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FRIGIDA LIKE 2 Is a Functional Allele in Landsberg *erecta* and Compensates for a Nonsense Allele of *FRIGIDA LIKE 1*

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FRIGIDA LIKE 2 Is a Functional Allele in Landsberg *erecta* and Compensates for a Nonsense Allele of *FRIGIDA LIKE 1*[1][W][OA]

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Abstract

The Landsberg *erecta* (L*er*) accession of Arabidopsis (*Arabidopsis thaliana*) has a weak allele of the floral inhibitor *FLOWERING LOCUS C* (*FLC*). *FLC*-L*er* is weakly up-regulated by the active San Feliu-2 (Sf2) allele of *FRIGIDA* (*FRI*-Sf2), resulting in a moderately late-flowering phenotype. By contrast, the Columbia (Col) allele of *FLC* is strongly up-regulated by *FRI*-Sf2, resulting in a very late-flowering phenotype. In Col, the *FRI*-related gene *FRI LIKE 1* (*FRL1*) is required for *FRI*-mediated up-regulation of *FLC*. It is shown here that in L*er*, the *FRL1*-related gene *FRI LIKE 2* (*FRL2*), but not *FRL1*, is required for *FRI*-mediated up-regulation of *FLC. FRL1*-L*er* is shown to be a nonsense allele of *FRL1* due to a naturally occurring premature stop codon in the middle of the conceptual protein sequence, suggesting that *FRL1*-L*er* is nonfunctional. Compared to *FRL2*-Col, *FRL2*-L*er* has two amino acid changes in the conceptual protein sequence. Plants homozygous for *FRI*-Sf2, *FLC*-L*er*, *FRL1*-L*er*, and *FRL2*-Col have no detectable *FLC* expression, resulting in an extremely early flowering phenotype. Transformation of a genomic fragment of *FRL2*-L*er*, but not of *FRL2*-Col, into a recombinant inbred line derived from these plants restores both *FRI*-mediated up-regulation of *FLC* expression and a late-flowering phenotype, indicating that *FRL2*-L*er* is the functional allele of

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FRL2. Taken together, these results suggest that in the two different Arabidopsis accessions Col and L*er*, either *FRL1* or *FRL2*, but not both, is functional and required for *FRI*-mediated up-regulation of *FLC*.

The timing of reproductive development is an important decision during the life cycle of flowering plants. The coordination of flowering time is vital for self-incompatible plant species, because they strongly need their sexual partners to flower at the same time. Coordinate regulation of flowering time is also required for the reproductive success of self-compatible species such as Arabidopsis (*Arabidopsis thaliana*). For instance, it can determine whether a population of dormant seeds or plants in a vegetative state will overwinter, because Arabidopsis has evolved both naturally occurring, early flowering summer-annual ecotypes and naturally occurring, late-flowering winter-annual ecotypes (Laibach, 1937). A major difference between the two growth habits is that winter-annual types overwinter as vegetative seedlings or plants, because they require vernalization (exposure to a prolonged cold period during winter) to flower in the next spring or summer. By contrast, summer-annual types generally, but not exclusively, produce seeds that remain dormant during winter and germinate the next year for a summer-annual growth habit (Nordborg and Bergelson, 1999). The requirement for vernalization in natural populations of Arabidopsis is mostly controlled by the synergistic interaction of two dominant genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). *FRI* encodes a plant-specific coiled-coil domain-containing protein required for the up-regulation of *FLC*, which produces a MADS domain-containing transcription factor that acts as a strong floral repressor. Vernalization is antagonistic to *FRI* and leads to the epigenetic down-regulation of *FLC* expression; that is, levels of *FLC* transcript remain low even after removal of the cold stimulus (Michaels and Amasino, 1999; Sheldon et al., 2000; Schläppi, 2001). *FRI*-mediated up-regulation of *FLC* is reset in the next generation when progeny plants become late flowering again (Amasino, 2004).

Until recently, *FRI* and *FLC* were considered the major determinants of flowering time in natural populations of Arabidopsis. This is because most early flowering accessions were shown to have either defects in *FRI* (Johanson et al., 2000; Le Corre et al., 2002;

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Gazzani et al., 2003), weak alleles of *FLC* (Koornneef et al., 1994; Lee et al., 1994; Sanda and Amasino, 1996; Schläppi, 2001; Gazzani et al., 2003; Michaels et al., 2003) or nonfunctional *FLC* transcripts (Shindo et al., 2005; Werner et al., 2005). However, recent studies have identified late-flowering Arabidopsis accessions that do not fit this pattern. Those accessions have either high levels of *FLC* expression in the absence of a functional *FRI* allele or are late flowering without a functional *FLC* allele (Schläppi, 2001; Werner et al., 2005). This suggests that there is naturally occurring variation in flowering time genes other than *FRI* and *FLC*. Through mutagenesis experiments with summer- and winter-annual ecotypes, several classes of flowering time genes were identified that might be candidates for natural variation in *FRI*- or *FLC*-independent late flowering. Those are the six genes of the autonomous floral promotion pathway, *LUMINIDEPENDENS*, *FCA*, *FLOWERING LOCUS D*, *FPA*, *FY*, and *FVE*, that repress up-regulation of *FLC* expression in the absence of *FRI* (Boss et al., 2004); or the three *FLC* paralogs, *FLOWERING LOCUS M*/*MADS AFFECTING FLOWERING 1* (*MAF1*), *MAF2*, and *MAF3*, that together with *FLC* have an additive effect on floral repression (Y. Pan and M.R. Schläppi, unpublished data) or repress flowering when overexpressed (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001). Another class of more pleiotropic suppressors of *FRI* activity and *FLC* up-regulation includes chromatin regulators such as encoded by *ACTIN-RELATED PROTEIN 6*, *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1*, or the *VERNALIZATION INDEPENDENCE* genes (Noh and Amasino, 2003; Oh et al., 2004; Choi et al., 2005; Deal et al., 2005).

In addition to finding accessions that are late flowering in the absence of active alleles of *FRI* or *FLC*, recent mutagenesis experiments led to the identification of *FRI LIKE 1* (*FRL1*), a *FRI*-related gene that is required for the winter-annual growth habit of Arabidopsis (Michaels et al., 2004). A single *frl1* mutant suppresses *FRI*-mediated late flowering and up-regulation of *FLC* expression in the Columbia (Col) ecotype of Arabidopsis. *FRL1* is part of a gene family of six *FRI*-related genes, including *FRI LIKE 2* (*FRL2*), which has some functional redundancy with *FRL1* in the Col ecotype (Michaels et al., 2004). In this study, it is shown that there is naturally occurring variation at *FRL1* and *FRL2* between the Col and Landsberg *erecta* (L*er*) accessions of Arabidopsis. In Col, *FRL1* is functional, whereas in L*er*, *FRL2* is shown to be functional but not vice versa. This suggests

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that natural variation at these two loci could potentially modify or even suppress *FLC* up-regulation and late flowering in Arabidopsis accessions that have functional alleles at both *FRI* and *FLC*.

Results

Genetic Identification of a Modifier Gene Required for FRI-Mediated Late Flowering in the Ler Background

In previous work, the effect of *FRI* on flowering time and its interaction with *FLC* was investigated in different genetic backgrounds of Arabidopsis (Schläppi, 2001). In a series of genetic experiments, control test crosses were performed between the very late-flowering Col-*FRI*-San Feliu-2 (Sf2) line (rosette leaf no. [RLN] range 51–72) and the moderately late-flowering L*er*-*FRI*-Sf2 line (RLN range 12–28), both of which contained the active *FRI*-Sf2 allele in the Col or L*er* background, respectively (Schläppi, 2001). While F_1 plants (RLN range 46–68) were almost as late flowering as the late Col-*FRI*-Sf2 parent, about 1/16th (17/255) very early flowering F_2 plants (RLN range 3–5) were recovered from this cross. These early flowering Col/L*er*-*FRI*-Sf2 F_2 plants were considered as transgressions, because they flowered significantly earlier than the earliest L*er*-*FRI*-Sf2 parent. Test crosses and mapping analyses showed that all early flowering F_2 plants were homozygous for the active and dominant *FRI*-Sf2 gene, the weak and recessive *FLC*-L*er* gene, and an unlinked recessive Col-specific suppressor gene of *FRI*-mediated late flowering (Schläppi, 2001). The flowering time phenotype suggested that the naturally occurring dominant L*er* variant of this suppressor gene was required for *FRI*-mediated late flowering in the L*er* background. Therefore, the gene was named *ACTIVATOR OF FRI-MEDIATED LATE FLOWERING IN LER* (*AFL*).

To determine the epistatic interaction between the naturally occurring Col and L*er* alleles of *AFL*, three randomly selected early flowering Col/L*er*-*FRI*-Sf2 plants containing *AFL*-Col (lines #1, #3, and #5; RLN range 3–4) were backcrossed with *AFL*-L*er*-containing L*er*-*FRI*-Sf2 (RLN range 18–27). As shown in Figure 1A, the flowering time phenotypes of F_1 plants (RLN range 5–18) from two of the three crosses were intermediate between that of the two parental lines,

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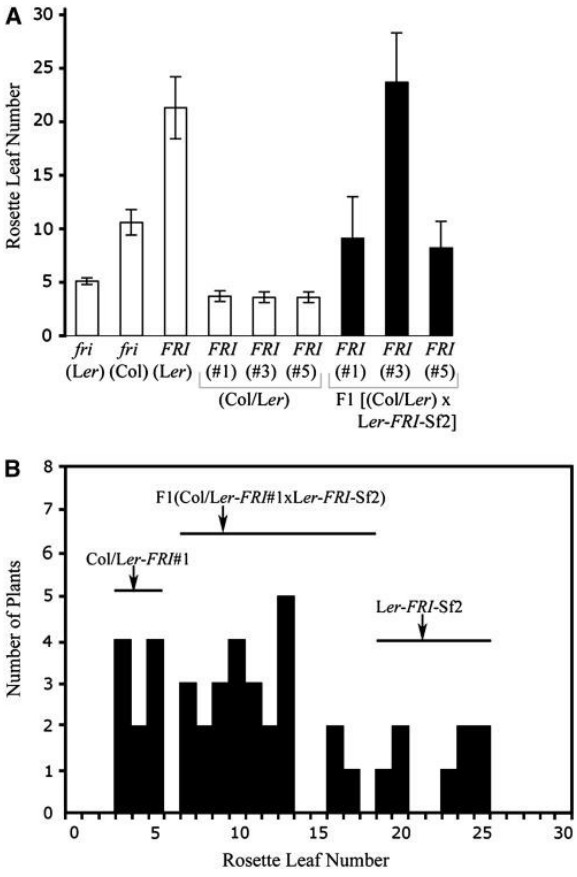
whereas F_1 plants from the third cross (RLN range 16–28) flowered as late as the L*er-FRI*-Sf2 parent. As shown in Figure 1B, F_2 plants from F_1 plants with an intermediate flowering time phenotype segregated 1:2:1 (χ^2 = 0.0697; $P > 0.9$) very early flowering (RLN range 3–5; 10 plants):intermediate late flowering (RLN range 7–13; 22 plants):late flowering (RLN range 16–25; 11 plants), suggesting that in this genetic background, *AFL*-L*er* regulated *FRI*-mediated late flowering in a dosage-dependent manner.

Figure 1

Genetic analysis of very early flowering F_2 plants Col/L*er-FRI*-Sf2#1, #3, and #5 from the cross of L*er-FRI*-Sf2 × Col-*FRI*-Sf2. Flowering time is expressed as the RLN at the time of bolting. Plants were grown in long-day photoperiods. Error bars indicate two sds. A, Flowering time of F_1 plants (black bars) from the cross of Col/L*er-FRI*-Sf2#1, #3, and #5 with late-flowering L*er-FRI*-Sf2. Flowering time of L*er* and Col is presented for comparison. B, Frequency distribution of flowering time (RLN) for an F_2 population from the cross of Col/L*er-FRI*-Sf2#1 × L*er-FRI*-Sf2. Flowering time ranges and means of parent plants and F_1 hybrids are shown by horizontal lines and arrows, respectively.



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AFL Is the Functional Ler Variant of FRL2

To identify the chromosomal position of *AFL*, bulked segregant analysis was done with the 17 very early flowering F_2 individuals and simple sequence length polymorphism (SSLP) molecular markers (Supplemental Fig. S1). This analysis suggested that *AFL* was linked to the nga248 marker on chromosome 1. Individual analysis of the 17 very early flowering plants using six molecular markers on chromosome 1 indicated that *AFL* was located about 2 cM south of *UNUSUAL FLORAL ORGANS* (*UFO*; Supplemental Table S1). While mapping of *AFL* was in progress, work on *FRI*-related genes was published, describing that *FRL1* on chromosome 5 was required for *FRI*-mediated up-regulation of *FLC* in the Col-*FRI*-Sf2 background (Michaels et al., 2004). Interestingly, the *AFL* locus mapped very closely to *At1g31814*, another *FRI*-related gene on chromosome 1. *At1g31814* single mutants had no effect on flowering time; however, *frl1 At1g31814* double mutants flowered slightly earlier than *frl1* single mutants (Michaels et al., 2004). Therefore, *At1g31814* was interpreted to be functionally redundant with *FRL1* and was named *FRL2*. Thus, the map location of *FRL2* and the absence of an *frl2* single mutant phenotype in Col raised the possibility that *FRL2*-Col was identical to the suppressor allele *AFL*-Col, and conversely, that *AFL*-L*er* was a functional allele of *FRL2*.

To test whether *AFL*-L*er* was a functional allele of *FRL2*, a molecular-genetic approach was taken. Toward this end, a partial *FRL2*-L*er* sequence was retrieved from the Monsanto L*er* single-pass shotgun sequencing database (Jander et al., 2002) and compared to *FRL2*-Col. Four nonsynonymous polymorphisms were found in the coding regions between *FRL2*-L*er* and *FRL2*-Col, one of which resulted in an *Alu*I site in the L*er* allele but not in the Col allele. As shown in Figure 2A, this *Alu*I polymorphism was a useful cleaved amplified polymorphic sequence (CAPS) marker to distinguish between the Col and L*er* alleles of *FRL2*. A prediction from the hypothesis that *AFL* is identical to *FRL2* was that the very early flowering Col/L*er*-*FRI*-Sf2 plants had the Col-specific suppressor allele of *FRL2*. Indeed, CAPS analysis of the 17 pooled very early flowering F_2 plants from the cross of Col-*FRI*-Sf2 with L*er*-*FRI*-Sf2 indicated that there was an apparent bias for *FRL2*-Col, the Col allele of *FRL2* (Fig. 2B; see result for Col/L*er*

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[#1–#17]). Lines Col/L*er*-*FRI*-Sf2#1, #3, and #5 used for backcrosses with L*er*-*FRI*-Sf2 were homozygous for *FRL2*-Col, as was early flowering F_2 progeny (plants 1, 2, and 3; Fig. 2B) from the backcross shown in Figure 1B. Conversely, late-flowering F_2 progeny (plants 40, 41, and 44; Fig. 2B) from the same backcross population was homozygous for *FRL2*-L*er*, whereas intermediate to late-flowering F_2 progeny (plants 14, 15, and 16; Fig. 2B) was heterozygous for both alleles. Individual CAPS analysis of the 17 very early flowering plants indicated that 16 out of 17 plants were homozygous for *FRL2*-Col and one plant was heterozygous. The heterozygous plant was subsequently shown to segregate early and late-flowering progeny, suggesting that it was misidentified during the initial selection (data not shown). Taken together, these results were an indication that the early flowering phenotype was associated with the Col allele of *FRL2*. To rule out that *FRL2*-Col was differentially expressed compared to *FRL2*-L*er*, semiquantitative reverse transcription (RT)-PCR was performed on RNA isolated from Col, L*er*, and *FRL2*-Col-containing, very early flowering Col/L*er*-*FRI*-Sf2 F_2 plants. As shown in Figure 2C, *FRL2* transcript levels were comparable in the L*er* and Col genetic backgrounds, indicating that *FRL2*-Col allele was not a hypomorph.

Figure 2

A, Graphic representation of the *Alu*I CAPS marker (Ala-132 site) for *FRL2* and an example of how *Alu*I cleaves the L*er*-specific PCR fragment. B, CAPS marker-based analysis of *FRL2* alleles in a pool of 17 and in three individual very early flowering Col/L*er*-*FRI*-Sf2 F_2 progeny plants from the cross of L*er*-*FRI*-Sf2 × Col-*FRI*-Sf2 (#1–#17 and #1, #3, and #5, respectively) and in randomly selected very early flowering F_2 plants (1, 2, and 3), late-flowering F_2 plants (40, 41, and 44), and intermediate to late-flowering F_2 plants (14, 15, and 16) from the cross of Col/L*er*-*FRI*-Sf2#1 × L*er*-*FRI*-Sf2. C, RT-PCR analysis of *FRL2* expression levels in Col, Col-*FRI*-Sf2, L*er*, L*er*-*FRI*-Sf2, and Col/L*er*-*FRI*-Sf2. *ACTIN2/8* (*ACT*) is shown as control for loading.

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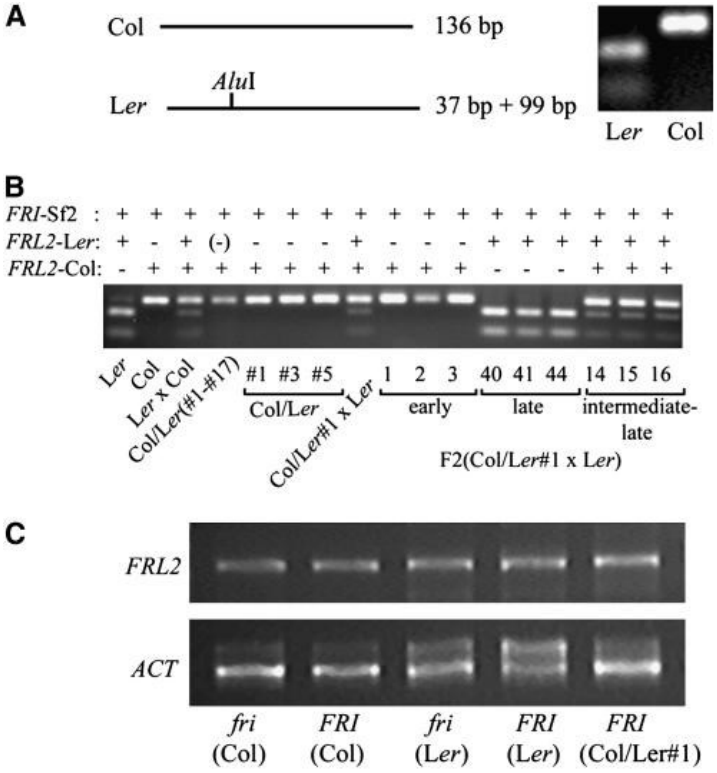

To directly show that *FRL2*-L*er* was required for *FRI*-mediated late flowering, a genomic clone of *FRL2*-L*er* was transformed into the very early flowering, recombinant inbred line (RIL) Col/L*er*-*FRI*-Sf2#1, which had been selfed and propagated from single seeds for five generations (Supplemental Table S2). To more easily identify transgenic plants that were later flowering than Col/L*er*-*FRI*-Sf2#1, but earlier flowering than L*er*-*FRI*-Sf2, the flowering time of 26 T_1 plants was analyzed under less inductive 12-h photoperiods. As shown in Figure 3A, individual T_1 plants had a range of flowering time phenotypes, from flowering as early as the Col/L*er*-*FRI*-Sf2#1 parent to as late as the L*er*-*FRI*-Sf2 control. Only six out of 26 T_1 plants flowered as early as Col/L*er*-*FRI*-Sf2#1, whereas the rest flowered later (Fig. 3A). As a control, a genomic clone of *FRL2*-Col was transformed into the same very early flowering RIL, and 14 T_1 plants were recovered. All 14 plants flowered as early as the RIL control, producing only four to five rosette leaves at the time of bolting (data not shown). Taken together, these experiments demonstrated that introduction of *FRL2*-L*er* into the very early flowering line Col/L*er*-*FRI*-Sf2#1 restored late flowering in a majority of T_1 plants.

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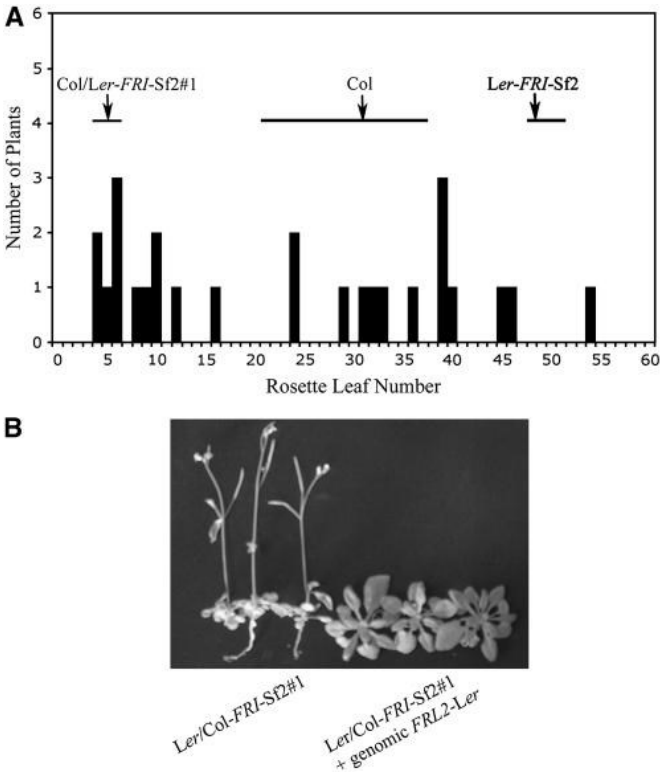
Figure 3

A, Frequency distribution of flowering time (RLN) for 26 Col/L*er*-*FRI*-Sf2#1 T_1 plants transformed with a genomic copy of *FRL2*-L*er*. Flowering time ranges and means of the untransformed parent Col/L*er*-*FRI*-Sf2#1 and of Col and L*er*-*FRI*-Sf2 control plants are shown by horizontal lines and arrows, respectively. T_1 plants and controls were grown in 12-h photoperiods. B, Late-flowering phenotype of T_2 plants from the *FRL2*-L*er*-transformed T_1 line 16 and untransformed control plants grown for 36 d in long-day photoperiods.



The late-flowering time phenotype of individual T_1 plants transformed with a genomic clone of *FRL2*-L*er* was generally maintained in T_2 progeny plants and cosegregated with the transgene. For instance, late-flowering T_1 line 8 had a single kanamycin resistance (Km^R) gene locus and thus a single *FRL2*-L*er* transgene locus. Kanamycin-selected T_2 progeny plants from the selfed T_1 parent were always late flowering, whereas unselected T_2 progeny plants segregated 3:1 late flowering:very early flowering (28 late:11 early; $\chi^2 = 0.21$; $P > 0.5$). The T_3 progeny of 10 unselected T_2 plants was subsequently analyzed for Km^R segregation, indicating that all chosen early flowering plants were kanamycin sensitive (three plants), and that the seven chosen late-flowering plants were either homozygous

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(two plants) or heterozygous (five plants) for Km^R. This suggested that the late-flowering phenotype cosegregated with the single *FRL2*-L*er* transgene locus. Moreover, as shown in Figure 3B, *FRL2*-L*er* transgene-containing T_2 progeny from T_1 line 16 that flowered late under 12-h photoperiods was also late flowering under long-day photoperiods. Conversely, T_2 progenies from T_1 plants that were early flowering remained early flowering. The T_2 flowering time distribution under long-day photoperiods of several transgenic plants is summarized in Table I. It is interesting to note that early flowering T_1 lines generally had a range of kanamycin-resistance cosuppression phenotypes in the T_2 generation, whereas selected T_2 progenies from late-flowering T_1 lines were fully kanamycin resistant.

Table I.

Flowering time analysis in long-day photoperiods

The flowering time of individual T_2 plants derived from the very early flowering RIL Col/L*er*-*FRI*-Sf2#1, transformed with a genomic copy of *FRL2*-L*er*, was determined by counting the RLN at the time of bolting. Plants were selected for Km^R (linked to the *FRL2* transgene) and grown in 16 h of light and 8 h of dark conditions. CLN, Number of cauline leaves.

Plant Lines	RLN, Mean ± SE (*n*)	CLN, Mean ± SE
Col/L*er*-*FRI*-Sf2#1 (RIL)	4.1 ± 0.3 (16)	1.2 ± 0.4
L*er* (CS20)	4.0 ± 0.0 (10)	1.0 ± 0.0
Col-0	10.3 ± 1.3 (8)	2.3 ± 0.7
L*er*-*FRI*-Sf2	36.4 ± 4.2 (9)	6.4 ± 0.9
T_2-3[a]	6.2 ± 2.1 (15)	–
T_2-11[a]	10.2 ± 2.9 (5)	–
T_2-12[a]	6.4 ± 3.2 (7)	–
T_2-8	32.1 ± 2.7 (15)	3.8 ± 0.6
T_2-14	27.7 ± 7.6 (10)	–
T_2-15	18.5 ± 5.4 (10)	–
T_2-16	22.8 ± 4.6 (10)	–
T_2-19	24.4 ± 5.4 (10)	–
T_2-22	18.3 ± 6.0 (10)	–
T_2-23	20.3 ± 6.0 (10)	–

[a] T_2 plants with a Km^R cosuppression phenotype.

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Allelic Variation at FRL2 and FRL1

When a partial genomic *FRL2*-L*er* sequence obtained from the Monsanto database (Jander et al., 2002) was compared with the standard *FRL2*-Col sequence, four nonsynonymous polymorphisms were found in the 473-amino acid sequence of FRL2. However, when the genomic *FRL2*-L*er* fragment used to transform plants was sequenced for this study, only the following two polymorphisms were confirmed: the functional L*er* allele has an Ala at position 132 (Ala-132; *Alu*I CAPS marker, Fig. 2) and a Leu at position 401 (Leu-401), whereas the nonfunctional Col allele has a Pro (Pro-132) and a Gln (Gln-401), respectively. It remains to be determined whether both Ala-132 and Leu-401 or only one of those substitutions is critical for the function of FRL2-L*er*. An alignment of the two FRL2 proteins and their polymorphisms together with FRI is shown in Figure 4.

Figure 4

CLUSTAL alignment of FRL2 and FRI. Polymorphisms in the amino acid sequence of the FRL2 proteins are indicated with the # sign. The underlined sequences above and below the alignment indicate the predicted coiled-coil domains of FRL2 and FRI, respectively. The COILS software at http://www.ch.embnet.org/software/COILS_form.html was used to predict the coiled-coil domains in FRL2 and FRI (Lupas et al., 1991).

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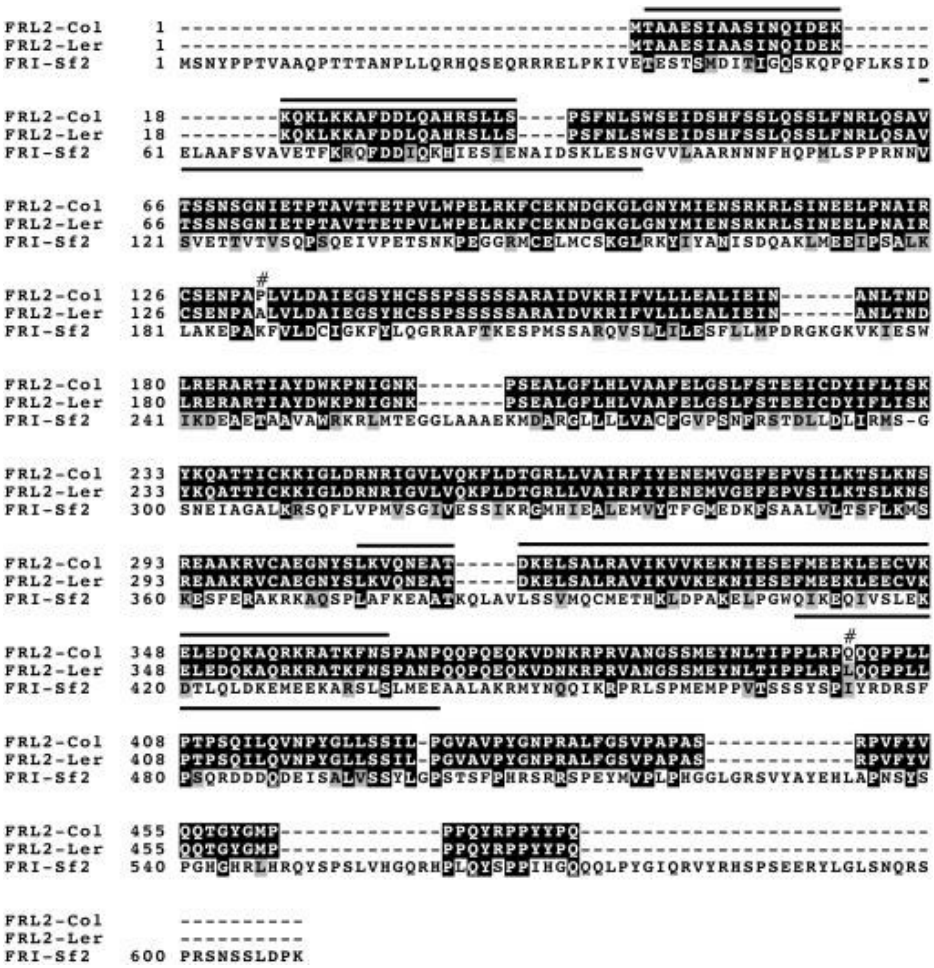

During mapping of the Col-specific suppressor gene, it appeared that besides *FLC* (near nga249), another L*er*-specific region linked to *FLC* on chromosome 5, near marker nga139, cosegregated with the very early flowering phenotype (Supplemental Table S1). Moreover, the previously reported complete absence of *FLC* expression in the RIL Col/L*er*-*FRI*-Sf2#1 (Schläppi, 2001) was similar to the phenotype of Col-*FRI*-Sf2 plants containing single *frl1* mutations (Michaels et al., 2004). This raised the possibility that the L*er* accession had a nonfunctional allele of *FRL1*. To determine whether *FRL1*-L*er* had a lesion, a partial *FRL1*-L*er* sequence from the Monsanto database (Jander et al., 2002) was compared to *FRL1*-Col. Interestingly, this comparison suggested that *FRL1*-L*er* contained a premature stop codon in the middle of the protein sequence; that is, the codon GAG [Glu-279] in *FRL1*-Col was changed to TAG [stop-279] in *FRL1*-L*er.* Cloning and sequencing of *FRL1*-L*er* confirmed this significant

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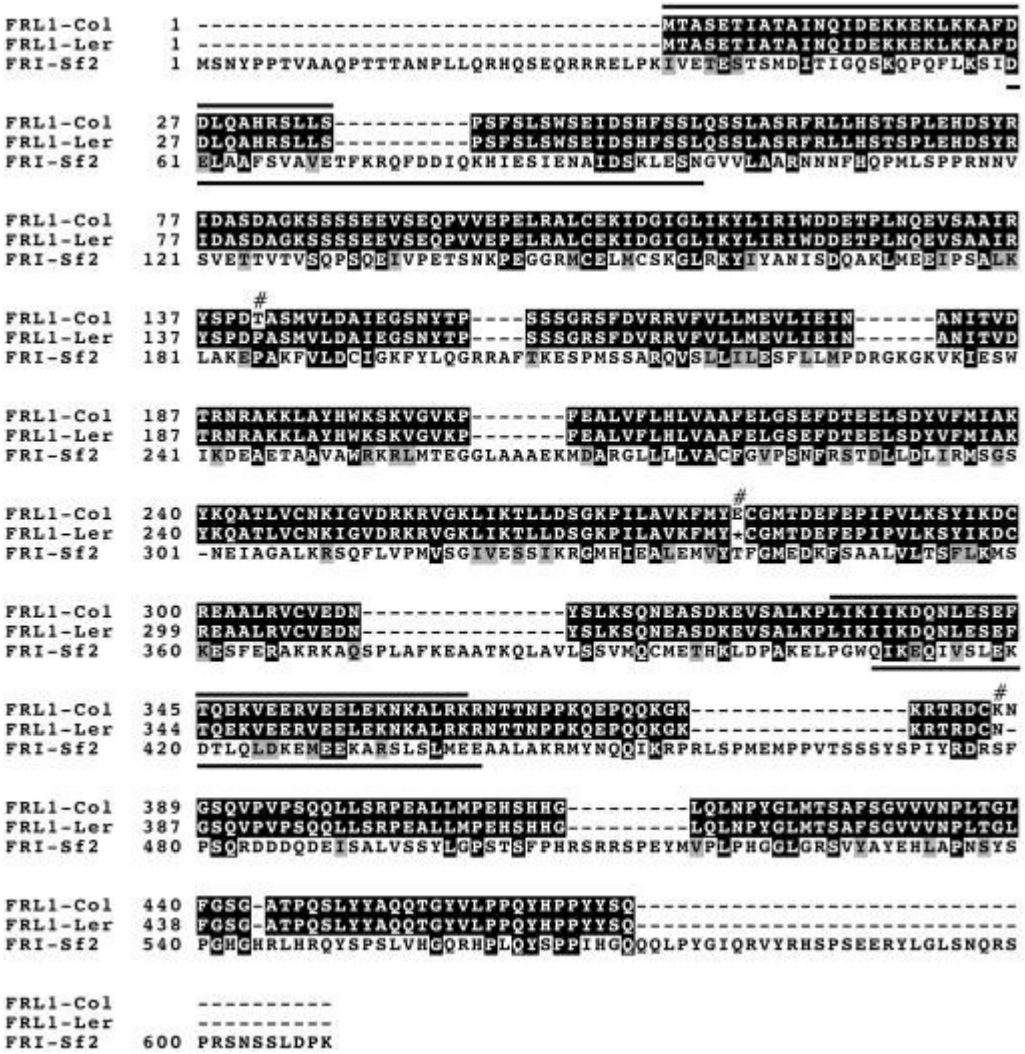
polymorphism, together with two other codon changes reported by the Monsanto sequence (a Pro instead of a Thr at position 141 and a deletion of a Lys at position 387). Whether the other two amino acid changes affect FRL1-L*er* function in the absence of the stop codon remains to be determined. An alignment of the two FRL1 proteins together with FRI is shown in Figure 5.

Figure 5

CLUSTAL alignment of FRL1 and FRI. Polymorphisms in the amino acid sequence of the FRL1 proteins are shown with the # sign. The stop codon in FRL1-L*er* is indicated by an asterisk. The underlined sequences above and below the alignment indicate the predicted coiled-coil domains of FRL1 and FRI, respectively.



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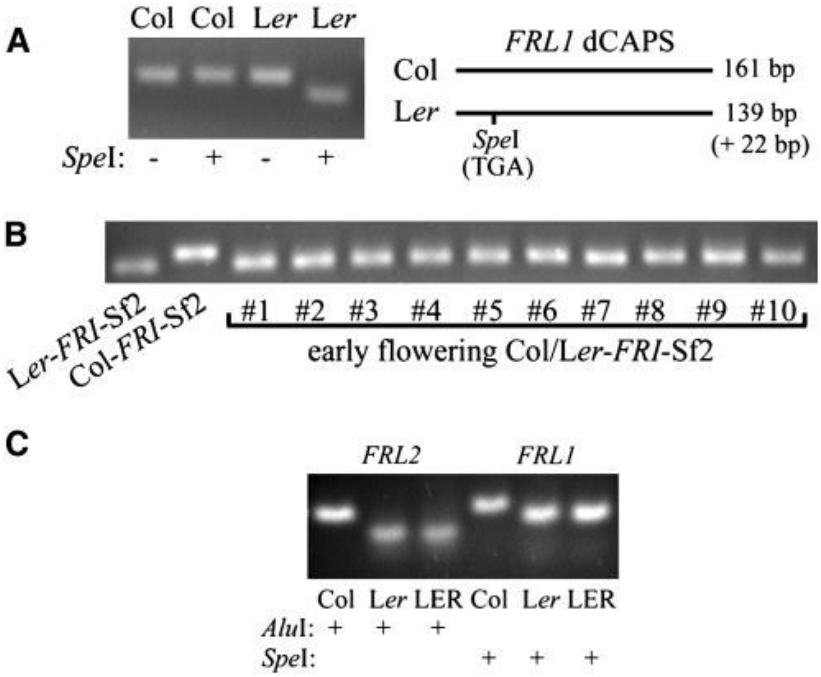
To genotype the stop codon in *FRL1*-L*er*, a derived CAPS (dCAPS) marker was generated that allows cleavage of *FRL1*-L*er*, but not of *FRL1*-Col, by the restriction enzyme *Spe*I (acTAGt recognition site). As shown in Figure 6A, *Spe*I indeed cleaved the dCAPS site in *FRL1*-L*er*, but not in *FRL1*-Col, indicating that the dCAPS marker was functional. A dCAPS marker analysis was then done to test whether early flowering Col/L*er*-*FRI*-Sf2 plants had the nonfunctional *FRL1*-L*er* allele, as predicted from their early flowering phenotypes. As shown in Figure 6B, all early flowering Col/L*er*-*FRI*-Sf2 plants assayed indeed had the *FRL1*-L*er* nonsense allele. Taken together, these data suggest that in *FRI*-containing L*er*, the functional *FRL2*-L*er* allele is the major *FRI*-related gene required for *FRI*-mediated late flowering.

Figure 6

A, Graphic representation of the *Spe*I dCAPS marker and an example of how *Spe*I cleaves the L*er*-specific PCR fragment at the internal stop codon (TGA). B, dCAPS marker-based analysis of *FRL1* alleles in very early flowering Col/L*er*-*FRI*-Sf2 plants (#1–#10; F_2 progeny from the cross of L*er*-*FRI*-Sf2 × Col-*FRI*-Sf2) and in late-flowering Col-*FRI*-Sf2 and L*er*-*FRI*-Sf2 controls. C, LER has the same polymorphisms as L*er* in *FRL1* and *FRL2*. CAPS marker-based analysis of *FRL2* alleles (*Alu*I digestion) and dCAPS marker-based analysis of *FRL1* alleles (*Spe*I digestion) in Col, L*er*, and LER accessions.



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To determine whether the nonsense allele of *FRL1*-L*er* was a result of mutagenesis in L*er*, dCAPS analysis on LER, the unmutagenized parent of L*er* (Rédei, 1962), was performed. The result indicated that *FRL1*-LER also has a premature stop codon and is thus a nonsense allele of *FRL1* (Fig. 6C). Moreover, a CAPS analysis of the LER allele of *FRL2* showed that it, too, contained the *Alu*I site observed in *FRL2*-L*er* (Fig. 6C). This suggests that the premature stop codon in *FRL1* and the *Alu*I polymorphism in *FRL2* are naturally occurring in L*er* and not the result of mutagenesis.

FRL2-Ler Promotes FRI-Mediated Activation of FLC

To determine whether introducing the genomic copy of *FRL2*-L*er* into the early flowering line Col/L*er*-*FRI*-Sf2#1 restored *FRI*-mediated up-regulation of *FLC*, as suggested by the late-flowering phenotypes of most transformants, a semiquantitative RT-PCR analysis using RNA from late-flowering T_1 plants and untransformed control plants was done. As shown in Figure 7, the level of *FLC* transcript was very low in line Col/L*er*-*FRI*-Sf2#1, comparable to L*er* controls lacking active *FRI*-Sf2. This was consistent with previous results from RNA gel-blot analyses (Schläppi, 2001). By contrast, compared to Col/L*er*-*FRI*-Sf2#1, three individual T_1 plants that were late flowering after transformation with *FRL2*-L*er* (Fig. 3; Table I) had higher levels of *FLC* transcript. This demonstrated that the late-flowering phenotype, after introducing a genomic copy of *FRL2*-L*er* into Col/L*er*-*FRI*-Sf2#1, indeed correlated with increased levels of *FLC* transcript. Taken together, the results of these experiments were in agreement with the hypothesis that *FRL2*-L*er* is a functional *FRI*-related gene and required for *FRI*-mediated up-regulation of *FLC* transcripts in L*er*.

Figure 7

Introducing a genomic copy of *FRL2*-L*er* into Col/L*er*-*FRI*-Sf2#1 restores *FRI*-mediated up-regulation of *FLC*. RT-PCR analysis of *FLC* expression in *FRL2*-L*er*-transformed T_1 lines 19, 22, and 25 and control plants. C, Col alleles of *FRL* genes; L, L*er* alleles of *FRL* genes. Functional alleles are shown in bold italics. *ACTIN2/8* (*ACT*) was included as loading control.

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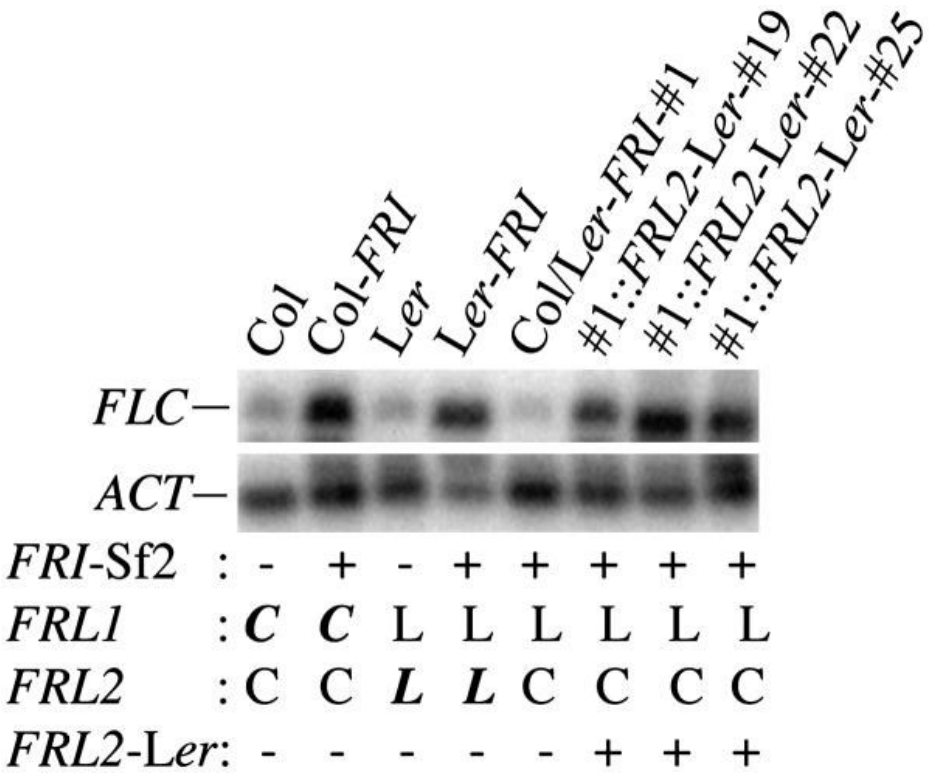

Discussion

The focus of this study was to characterize *AFL*, a gene required for *FRI*-mediated late flowering and *FLC* up-regulation in the L*er* genetic background of Arabidopsis. The activity of *AFL* was discovered, because 1/16th of F_2 plants from the cross of Col-*FRI*-Sf2 with L*er*-*FRI*-Sf2 were very early flowering, even in the presence of active *FRI*-Sf2 (Schläppi, 2001). Based on phenotype and *FLC* expression, these F_2 plants were determined to be transgressions, because they flowered significantly earlier than the earliest L*er*-*FRI*- Sf2 parent plant and had no detectable levels of *FLC* transcript (Schläppi, 2001; Fig. 7). This suggested that at least two naturally occurring recessive genes, one derived from Col and one from L*er*, recombined in those F_2 plants, which resulted both in suppression of *FRI*-mediated late flowering and *FLC* up-regulation. The L*er* component was mapped to the vicinity of *FLC* at the top of chromosome 5, and the Col component to the vicinity of *UFO* at the top of chromosome 1. This initially suggested that the L*er*-specific component was the weak *FLC*-L*er* allele and that the Col-specific suppressor of *FRI*-mediated up-regulation of *FLC*-L*er* was located near *UFO* on chromosome 1. A candidate gene for *AFL* was

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FRL2, because it was previously reported that this gene was tightly linked to *UFO* (Michaels et al., 2004). Consistent with this idea, all true-breeding, very early flowering transgressions tested were homozygous for the Col allele of *FRL2*. Therefore, a L*er*-specific genomic fragment of *FRL2* was genetically transformed into Col/L*er*-*FRI*-Sf2#1, an RIL derived from one of the very early flowering transgressions, which resulted in restoration of a late-flowering phenotype and *FLC* up-regulation in a majority of T_1 plants (Figs. 3 and and7).7). This suggested that *AFL* is identical to the L*er* allele of *FRL2* and was thus renamed *FRL2*-L*er*. By contrast, transformation of a Col-specific genomic fragment of *FRL2* did not restore late flowering in the Col/L*er*-*FRI*-Sf2#1 RIL, indicating that the L*er* allele of *FRL2* is functional and that the Col variant *FRL2*-Col is nonfunctional. This interpretation explains, at least in part, why a single *frl2* mutant does not suppress the very late-flowering phenotype of Col-*FRI*-Sf2 (Michaels et al., 2004). Taken together, these results suggest that *FRL2* is an active *FRI*-related gene in the L*er* genetic background and required for *FRI*-mediated late flowering.

The reason why *FRL2*-L*er*, but not *FRL2*-Col, is an active allele of *FRL2* is not known at the moment. It does not appear that *FRL2*-Col is expressed at lower levels than the *FRL2*-L*er* allele (Fig. 2), and it is thus more likely that either one or both of the amino acid substitutions in FRL2-Col has a negative effect on FRL2 function. It is, however, interesting to speculate that the Ala-132 to Pro-132 change has a more dramatic effect on FRL2 function than the other substitution. This is because Ala/Pro-132 is located between the two putative protein interacting coiled-coil domains of FRL2 (Lupas et al., 1991; Fig. 4). As shown in Figure 8, the closest homologs of FRL2 in Arabidopsis and other plants all have a very conserved Pro two amino acids prior to Ala/Pro-132, but none of them has an additional Pro in a Pro-X-Pro sequence as FRL2-Col does. It is, therefore, conceivable that an additional Pro at this position might change overall protein conformation of FRL2-Col and affect its ability to interact with protein partners. Because the closest FRL2 homologs have similarity to ABI3-interacting protein 2 (AIP2) in other plants (Fig. 8), it is intriguing to speculate that the proposed Pro-induced conformational change of FRL2-Col compromises its ability to interact with an ABI3-type protein in Arabidopsis. These questions can be addressed in future experiments using chimeras between the two protein sequences and

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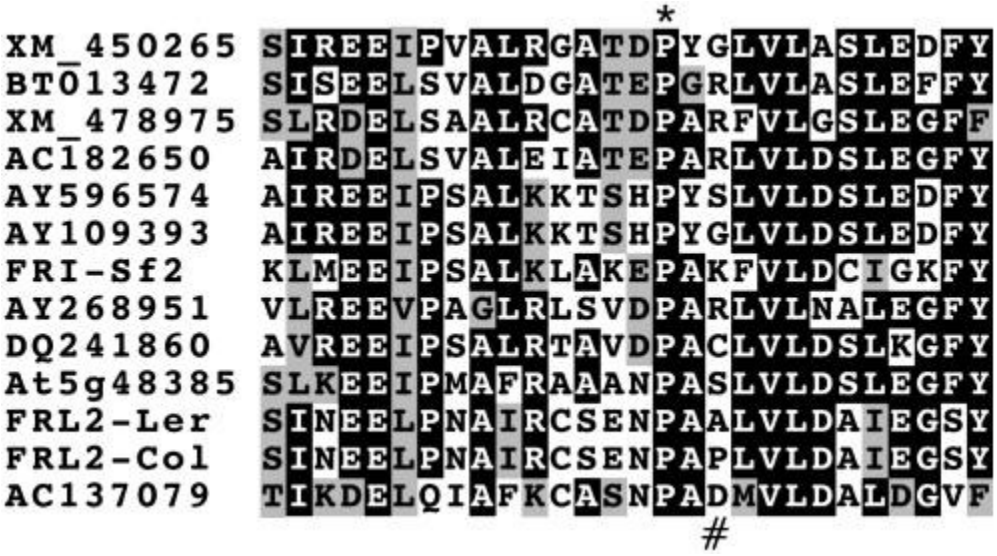
might define a region or regions critical for the function of FRI-related proteins and potentially for FRI itself.

Figure 8

CLUSTAL alignment of FRL2 alleles and related proteins. The 28-amino acid sequence surrounding the Ala/Pro-132 polymorphism of FRL2 is shown (position 115–142). The site of a conserved Pro (P) is shown by an asterisk, and the site of the Ala/Pro-132 polymorphism between FRL2-L*er* and FRL2-Col is shown by the # sign. AC137079, *Medicago truncatula* AIP2; AC182650, *Populus trichocarpa* clone Pop1-117O4; AY109393, *Zea mays* clone CL6746_2; AY268951, *Chamaecyparis nootkatensis* AIP2 (CnAIP2); AY596574, *Saccharum officinarum* clone SCCCLR1072A04; BT013472, *Lycopersicon esculentum* clone 132133F; DQ241860, *Solanum tuberosum* clone 021F10; XM_450265, *Oryza sativa* putative AIP2; and XM_478975, *Oryza sativa* putative AIP2 (CnAIP2).



Contrary to the absence of a phenotype for single *frl2* mutants in Col-*FRI*-Sf2, mutations in the *FRL2*-related gene *FRL1* have a strong effect and significantly suppress late flowering in Col-*FRI*-Sf2, indicating that *FRL1* is an active *FRI*-related gene in the Col genetic background (Michaels et al., 2004). By contrast, the premature stop codon in the middle of *FRL1*-L*er* indicates that it is a nonsense allele in the L*er* genetic background and suggests that it is nonfunctional. It is interesting to note, however, that FRL1-L*er* has a similar Pro substitution as FRL2-Col in the region between the two putative coiled-coil domains (Fig. 5), leading to a Pro-X-Pro sequence unique for FRL2-Col and FRL1-L*er*. It is possible that this polymorphism leads to a nonfunctional protein even in the absence of the premature stop codon

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further downstream. This possibility, or whether the deletion of a Lys in the C terminus has an additional effect on protein function, can be addressed in future studies using protein chimeras.

The *frl1* and *frl2* single mutant phenotypes thus suggest that active *FRL1* is the main requirement for *FRI*-mediated late flowering in the Col genetic background. However, *FRL2*-Col may have at least some partially overlapping function with *FRL1*, because it was reported that the *frl1frl2* double mutant was slightly earlier flowering than the *frl1* single mutant (Michaels et al., 2004). If *FRL2*-Col is indeed partially functional in Col, then its activity may not be strong enough for *FRI*-mediated up-regulation of the weak *FLC*-L*er* allele. Therefore, if the strong *FLC*-Col allele requires mainly *FRL1* activity to affect its *FRI*-mediated up-regulation, is it then possible that *FLC*-L*er* is so weak that it requires the cooperation of both *FRL1* and *FRL2* for its *FRI*-mediated *FLC* up-regulation? From this study, the simple answer is no, because *FRL1*-L*er* has a premature stop codon at position 279 (Figs. 5 and and6)6) and is, therefore, an apparent null allele of *FRL1*. This suggests that *FRL2*, but not *FRL1*, is necessary for *FRI*-mediated up-regulation of *FLC* transcripts and late flowering in L*er*, and, conversely, that *FRL1*, but not *FRL2*, is active in Col. It is important to note that both the L*er*-type nonsense allele of *FRL1* and the *Alu*I polymorphism of functional *FRL2*-L*er* were also found in the LER accession (Fig. 6), the unmutagenized parent of L*er* (Rédei, 1962). This indicates that the described variations at *FRL1* and *FRL2* in L*er* are naturally occurring and not the result of mutagenesis.

That *FRL1* is nonfunctional in L*er* is most likely the reason why about 1/16th of the F_2 progeny from the cross of Col-*FRI*-Sf2 with L*er*-*FRI*-Sf2 were very early flowering, because nonfunctional *FRL1*-L*er* is closely linked to weak *FLC*-L*er*. Thus, early flowering transgressions homozygous for *FRL2*-Col are probably not only produced in the presence of a weak *FLC*-L*er*, as assumed before (Schläppi, 2001), but rather because *FRL1* is linked to *FLC* on chromosome 5. The most likely scenario, therefore, is that *FRL1*-L*er* and *FRL2*-Col are the two recessive genes with complementary gene action necessary for an early flowering transgression phenotype. However, *FLC*-L*er* always cosegregated with *FRL1*-L*er* in the limited number of transgressions tested here and was thus responsible for the very early flowering phenotype of those plants. If this interpretation is correct, then slightly

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later-flowering transgressions with recombination events between *FLC*-Col and *FRL1*-L*er* should be identified when larger populations of early flowering transgressions are analyzed in future studies.

The observation that neither Col nor L*er* have fully active alleles of both *FRL1* and *FRL2* may also explain, at least in part, why the *FLC*-Col allele appeared dominant in F_1 plants from the cross of Col-*FRI*-Sf2 with L*er*-*FRI*-Sf2 but semidominant in other crosses (Lee et al., 1994; Schläppi, 2001). The reason for this dominance may be that F_1 plants from the Col × L*er* cross have active alleles of both *FRL1* and *FRL2*, which together might effect stronger up-regulation of either *FLC*-Col, *FLC*-L*er*, or possibly both, thus compensating for the weak *FLC*-L*er* copy in the F_1 hybrid. This question can be addressed in future experiments designed to determine whether *FRL1*-Col alone or a combination of *FRL1*-Col and *FRL2*-L*er* enhances *FRI*-mediated up-regulation of weak *FLC*-L*er* expression. Conversely, it can also be determined whether a combination of both active alleles produces very late-flowering Col-*FRI*-Sf2 plants that need a longer vernalization period to induce early flowering or whether both active alleles partially up-regulate *FLC* even in the absence of active *FRI*. It is also interesting to note that some F_1 plants from backcrosses between very early flowering transgressions and the late-flowering L*er*-*FRI*-Sf2 tester had flowering times between the two parents, whereas other F_1 plants were as late as L*er*-*FRI*-Sf2 (Figs. 1 and and2).2). One explanation for this observation is that *FRL2*-L*er* may be semidominant or that the nonfunctional FRL2-Col protein somehow interferes with full FRL2-L*er* activity in some crosses. This does not explain, however, why in other crosses *FRL2*-L*er* can be fully dominant (Fig. 1). An alternative explanation is that accession-specific variants of other *FRI*-related genes such as *At1g14900*, *At2g22440*, *At5g27230*, and *At5g48385* (Michaels et al., 2004) or other flowering time genes interact with *FRL2* and thus regulate its activity in a dosage-dependent manner.

In summary, this study presents an example that naturally occurring variation of flowering time genes in Arabidopsis can be uncovered in very well-studied laboratory strains such as Col and L*er*, which were previously used for quantitative trait loci mapping of flowering time loci (Alonso-Blanco et al., 1998; Koornneef et al., 1998; Alonso-Blanco and Koornneef, 2000). It is thus possible that *FRL1* and *FRL2* correspond to some previously identified quantitative trait loci

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such as *FLG* on chromosome 5 or AD.121C on chromosome 1, which were identified in crosses of L*er* to the Cape Verde Island accession of Arabidopsis (Alonso-Blanco et al., 1998). It is important to point out, however, that the large effect on flowering time of these naturally occurring suppressors of *FRI*-mediated late flowering in Col and L*er* was uncovered in this study only because the active *FRI*-Sf2 allele had been introgressed into these laboratory stains (Lee et al., 1994). From this Col × L*er* analysis it appears that there is some selection pressure to maintain an active copy of at least one *FRI*-related gene, even in the absence of an active *FRI* allele. It is thus likely that some of the previously observed, *FRI*-independent, flowering time variations (Gazzani et al., 2003; Werner et al., 2005) could be attributed to natural variation in *FRL1* and *FRL2*. This may be especially true in the case of F_2 plants from the L*er* × Col cross where earliness was linked to the recessive *ms1* allele of L*er*, which maps near *FRL1* (Koornneef et al., 1994). It is now possible to test in future studies whether the *FRL1* and *FRL2* polymorphisms identified here can be correlated with flowering time differences and the adaptation to ecological niches of the large number of available Arabidopsis accessions collected from the wild.

Materials and Methods

Plant Material

Early flowering Arabidopsis (*Arabidopsis thaliana*) accessions L*er* and wild-type LER (Michaels et al., 2003) were kindly provided by T-p. Sun (Duke University) and R. Amasino (University of Wisconsin-Madison), respectively. A Col-0 accession and lines Col-*FRI*-Sf2 and L*er*-*FRI*-Sf2 containing the dominant *FRI*-Sf2 were kindly provided by R. Amasino and were described previously (Lee et al., 1993, 1994). Col/L*er*-*FRI*-Sf2#1, #3, and #5 lines were homozygous for *FRI*-Sf2 and *FLC*-L*er* and were derived from very early flowering F_2 plants of crosses between Col-*FRI*-Sf2 and L*er*-*FRI*-Sf2. Col/L*er*-*FRI*-Sf2#1 was described previously (Schläppi, 2001). A RIL was generated with Col/L*er*-*FRI*-Sf2#1 by selfing the plant and single seed propagation for five consecutive generations (Supplemental Table S2).

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Plant Growth Conditions

Per sterile petri dish (90-mm plate), about 100 surface-sterilized seeds were grown on 0.8% agar-solidified medium (Difco) containing half-strength Murashige and Skoog (Gibco BRL) salts without Suc. Petri dishes were placed at 4°C for up to 2 d to break seed dormancy, then grown under cool fluorescent light with a 16-h-light/8-h-dark long-day photoperiod or a 12-h-light/12-h-dark short-day photoperiod with approximately 100 μmol m^{-2} s^{-1} photon flux and about 22°C day/night temperature. After 10 to 14 d, plantlets were transferred from petri dishes to soil (2:1:1 mix of peatmoss:vermiculite:perlite) into 2-inch pots (four plants/pot; 32 pots/flat) and grown under cool fluorescent light with a 16-h-light/8-h-dark long-day photoperiod, 20°C ± 1°C day/night temperature, and about 60% to 70% relative humidity. Flats were watered three times per week with 0.1 g/L 15-16-17 Peters fertilizer (Grace Sierra).

Flowering Time Analysis

Flowering time of individual plants was measured as the RLN produced by the main shoot when its floral bolt was 0.5 to 1 cm high. For some plants, the number of cauline leaves on the floral bolt was also recorded (Table I).

Isolation of Genomic DNA, Cloning, Sequencing, and Plant Transformation

Genomic fragments of *FRL2* were isolated from L*er* or Col DNA using the Ex*Taq* DNA polymerase (Takara) and primers 5′-AAGAAAAGGTACCATGTCGTCGT-3′ (*Kpn*I site underlined) and 5′-ATTGGCTTATTCGGATCCGTATG-3′ (*Bam*HI site underlined). The primers were designed to include most of the 5′ and 3′ noncoding region between *FRL2* and the neighboring genes (525-bp 5′ upstream from the start codon and 666-bp 3′ downstream from the stop codon). The 2.6-kb PCR fragment was ligated into pGEM-T (Promega). Cloned *FRL2* DNA was sequenced using the MGW sequencing service (MGW-Biotech). The *FRL2*-L*er* genomic fragment was removed as a *Kpn*I-*Bam*HI fragment from pGEM-T and ligated into the binary vector pPZP211 (Hajdukiewicz et al., 1994), cloned in *Escherichia coli*, and

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introduced into *Agrobacterium tumefaciens* strain ABI using the freeze-thaw method (Chen et al., 1994). Genomic *FRL2*-L*er* and *FRL2*-Col fragments were transformed into very early flowering line Col/L*er*-*FRI*-Sf2#1 using the floral dip method (Clough and Bent, 1998). A *FRL1*-L*er* genomic fragment was isolated from L*er* DNA using primers 5′-AGCCAAAGAAATCTTAGAGATC-3′ and 5′-TAAGATCTTATTGTCGAGATGC-3′ and sequenced using the Agencourt sequencing service (Agencourt Bioscience).

RNA Isolation and Analysis

RNA was isolated from petri dish-grown whole seedlings or leaves of adult, soil-grown plants. Plant tissue was ground to a fine powder on dry ice in a mortar and pestle with added liquid N_2. Total RNA was isolated by a modified miniprep procedure as described previously (Schläppi, 2001).

For RT-PCR analysis, first-strand cDNA synthesis was performed on 1 to 2 μg of total RNA using primer 5′-GGCCACGCGTCGACTAC$(T)_{17}$-3′ and Superscript II reverse transcriptase according to the manufacturer's instruction (Invitrogen). *FLC* was amplified using intron-spanning primers 5′-GAAATCAAGCGAATTGAGAAC-3′ and 5′-TAAGATTCTCAACAAGCTTCAAC-3′; *ACT2/8* was amplified using intro-spanning primers 5′-ATGAAGATTAAGGTCGTGGCA-3′ and 5′-TCCGAGTTTGAAGAGGCTAC-3′; *FRL2* was amplified using the gene-specific primer 5′-TACCGCCCACCGTACTATCCT-3′ and, because the gene is intronless, the 3′-RACE primer 5′-GGCCACGCGTCGACTAC-3′. After 3 min denaturing of DNA at 95°C, hot-start PCR was performed using the *Taq* DNA polymerase (Promega) and the following cycles: 25 cycles of 95°C for 15 s, 53°C for 15 s, and 72°C for 30 s for *ACT2/8*; 28 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s for *FRL2*; and 30 cycles of 95°C for 15 s, 54°C for 15 s, and 72°C for 30 s for *FLC*. PCR fragments were separated on a 1.2% agarose gel and either visualized by ethidium bromide staining (Sambrook et al.,1989) or blotted onto Protran nitrocellulose membranes (Schleicher & Schuell), hybridized with radiolabeled probes at a concentration of 1×10^6 cpm/mL as described previously (Schläppi, 2001), and visualized using a Storm PhosphorImager (Molecular Dynamics/Amersham Bioscience).

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SSLP Mapping and CAPS/dCAPS Analysis

A total of 18 SSLP markers (Bell and Ecker, 1994; Lukowitz et al., 2000) were used to map *AFL*-Col to the top of chromosome I, about 2 cM south of *UFO* (Supplemental Fig. S1; Supplemental Table S1). For CAPS analysis of *FRL2* alleles, PCR primers 5′-AGCTTCCTAATGCGATTCGAT-3′ and 5′-CAAAACGAAGATCCTCTTCAC-3′ were used to amplify a 136-bp product spanning a L*er*-specific *Alu*I site. After digestion with *Alu*I (New England Biolabs), the L*er*-specific product was cleaved into 99-bp and 37-bp fragments, while the Col-specific product remained intact. Cleavage products were separated on a 2% agarose gel and visualized by ethidium bromide staining. For dCAPS (Neff et al., 1998) analysis of *FRL1* alleles, dCAPS forward primer 5′-TTTTAGCAGTCAAATTCATGTAC-3′ (mismatch underlined) and regular reverse primer 5′-TCTTTATCAGAGGCTTCGTTC-3′ were used to amplify a 161-bp fragment. The C nucleotide at the end of the dCAPS primer introduced a *Spe*I site spanning a stop codon in *FRL1*-L*er* (ACTAGT) but not in *FRL1*-Col (ACGAGT). After digestion with *Spe*I, the L*er*-specific product was cleaved into 139-bp and 22-bp fragments, while the 161-bp Col-specific product remained intact. Cleavage products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF052677 and EF052678.

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Supplemental Data

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** SSLP mapping of *AFL*-Col.

Mappig of *AFL*-Col using 17 individual very-early-flowering F2 lines from the cross of L*er-FRI*-Sf2 x Col-*FRI*-Sf2			
Chrom. I markers	**# of Col chromosomes**	**# of Ler chromosomes**	**Recomination frequency**
nga63	25	9	35
ciw12	30	4	12.5
nga248	31	3	9
UFO	32	2	6
nga280	23	11	32
nga111	16	18	53
Mappig of the early L*er* component using 17 individual very-early-flowering F2 lines			
Chrom. V markers	**# of Col chromosomes**	**# of Ler chromosomes**	**Recomination frequency**
CTR1	3	31	9
nga249	1	33	3
nga139	3	31	9

-

- **Supplemental Table S1.** Recombination frequencies.

Characterization of near isogenic (F5 generation) recombinant inbred line Col/L*er*-*FRI*-Sf2#1:		
Chromosome	**Marker**	**Genotype (C = Columbia; L = Ler)**
I	nga63	C
I	ciw12	C
I	nga248	C
I	*UFO*	C
I	*FRL2*	C
I	nga280	C
I	nga111	C
II	ciw2	L
II	ciw3	L
II	*ER*	C
II	nga163	C
III	nga172	C
III	nga162	L
III	nga6	C
IV	nga8	C
IV	nga1107	L
V	CTR1	L
V	nga249	L
V	*FRL1*	L
V	ciw9	C
V	ciw10	C

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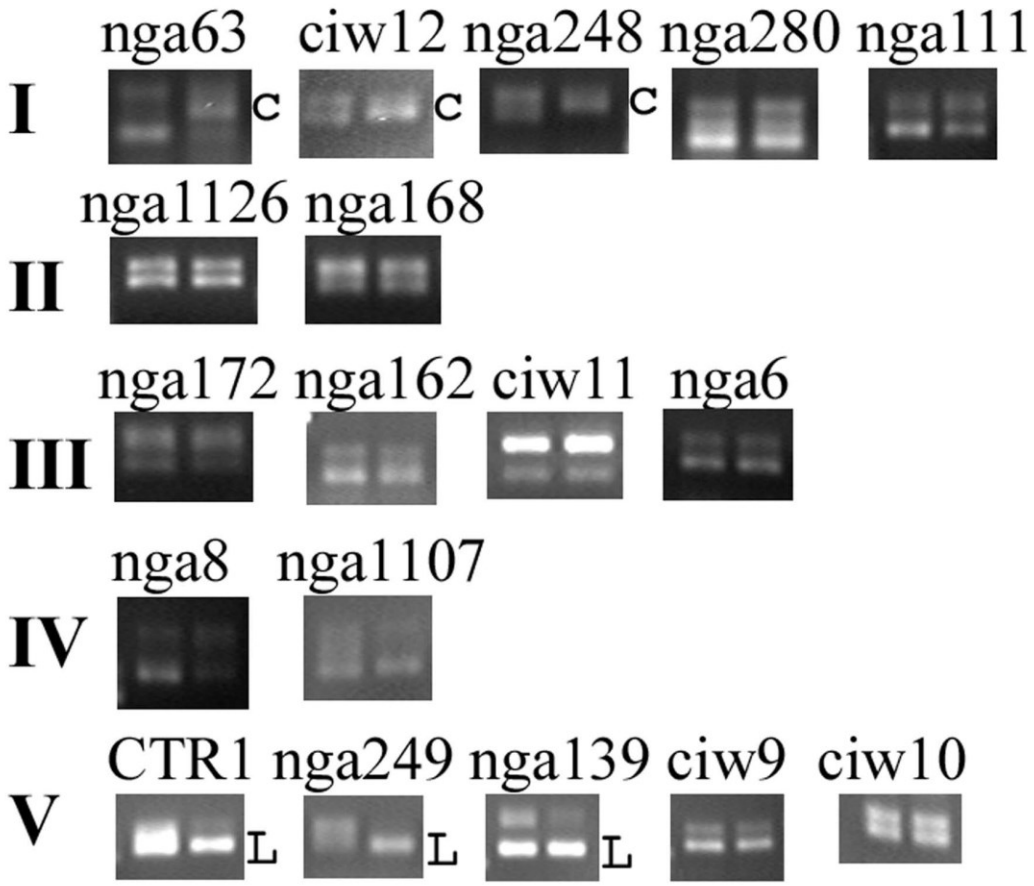
- **Supplemental Table S2.** RIL characterization.



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Notes

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