Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*

Scott D. Michaels, Yuehui He, Katia C. Scortecci, and Richard M. Amasino*

Department of Biochemistry, University of Wisconsin, Madison, WI 53706

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Plant species have evolved a wide variety of flowering habits, each adapted to maximize reproductive success in their local environment. Even within a species, accessions from different environments can exhibit markedly different flowering behavior. In *Arabidopsis*, some accessions are rapid-cycling summer annuals, whereas others accessions are late flowering and vernalization responsive and thus behave as winter annuals. Two genes, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), interact synergistically to confer the winter-annual habit. Previous work has shown that many summer-annual accessions contain null mutations in the *FRI* gene; thus it appears that these summer-annual accessions have arisen from winter-annual ancestors by losing *FRI* function. In this work we demonstrate that naturally occurring allelic variation in *FLC* has provided another route to the evolution of summer-annual flowering behavior in *Arabidopsis*. We have identified two summer-annual accessions, Da (1)-12 and Shakhdara, that contain functional alleles of *FRI*, but are early flowering because of weak alleles of *FLC*. We have also determined that the weak allele of *FLC* found in Landsberg *erecta* is naturally occurring. Unlike accessions that have arisen because of loss-of-function mutations in *FRI*, the *FLC* alleles from Da (1)-12, Shakhdara, and Landsberg *erecta* are not nulls; however, they exhibit lower steady-state mRNA levels than strong alleles of *FLC*. Sequence analysis indicates that these weak alleles of *FLC* have arisen independently at least twice during the course of evolution.

FRIGIDA | vernalization | winter annual | natural variation

Identification of the genetic mechanisms that underlie differences in life history is key to understanding the evolution of adaptive traits. Many plants species occur over a wide range of latitudes, and within a species, accessions from different regions can differ substantially in flowering behavior (1). As an adaptation to seasonal fluctuations in temperature experienced in temperate climates, flowering in many species is promoted by prolonged exposure to cold temperatures (vernalization) (2). Many accessions of *Arabidopsis* from temperate climates are relatively late flowering unless vernalized (3). This vernalization requirement prevents flowering before winter and promotes rapid flowering in the spring, and thus these accessions behave as winter annuals. Summer-annual *Arabidopsis* accessions, in contrast, flower rapidly in the absence of vernalization, and these accessions predominate in warmer climates that do not experience the cold temperatures required for vernalization (3).

The late-flowering phenotype of winter-annual accessions of *Arabidopsis* is created by the interaction of two genes, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (2). *FLC* encodes a MADS domain-containing transcription factor whose expression is sufficient to inhibit flowering (4, 5); however, the native *FLC* gene is only expressed to high levels in the presence of *FRI*, which encodes a plant-specific gene of unknown biochemical activity (3). Thus, loss-of-function mutations in either gene eliminate the late-flowering phenotype. Vernalization promotes flowering in winter-annual strains of *Arabidopsis* by causing an epigenetic down-regulation of *FLC* (4, 5).

Genetic analysis of winter- and summer-annual accessions has revealed that variation at the *FRI* locus was a common determinate of flowering habit (6–9). Subsequent sequence analysis of the *FRI* gene from summer-annual accessions of *Arabidopsis* revealed that many contained lesions that create null alleles of *FRI* (3, 10). Thus, it appears that these summer-annual accessions were derived from winter-annual ancestors by losing *FRI* activity, and that *FRI* activity was lost multiple times during the course of evolution.

Here we describe a second mechanism by which the vernalization requirement of winter-annual *Arabidopsis* has been lost to produce a summer-annual flowering habit. We have identified summer-annual accessions of *Arabidopsis* that contain active alleles of *FRI*, but are early flowering because of novel weak alleles of *FLC*. Unlike the null alleles of *FRI* present in most summer-annual accessions, these alleles of *FLC* are not nulls; they are expressed and contain no nonsense or missense mutations. These alleles, however, cannot be effectively up-regulated by *FRI*.

Materials and Methods

Plant Materials. *Arabidopsis* accessions were obtained from the *Arabidopsis* Biological Resource Center: Abd-0 (CS932), Bla-2 (CS6194), Cnt-1 (CS1635), Co (CS3180), Da (1)-12(CS917) Kondara (CS916), Di-G (CS910), Di-M (CS919), Ema-1 (CS1637), ENF (CS8141), En-D (CS920), En-T (CS921), Est (CS911), Gr3 (CS3179), H55 (CS923), Je54 (CS924), Li5 (CS3178), Limeport (CS8070), LIN (CS8144), Litva (CS925), M3385S (CS3111), Nd-1 (CS1636), Oy-1 (CS1643), Petergof (CS926), Santa Clara (CS8069), Shakhdara (CS929), and Wei-0 (CS3110). *FRI*-SF2 in the Columbia (Col) background, *FRI*-SF2 in the Landsberg *erecta* (L*er*) background, and *FLC*-Col in the L*er* background have been described (11). *FRI*-SF2/*FLC*-Col in the L*er* background was obtained from an F_2 population generated by crossing *FRI*-SF2 in L*er* and *FLC*-Col in L*er*. *flc*-2, *flc*-3, *FRI*-SF2/*flc*-3 (4), *fpa*-7/*flc*-3, and *ld*-1/*flc*-3 (12) are all in the Col background and have also been described. LER was kindly provided by Maarten Koornneef.

Plant Growth Conditions. All plants were grown at 22°C under long-days consisting of 16 h of cool-white fluorescent light followed by 8 h of darkness. For experiments involving vernalization, seeds plated on agar-solidified medium containing 0.65 g/liter Peters Excel 15-5-15 fertilizer (Grace Sierra, Milpitas, CA) and were kept at room temperature overnight to allow seeds to become metabolically active before being transferred to 2°C for 50 days. During cold treatment, samples were kept under short-day conditions (8 h light/16 h dark).

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Abbreviations: Col, Columbia; L*er*, Landsberg *erecta*.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY303833–AY303835).

*To whom correspondence should be addressed. E-mail: amasino@biochem.wisc.edu.

RNA-Blot Analysis. Total RNA was isolated by using RNA Isolator (Genosys Biotechnologies, The Woodlands, TX) according to the manufacturer's instructions. For RNA blots, 15 μg of RNA was separated by denaturing-formaldehyde-agarose gel electrophoresis as described (13). RNA blots were probed with a deoxyadenosine 5′-[α-^{32}P]triphosphate-labeled cDNA fragment that did not contain the conserved MADS-box domains of *FLC*. Blots were also probed with an 18S rRNA probe as a control for the quantity of RNA loaded.

***FLC* Constructs.** The full-length *FLC* clone consisted of an 8.1-kb Col genomic fragment containing 1.7 kb 5′ of the start site of translation and 0.5 kb 3′ of the translational stop in the binary vector pPZP211 (14). Promoter/coding region chimerics were created by using an *Nco*I site that occurs at the start site of translation of *FLC*. Sequences 5′ or 3′ of the *Nco*I site were PCR amplified from L*er*, Da (1)-12, and Shakhdara and exchanged with the corresponding fragment in the full-length *FLC* clone from Col. Chimeric full-length Col *FLC* clones containing the 30-bp deletion or the 1.2-kb insertion from L*er* were created by exchanging either an *Eco*RI/*Eco*RV fragment containing the 30-bp deletion or a *Afe*I/*Sph*I fragment containing the 1.2-kb insertion with the corresponding Col fragments. Constructs were verified by sequencing.

Results and Discussion

Identification of Candidate Accessions with Weak Alleles of *FLC*. The late-flowering vernalization-responsive habit of winter-annual *Arabidopsis* requires active alleles of both *FRI* and *FLC*. Recent work with naturally occurring early-flowering accessions has shown that most contain null alleles of *FRI*, suggesting that summer-annual accessions arose from winter annuals by loss of *FRI* function (3). In the laboratory, however, we have shown that loss-of-function mutations in either *FRI* or *FLC* results in an equivalent early-flowering phenotype (4). This led us to investigate whether any naturally occurring summer-annual accessions have arisen because of natural allelic variation at *FLC*. *FRI* and *FLC* function was evaluated in 27 randomly chosen accessions by crossing to early-flowering tester lines containing an SF-2 allele of *FRI* with a null allele of *flc* (*FRI/FRI;flc-3/flc-3* in the Col background) or *FLC* with a null allele of *fri* (*fri/fri;FLC/FLC*, which is wild-type Col). (The *FRI* allele from the winter-annual accession SF-2 and the *FLC* allele from Col are considered to be active alleles and are used as reference alleles in the evaluation of *FRI* and *FLC* alleles from other accessions.) Accessions containing functional *FLC* alleles will give rise to late-flowering F_1 plants when crossed to the *FRI*-containing tester; likewise, accessions containing functional *FRI* alleles will give rise to late-flowering F_1 plants when crossed to the *FLC*-containing tester.

The majority of accessions (Abd-0, Bla-2, Cnt-1, Co, Di-G, Di-M, Ema-1, ENF, En-D, En-T, Est, Gr3, H55, Je54, Li5, Limeport, LIN, Litva, M3385S, Nd-1, Oy-1, Petergof, Santa Clara, and Wei-0) gave rise to late-flowering F_1 progeny only when crossed to the *FRI*-containing tester, indicating that these accessions likely contain functional alleles of *FLC* and nonfunctional alleles of *FRI*. Kondara, Da (1)-12, and Shakhdara, however, only gave rise to late-flowering progeny in crosses to the *FLC*-containing tester (Fig. 1*A*), indicating that these lines may contain weak or nonfunctional alleles of *FLC* and functional alleles of *FRI*. (Independent work using recombinant inbred lines has also suggested that Shakhdara contains a weak allele of *FLC* and a functional allele of *FRI*; ref. 15) Consistent with these accessions containing functional alleles of *FRI*, none of the lesions previously described in *FRI* genes from naturally occurring summer-annual accessions are present in Kondara, Shakhdara (3) or Da (1)-12 (data not shown). To confirm that the late-flowering phenotype observed in crosses to the *FLC*-containing tester was caused by the restoration of *FLC* activity, Kondara, Da (1)-12 and Shakhdara were crossed to a tester line with null alleles of both *flc* and *fri* (*fri/fri;flc-3/flc-3* in Col). F_1 progeny of this cross were early flowering (Fig. 1*A*), indicating that the late flowering observed in crosses to the *FLC*-containing tester is indeed caused by the restoration of *FLC* activity in the F_1.

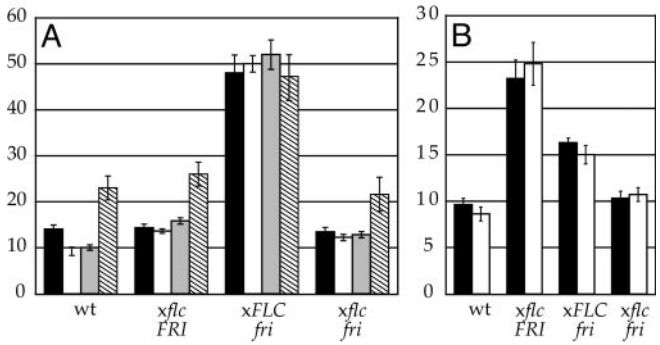

Fig. 1. Determination of *FRI* and *FLC* activity in early-flowering accessions. (*A*) Da (1)-12 (open bars), Shakhdara (gray bars), and Kondara (cross-hatched bars) were crossed to tester lines homozygous for the indicated genotypes, and the rosette leaf numbers of the resulting F_1 plants are presented. A line homozygous for *FRI* and *flc*-3 was used as a control (black bars). (*B*) L*er* (black bars) and LER (open bars) were crossed to tester lines homozygous for the indicated genotypes, and the rosette leaf numbers of the resulting F_1 plants are presented.

Among the accessions containing weaker alleles of *FLC*, Kondara formed approximately twice as many rosette leaves before flowering as Da (1)-12 and Shakhdara. One possible explanation for the later flowering of Kondara is that it contains an *FLC* allele that is intermediate in strength relative to the strong Col allele and the weak Da (1)-12 and Shakhdara alleles. Because Da (1)-12 and Shakhdara were likely to have most severely reduced *FLC* function, these accessions were chosen for further analysis. To determine whether Da (1)-12 and Shakhdara are closely related accessions, PCR was performed by using 12 simple sequence length polymorphism markers that detect polymorphisms between Col and L*er* (16). Da (1)-12 and Shakhdara showed polymorphisms for 8 of the 12 markers tested (data not shown), indicating that these two accessions are not closely related.

Restoration of *FLC* Activity Is Sufficient to Confer Winter-Annual Behavior in Da (1)-12 and Shakhdara. If the summer-annual strains Da (1)-12 and Shakhdara arose from winter-annual ancestors by an attenuation of *FLC* function, then introducing a functional allele of *FLC* into these accession should restore the winter-annual habit. To test this hypothesis, Da (1)-12 and Shakhdara were transformed with a construct containing the *FLC* genomic region from Col (4). The majority of the resulting T_1 plants were late flowering and vernalization responsive (Fig. 2). Thus, transformation with an active allele of *FLC* is able to convert Da (1)-12 and Shakhdara from summer to winter annuals.

L*er* Contains a Naturally Occurring Weak Allele of *FLC*. *FLC* was first identified in crosses of late-flowering lines containing *FRI* or mutations in the autonomous-pathway gene *LUMENIDEPENDENS* to the L*er* strain of *Arabidopsis* (11, 17). L*er* contains a weak allele of *FLC* that is able to partially suppress the late-flowering phenotype of *FRI* and autonomous-pathway mutants (18). Thus, the L*er* allele of *FLC* behaves similarly to the *FLC* alleles of Da (1)-12 and Shakhdara in that it is able to suppress the late-flowering phenotype of *FRI*. L*er*, however, was isolated from a mutagenized population of heterogeneous plants (19)

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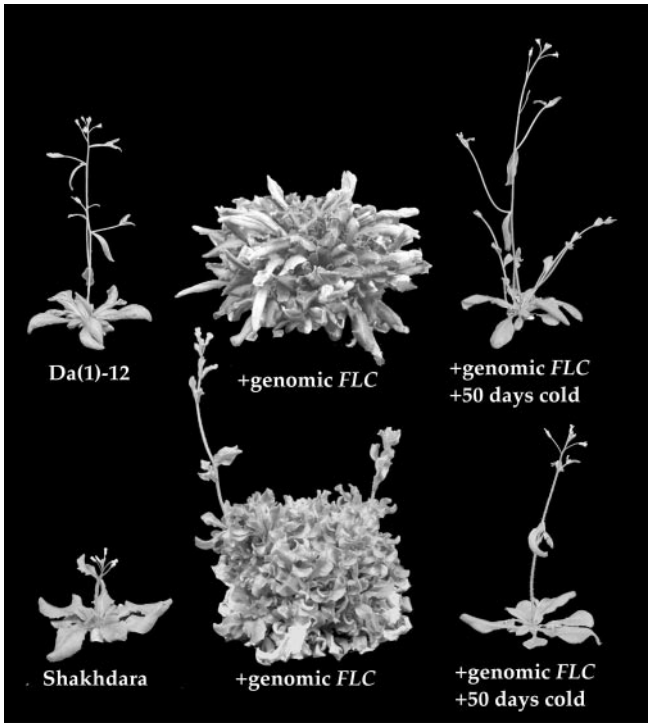

Fig. 2. Transformation with a genomic *FLC* construct is sufficient to transform Da (1)-12 and Shakhdara from summer annuals to winter annuals.

and it was therefore unclear whether the weak allele of *FLC* present in L*er* is naturally occurring or the result of mutagenesis.

To address the origin of the *FLC* allele present in L*er,* we crossed L*er* and a line thought to be the wild-type, unmutagenized parent of L*er* (LER) to the same set of tester lines used above for the analysis of Da (1)-12 and Shakhdara. If the weak allele of *FLC* found in L*er* is naturally occurring, then both lines should behave similarly in their interaction with *FRI*; however, if it is the result of mutagenesis, then the wild-type parent of L*er* should contain a stronger allele of *FLC*. In all crosses the LER parent behaved similarly to L*er* (Fig. 1*B*), demonstrating that both lines contain weak alleles of *FLC*. Thus, the weak allele of *FLC* present in L*er* is naturally occurring and not the result of mutagenesis. To confirm that LER was in fact the parent of L*er*, both lines were tested with 12 highly polymorphic sequence length polymorphism markers. If LER is the L*er* parent, the markers from both lines should be identical in size. Indeed, for all 12 markers, LER and L*er* gave identical products, indicating that these strains are closely related. This result is consistent with previous amplified fragment length polymorphism analysis performed on LER and L*er* (20).

The Da (1)-12, Shakhdara, and L*er* Alleles of *FLC* Do Not Contain Missense or Nonsense Mutations. As discussed above, summer-annual *Arabidopsis* accessions with null alleles of *FRI* have been previously described. To determine whether the weak alleles of *FLC* present in naturally occurring accessions are likewise the result of loss-of-function mutations, the *FLC* cDNA sequence was determined for Da (1)-12, Shakhdara, and L*er*. As has been previously reported, L*er* contains no missense or nonsense mutations in *FLC* (21) when compared with the Col sequence. We found that L*er* contains only a single nucleotide difference located 369 bp 3′ of the start of translation (Col:ACACCTT; L*er*:ACATCTT). The cDNA sequences from Da (1)-12 and Shakhdara were identical to Col throughout the coding region. Thus, the weak alleles of *FLC* present in Da (1)-12, Shakhdara, and L*er* are not caused by alterations in the coding regions of *FLC*.

***FLC* Alleles from Da (1)-12, Shakhdara, and L*er* Result in Lower Steady-State Levels of *FLC* RNA.** *FLC* steady-state mRNA levels are closely correlated with flowering time (4, 5). *FLC* expression is low in early-flowering summer-annual accessions and is up-regulated by *FRI* in winter annuals. Vernalization of winter-annual accessions causes in an epigenetic reduction of *FLC* levels and results in early flowering. Because the regulation of *FLC* levels plays a critical role in the regulation of flowering time, we investigated whether the weak *FLC* alleles of Da (1)-12, Shakhdara and L*er* might exhibit reduced steady-state mRNA levels.

The expression levels of the L*er* allele of *FLC* were initially compared with the alleles from Da (1)-12, Shakhdara, and Col by crossing L*er* to Da (1)-12, Shakhdara, and Col and examining *FLC* expression in the resulting F_1 plants by allele-specific RT-PCR. The coding regions of the four alleles are identical except for a single base change in L*er* that creates a unique Hpy188III site. By performing RT-PCR with primers that flank the base change and digesting the product with Hpy188III, expression of the L*er* allele can be distinguished from the Da (1)-12, Shakhdara, and Col alleles in the F_1 plants. This approach has the advantage that both *FLC* alleles are in an identical F_1 genetic background; thus, differences in expression must be caused by locus-specific effects. In the F_1 generated from a L*er*/Col cross the Col allele is expressed at much higher levels than the L*er* allele (Fig. 3*A*). In contrast, the L*er*, Da (1)-12, Shakhdara alleles are expressed to similar levels in the F_1 progeny. Thus, the strong allele of Col is expressed to higher levels than the weak alleles from L*er*, Da (1)-12, and Shakhdara.

To further investigate the relationship between *FLC* allele strength and steady-state mRNA levels, RNA blot analysis was also performed on L*er*, Da (1)-12, Shakhdara and Col, and near isogenic lines in the L*er* background (see Methods) containing either *FRI*-SF2, *FLC*-Col, or both *FRI*-SF2 and *FLC*-Col (Fig. 3*B*). As with the allele-specific RT-PCR, RNA blot analysis indicates that the weak alleles of *FLC* are expressed to lower levels than the strong Col allele in similar genetic backgrounds. As shown previously, in the near isogenic lines, the L*er* allele of *FLC* is expressed to lower levels than the Col allele both in the absence (Fig. 3*B* lanes 1 and 3) or presence of *FRI* (Fig. 3*b* lanes 4 and 5) (5, 22). Consistent with Da (1)-12 and Shakhdara having strong alleles of *FRI* and weak alleles of *FLC*, Da (1)-12 and Shakhdara show levels of *FLC* expression similar to the near isogenic line containing *FRI*-SF2 in the L*er* background (Fig. 3*B* lanes 4, 6, and 7). L*er* and the near isogenic line containing *FLC*-Col were also crossed to a line containing *FRI*-SF2 and *flc*-2 in the Col background and *FLC* expression was examined by RNA blot analysis (Fig. 3*C*). *flc*-2 is a deletion allele that completely eliminates the *FLC* gene. Thus, F_1 plants are hemizygous for either the L*er* or Col allele of *FLC*. In this case, the Col allele of *FLC* is also expressed to higher levels than the L*er* allele.

Sequences Downstream of the Start Site of Translation Are Responsible for the Weak Nature of the Ler, Da, and Shakhdara *FLC* Alleles. Because the weak nature of the L*er*, Da (1)-12, and Shakhdara alleles of *FLC* is caused by reduced expression rather than changes in the coding region of the gene, it was of interest to localize the region of the gene responsible for the relatively low level of expression. The translational start site of *FLC* is located in an *Nco*I restriction enzyme site. This *Nco*I site was used to create chimeric genomic constructs containing the Col 5′ upstream region fused to the coding regions from L*er*, Da (1)-12, and Shakhdara and, conversely, constructs containing the 5′ upstream regions from L*er*, Da (1)-12, and Shakhdara fused to the Col coding region. The constructs were used to transform a

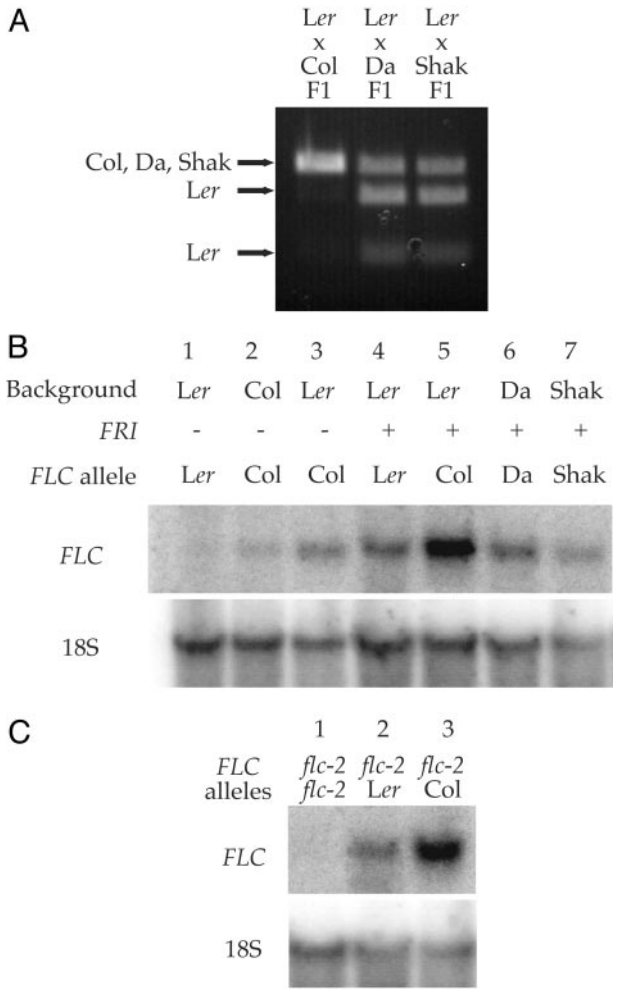

Fig. 3. Analysis of *FLC* mRNA levels. (*A*) Allele-specific RT-PCR was used to examine the relative expression levels of different *FLC* alleles in F_1 plants resulting from the indicated crosses. RT-PCR products were digested with Hpy188III, resolved on a 1.5% agarose gel, and visualized by staining with ethidium bromide. A unique Hpy188III site in the L*er* RT-PCR product allows the L*er* product to be separated from the Col, Da (1)-12, and Shakhdara products. (*B*) RNA blot analysis of *FLC* expression in L*er*, Col, Da (1)-12, and Shakhdara, and in near isogenic lines containing *FRI*, *FLC*-Col, or *FRI* and *FLC*-Col in the L*er* genetic background. (*C*) RNA blot analysis of *FLC* expression in F_1 plants resulting from crosses between a line containing *FRI* and *flc*-2 in the Col background and L*er* or a near isogenic line containing *FLC*-Col in the L*er* genetic background. The *flc*-2 allele of *FLC* is a large deletion that completely removes the *FLC* gene.

line homozygous for *FRI*-SF2 and *flc*-3 in the Col background, and the flowering time of the resulting T_1 plants was determined (Table 1). All three of the weak alleles of *FLC* behaved similarly in the chimeric constructs. Constructs containing the Col 5′ upstream region fused to the L*er*, Da (1)-12, and Shakhdara coding regions were early flowering, whereas constructs containing the 5′ upstream regions from L*er*, Da (1)-12, and Shakhdara fused to the Col coding region were late flowering. Thus, the weak nature of the L*er*, Da (1)-12, and Shakhdara *FLC* allele is caused by sequences downstream of the start site of translation.

The Weak Behavior of the L*er* allele of *FLC* Is Caused By an Insertion in the First Intron. The ability of the L*er* allele of *FLC* to suppress the late-flowering vernalization-responsive phenotype of *FRI* and autonomous-pathway mutations has been extensively characterized (11, 17, 18). Thus, it was of interest to identify the region downstream of the start codon that was responsible for the weak nature of the L*er* allele. The first intron of *FLC* was chosen as a candidate region and was sequenced from L*er* (GenBank accession AY303833). Like many MADS-box transcription factors in *Arabidopsis*, the Col allele of *FLC* has a small first exon (180 bp) and a relatively large first intron (3.5 kb). Two major differences were observed between the L*er* and Col intron I sequences (Fig. 4). First, Col contains a direct repeat of the 30-bp sequence 5′-CACAACCTTTGTATCTCGTGTCTTTT-GTCA-3′, whereas L*er* contains only a single copy of this element. Second, L*er* contains an insertion near the 3′ end of the intron, 5,236 bp from the translational start site. The 1,224-bp insertion is defined by imperfect inverted repeats and is flanked by an apparent 9-bp target site duplication. These structural features suggest that this element represents a miniature inverted repeat transposable element (MITE) (23, 24), which are nonautonomous Class II transposable elements (24, 25). Elements related to the L*er* insertion are found on all five chromosomes of the Col genome. Related elements on bacterial artificial chromosomes T32N4, MBK23, and F24H14 are >90% identical to the L*er* insertion, and ≈15 other sequences exhibit a clear degree of relatedness.

To determine whether either of these polymorphisms is responsible for the weak nature of the L*er* *FLC* allele, a Col genomic *FLC* clone was engineered to contain either the 30-bp deletion or the 1.2-kb insertion from L*er*. To assay *FLC* activity of the constructs, they were transformed into a line homozygous for *FRI* and *flc*-3 (*FRI*/*FRI*;*flc-3*/*flc-3*) and the flowering time of the resulting T_1 plants was determined (Fig. 5). The untransformed *FRI*/*FRI*;*flc-3*/*flc-3* line is early flowering because of the lack of *FLC* activity (Fig. 5*A*). Transformation with the wild-type Col genomic *FLC* clone or the chimeric clone containing the 30-bp deletion from L*er* (Fig. 5 *B* and *C*) gave rise to a similar range of late-flowering plants. Thus, the 30-bp deletion does not have a significant effect on *FLC* activity. Transformed plants containing the 1.2-kb insertion, however, were uniformly early flowering indicating that the 1.2-kb insertion is sufficient to dramatically reduce *FLC* function (Fig. 5*D*).

Because the L*er* allele of *FLC* has been shown to suppress the late-flowering phenotype of both *FRI* and autonomous-pathway

Table 1. Analysis of chimeric *FLC* constructs

Promoter	Coding	T_1 flowering time*
Col	Col	55.5 (13.2)
Col	L*er*	11.0 (0.8)
Col	Da	11.0 (1.1)
Col	Shahkdara	12.4 (1.7)
L*er*	Col	50.9 (12.2)
Shahkdara	Col	51.7 (15.8)
Da	Col	48.1 (19.8)

*Rosette leaves formed before flowering (SD).

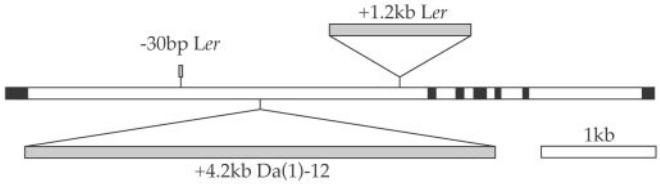

Fig. 4. Summary of the polymorphisms detected in the first intron of *FLC* in L*er* and Da (1)-12. The structure of the Col allele of *FLC* is shown with exons as black boxes and introns as open boxes. L*er* and Da (1)-12 insertions and the L*er* 30-bp deletion are shown as gray boxes.

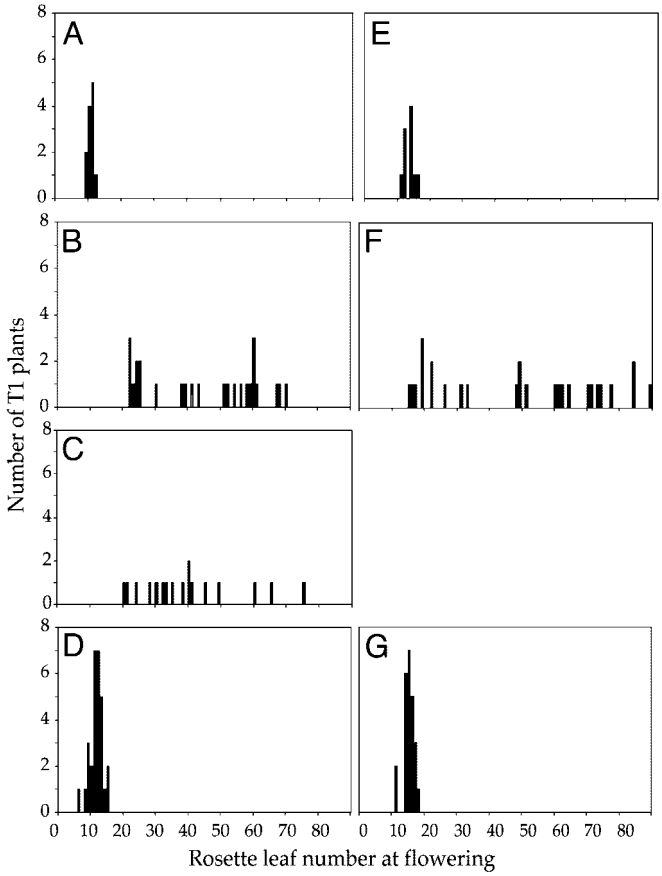

Fig. 5. Effect of L*er* polymorphisms on *FLC* activity in a *FRI*-containing or autonomous-pathway-mutant background. The number of rosette leaves formed by the primary meristem was determined for a line homozygous for *FRI* and *flc*-3 in the Col background alone (*A*) or after transformation with a wild-type Col *FLC* genomic construct (*B*), a Col *FLC* genomic construct containing the 30-bp deletion found in L*er* (*C*), or a Col *FLC* genomic construct containing the 1.2-kb insertion found in L*er* (*D*). In addition, rosette leaves formed before flowering were determined for a line homozygous for *fpa*-7 and *flc*-3 either alone (*E*) or after transformation with a wild-type Col *FLC* genomic construct (*F*) or a Col *FLC* genomic construct containing the 1.2-kb insertion found in L*er* (*G*).

mutations, we also tested the affect of the 1.2-kb insertion in the autonomous-pathway-mutant *fpa*-7. A line homozygous for *fpa*-7 and *flc*-3 was transformed with the wild-type Col genomic *FLC* clone or the chimeric clone containing the 1.2-kb insertion from L*er,* and the flowering time of the resulting T_1 plants was determined. Similar to the *FRI*/*flc*-3 line, the *fpa*-7/*fpa*-7;*flc*-3/*flc*-3 line is early flowering because of the lack of *FLC* activity (Fig. 5*E*) (12). Late flowering is restored by the wild-type Col genomic clone but not by the chimeric clone containing the 1.2-kb insertion from L*er.* Similar results were also obtained in the *ld*-1 autonomous-pathway mutant background (data not shown). Thus, the 1.2-kb insertion present in L*er* is sufficient to dramatically reduce the activity of the Col allele of *FLC* in either a *FRI*-containing line or an autonomous-pathway mutant background.

It is interesting to note that although *FRI* and autonomous-pathway mutations are modestly late flowering in the L*er* background, the chimeric Col clone containing the 1.2-kb insertion from L*er* has little effect on flowering time in the *FRI*, *fpa*, or *ld* backgrounds. Thus, the chimeric clone appears to be weaker than the L*er* allele of *FLC*. One possible explanation for this observation is that, in addition to the 1.2-kb insertion, which has a negative impact on *FLC* expression, the L*er* allele of *FLC* may also contain other sequence differences relative to Col that enhance *FLC* expression. Such polymorphisms may allow partial activity in the L*er* *FLC* allele despite the 1.2-kb insertion event.

The 1.2-kb Insertion Responsible for the Weak Nature of the Ler Allele of *FLC* Is Not Found in Da (1)-12 or Shakhdara. Because Da (1)-12 and Shakhdara contain *FLC* alleles that behave similarly to the L*er* allele and, like L*er*, the sequences that confer the weak behavior are located downstream of the translational start site, it was of interest to determine whether Da (1)-12 or Shakhdara might contain L*er*-like insertions in the first intron of *FLC*. Thus, the first intron of *FLC* from Da (1)-12 and Shakhdara was amplified, sequenced, and compared with the sequences from L*er* and Col. Neither Da (1)-12 nor Shakhdara (GenBank accession nos. AY303834 and AY303835, respectively) contains an insertion event in the region where the 1.2-kb L*er* insertion is found; in this region the Da (1)-12 and Shakhdara sequences are similar to Col. Thus, the sequences responsible for the weak behavior of the *FLC* alleles from Da (1)-12 and Shakhdara appear to be different from that of the L*er* allele. Shakhdara did not contain any large insertions or deletions; however, Da (1)-12 contains a 4.2-kb insertion in approximately the middle of the first intron of *FLC,* 4,009 bp from the translational start site (Fig. 4). The sequence of the insertion in Da (1)-12 is unrelated to the 1.2-kb insertion in L*er* and exhibits similarity to copia-type retrotransposons (26). Two similar sequences are present in the Col genome on BACs F9O13 and F8A12 that are >95% identical to the insertion in Da (1)-12. Given that the 1.2-kb insertion found in L*er* is sufficient to strongly inhibit *FLC* function, perhaps this 4.2-kb insertion has a similar attenuating effect on the Da (1)-12 allele of *FLC*.

In addition to Da (1)-12 and Shakhdara, the C24 accession of *Arabidopsis* has also been reported to contain a functional *FRI* allele and a weak or nonfunctional *FLC* allele (27). The sequence and expression of the C24 allele of *FLC* has been determined (5), and it is worth noting that, like Shakhdara, it is expressed at relatively low levels and does not contain any large insertions or deletions relative to Col (the one exception is that the C24 allele contains a 30-bp deletion in the first intron of *FLC* that is identical to that found in the L*er*). Whether C24 should be considered a naturally occurring accession, however, is unclear. Its origin is unknown and the glabrous phenotype of C24 suggests that it may have been isolated from a mutagenized population (27).

To date, four accessions that contain weak alleles of *FLC* have been identified. L*er* and Da (1)-12 contain insertion events, whereas Shakhdara and C24 do not contain any large insertions or deletions relative to the Col allele. How these polymorphisms lead to *FLC* attenuation is not known. One possibility is that certain polymorphisms lead to general defects in RNA processing. For example the large insertions in L*er* and Da (1)-12 may reduce splicing efficiency. Another possibility is that polymorphisms affect *cis*-acting regulatory elements that are required for high levels of *FLC* expression.

Evolution of Weak Alleles of *FLC*. Analysis of the intron I sequences of the L*er*, Da (1)-12, and Shakhdara alleles of *FLC* provides evidence that attenuation of *FLC* activity has occurred at least twice during evolution. Because Da (1)-12 and Shakhdara do not contain the 1.2-kb insertion responsible for the weak nature of the L*er* allele of *FLC*, they likely arose independently from the L*er* allele. The Da (1)-12 and Shakhdara alleles of *FLC* may have also arisen independently from each other. The 4.2-kb insertion present in Da (1)-12 is not present in Shakhdara; further Da (1)-12 and Shakhdara do not have any sequence polymorphisms in common in intron I. Thus, it is possible that the L*er*, Da (1)-12, and Shakhdara alleles of *FLC* have all arisen independently.

In is interesting to note that, in contrast to summer-annual

accessions that have arisen by *FRI* loss-of-function mutations, the *FLC* alleles from L*er*, Da (1)-12, and Shakhdara are not nulls; they contain no nonsense or missense codons and have detectable levels of transcription. The explanation of why the sequence changes responsible for the weak nature of these *FLC* alleles have arisen in the noncoding regions of the gene may be simply statistical; the six introns of *FLC* account for 89.5% of the genomic sequence of the gene. Thus, it is much more likely that a mutation would take place in an intron rather than an exon. Another possibility, however, is that a total loss of *FLC* activity is detrimental. *FLC* plays a central role in the regulation of flowering time in *Arabidopsis* and is positively regulated by *FRI*, negatively regulated by the autonomous floral promotion pathway, and is epigenetically down-regulated by vernalization (2). *FLC* may also have roles other than the regulation of flowering time. For example, it has been shown that allelic variation at the *FLC* locus is strongly associated with circadian period in quantitative trait locus mapping studies using recombinant inbred lines derived from a L*er*/Col cross, and that *flc* loss-of-function mutations have shorter circadian periods (28).

The work presented here, along with previous work, indicates that the vernalization requirement of winter-annual accessions of *Arabidopsis* has been lost several times during evolution by either loss-of-function mutations in *FRI* or reduction of *FLC* function. It is interesting to note, however, that among the summer-annual accessions thus far examined, L*er* is unique in that it contains both a weak allele of *FLC* and a null allele of *FRI*. Two other accessions, Dijon and Gr, contain L*er*-like lesions in *FRI* (3), but contain strong alleles of *FLC* (this work). Thus, it appears that *FRI* function was lost in a common ancestor of L*er*, Dijon, and Gr, and that *FLC* activity was subsequently attenuated in the lineage that gave rise to L*er*. Presumably, during the course of evolution, there was a pressure for earlier flowering that selected for the loss of *FRI* activity. The same evolutionary pressure may have also selected for a reduction of *FLC* activity. A near isogenic line containing the Col allele of *FLC* in the L*er* background flowers after forming approximately six more rosette leaves than L*er* (11). Thus, if L*er* originally contained a Col-like *FLC* allele, the attenuation of *FLC* activity in a *fri* background would have resulted in a significant additional acceleration of flowering time.

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