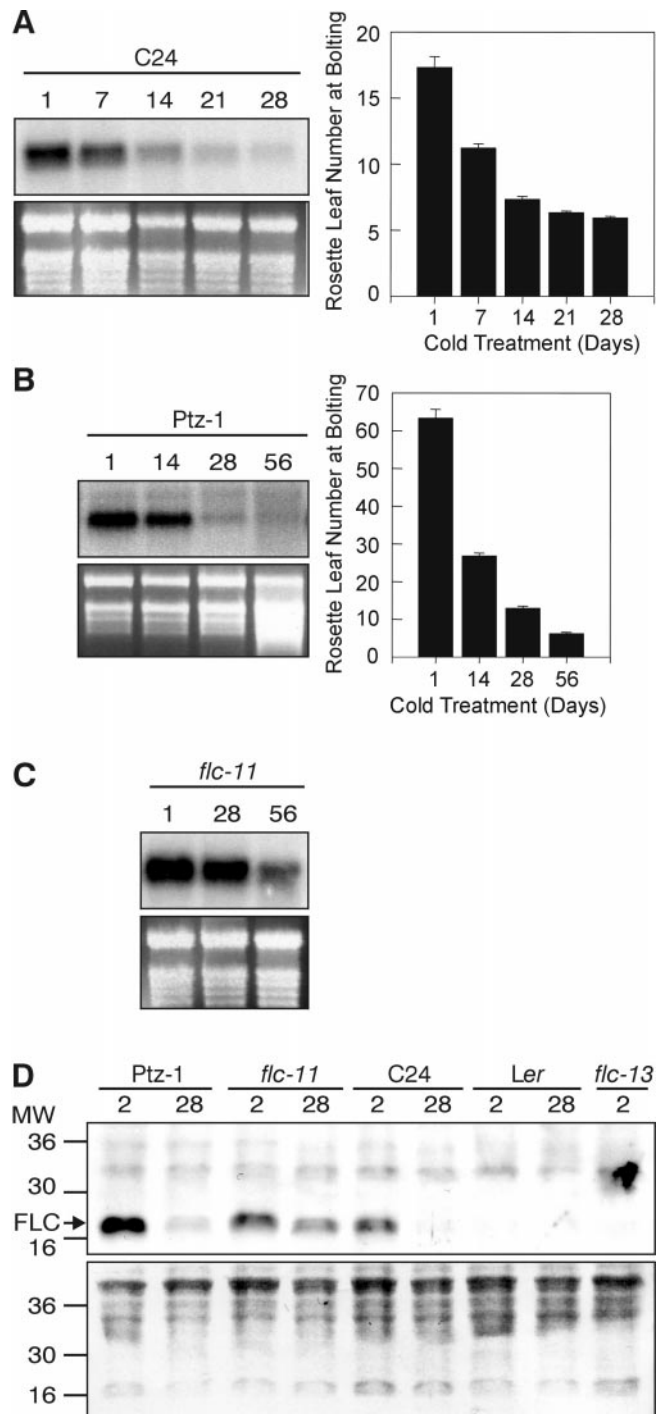


Fig. 4. The down-regulation of *FLC* and the decrease in flowering time is proportional to the duration of the cold treatment. (A) *FLC* expression and flowering time, expressed as rosette leaf number, in C24 seedlings that were subjected to a cold pretreatment of 1, 7, 14, 21, or 28 days, as indicated, before growth at 21°C. Error bars represent the SE in A and B, and the ethidium bromide-stained ribosomal bands are shown as loading controls in A–C. (B) *FLC* expression and flowering time, expressed as rosette leaf number, in Ptz-1 seedlings that were subjected to a cold pretreatment of 1, 14, 28, or 56 days, as indicated, before growth at 21°C. (C) *FLC* expression in *flc-11* seedlings that were subjected to a cold pretreatment of 1, 28, or 56 days, as indicated, before growth at 21°C. (D) *FLC* protein levels in the soluble fraction from Ptz-1, *flc-11*, C24, *Ler*, and *flc-13* seedlings, cold-treated for either 2 or 28 days, as indicated. The amido black-stained filter is shown below as a loading control. The migration of the molecular weight markers ($\times 1000$) is shown to the left. A band detectable with the *FLC* antibody, but not with the preimmune serum, is indicated as *FLC*.

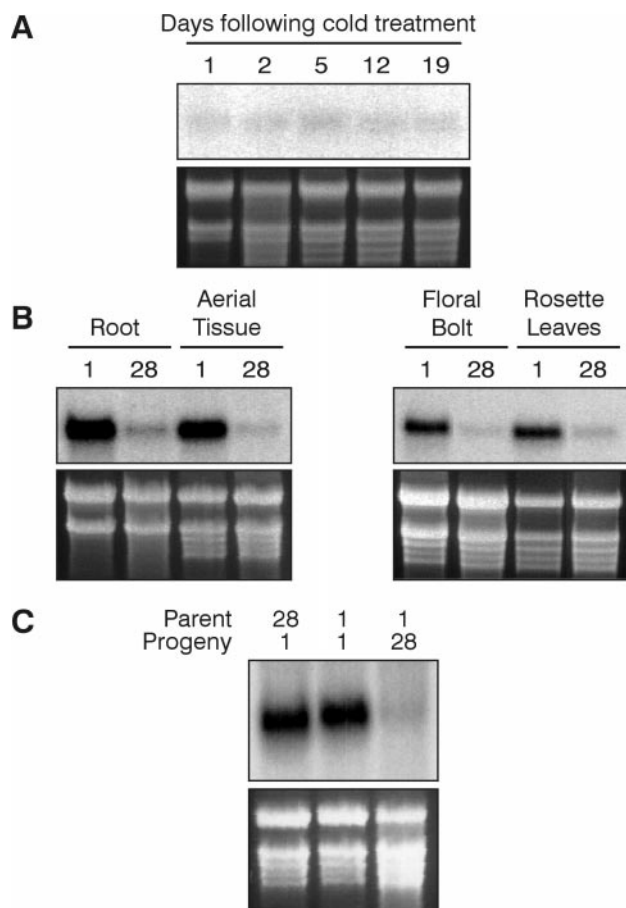


Fig. 5. The down-regulation of *FLC* transcript by cold treatment is mitotically stable, but is reset in progeny. (A) *FLC* expression in 28-day cold-treated C24, harvested after 1, 2, 5, 12, or 19 days of growth at 21°C, as indicated above the figure. The majority of the plants were bolting at 19 days. (B) *FLC* expression in tissues of 1- and 28-day cold-treated C24 plants. Root and aerial tissue were from one set of seedlings, and floral bolts (2–3 cm in height) and rosette leaves were derived from another set of plants. (C) *FLC* expression in C24 seedlings that were the progeny of plants that were either cold-treated for 28 days (lane 1) or for 1 day (lanes 2 and 3), which, in turn, themselves were either cold-treated for 1 day (lanes 1 and 2) or for 28 days (lane 3). The ethidium bromide-stained ribosomal bands are shown below the blots as loading controls.

flc-11 mutant also requires a longer period of cold treatment to decrease *FLC* transcript levels as compared with C24 (Fig. 4C).

Vernalization Reduces the Level of FLC Protein. FLC protein was detected on Western blots by using antibodies raised to a bacterially expressed protein lacking the MADS domain (Fig. 4D). The amount of FLC protein decreased dramatically after 28 days of cold treatment in C24 and Ptz-1. Consistent with both the flowering-time data and the RNA-expression data, the decrease in the *flc-11* mutant was not as great as in the two ecotypes. There was no detectable FLC protein in either the *Ler* ecotype or the *flc-13* loss-of-function mutant, again consistent with the RNA data. Thus, the level of protein expression follows that of the mRNA, indicating that the regulation of *FLC* occurs predominantly at the transcriptional level.

The Down-Regulation of FLC Transcript by Cold Treatment Is Mitotically Stable, but Is Reset in Progeny. The exposure to low temperature often occurs early in a plant's development, and flowering may occur after many weeks or months of growth in warmer temperatures. This situation indicates that the vernalization

signal is maintained through a number of cell divisions after the cessation of the low temperature (20). We investigated *FLC* transcript levels in C24 plants during growth at 21°C after a 4-week cold treatment. Fig. 5A shows the same low level of *FLC* transcript is present in vernalized plants harvested at 1, 2, 5, 12, and 19 days after transfer to 21°C growth conditions. These data indicate that the decrease in *FLC* transcript is mitotically stable; once transcript levels are decreased by vernalization, they remain low during the subsequent growth of the plant. The *FLC* transcript in both roots and floral bolts is decreased by cold treatment in a manner similar to that in rosette leaves (Fig. 5B). This result indicates that the cold treatment affects the meristems giving rise to both shoots and roots, and that the vernalization-induced reduction in *FLC* transcript is mitotically stable even after the transition to flowering is effected.

The vernalized state is not transmitted to the progeny, and each generation of plants must be exposed to low temperature for early-flowering to occur (20). This finding is paralleled by the activity of the *FLC* gene (Fig. 5C). Progeny of both vernalized and nonvernalized plants have the same level of *FLC* transcript, which means that the *FLC* transcript level, as well as the vernalization requirement, is reestablished to the ground state in the progeny of a vernalized plant.

Conclusion: The Key Role of FLC in the Initiation of Flowering. We have demonstrated a number of parallels between the reduction in flowering time in response to vernalization and the reduction in the level of *FLC* expression, including the proportionality between the duration of cold treatment and the reduction in both flowering time and *FLC* activity, and the mitotically stable inheritance and the need for cold treatment each generation. We conclude that *FLC* is central to both the control of the transition to flowering and the response to vernalization.

We have shown previously that the mutants of the photoper-

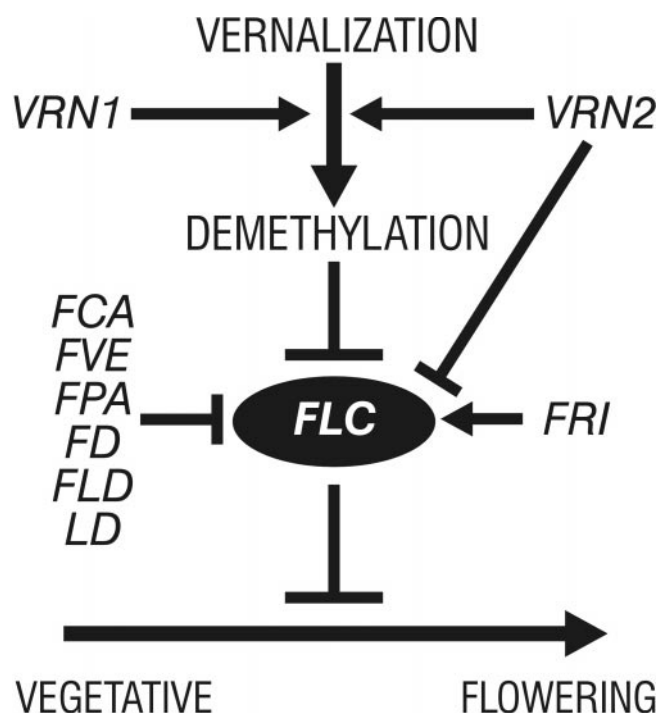


Fig. 6. Model indicating the role of *FLC* in the transition to flowering and its regulation by vernalization, methylation, and genes involved in controlling flowering time, based on data presented here and in refs. 1 and 7. The down-regulation of *FLC* expression by demethylation may be direct or may be through a regulator of *FLC*.

riod-responsive pathway do not have altered levels of *FLC* mRNA (1), and alteration of the photoperiod does not affect *FLC* expression (ref. 5 and data not shown). In support of this, the *flc-13* mutant, which lacks *FLC* expression, maintains a photoperiod response (Fig. 1A) with the time-to-flower in a 16-h photoperiod being $\approx 75\%$ of that in an 8-h photoperiod (about the same ratio as for the wild-type C24). Although *FLC* has a central role in the initiation of flowering in response to a low-temperature treatment, the change in photoperiod from 8 h to 16 h appears to induce flowering independently of *FLC*.

We have identified at least eight genes that regulate *FLC* expression, acting to either increase or decrease transcript levels. Together with low temperature-induced down-regulation of *FLC*, these genes serve to fine-tune the expression of *FLC* (Fig. 6). This regulation of *FLC* expression results in the sensitive control of flowering time in wild populations, allowing the matching of the time of flowering to the environmental conditions and the developmental state of the plant. Two of the genes that regulate *FLC* expression have been characterized. *LD* encodes a putative transcription factor (16) and may act directly to regulate *FLC* transcription. *FCA* encodes an RNA binding protein (21), which may regulate *FLC* at a posttranscriptional level. The identification of many genes that regulate *FLC* expression (Fig. 6) suggests that they may not all act directly on the *FLC* gene, but rather that some may act within the same regulatory cascade.

Vernalization can override the up-regulation of *FLC* transcript that occurs when *FCA*, *FVE*, *FPA*, *FLD*, or *LD* are mutated. We have proposed previously (6, 7) that vernalization acts through demethylation of specific sites of genomic DNA. *FLC* expression is reduced in transgenic plants that have a reduced level of DNA methylation (1), indicating that either *FLC* or a regulator of *FLC* is controlled by the DNA methylation status. If the *FLC* gene were regulated directly by its methylation status, for example, if a transcriptional activator bound to a motif only within a methylated promoter, then the reduction of DNA methylation by vernalization could prevent transcription and override control by other regulators.

FLC encodes a member of the MADS-box class of transcription factors, so it is likely to effect its repression of reproductive development by regulating the transcription of genes whose products are operative in the pathway to flowering. Gibberellins can promote flowering in *Arabidopsis* (22), but have no effect on *FLC* expression (1), suggesting that they may act downstream of *FLC*. We have proposed previously that *FLC* may act by antagonizing the promotive effect of gibberellins on flowering (1). We need to identify which, if any, of the gibberellin biosynthetic or signal-transduction genes is involved in mediating the action of *FLC* in the pathway to flowering.

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