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

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The Landsberg erecta (Ler) accession of Arabidopsis (Arabidopsis thaliana) has a weak allele of the floral inhibitor FLOWERING LOCUS C (FLC). FLC-Ler 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 Ler, the FRL1-related gene FRI LIKE 2 (FRL2), but not FRL1, is required for FRI-mediated up-regulation of FLC. FRL1-Ler 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-Ler is nonfunctional. Compared to FRL2-Col, FRL2-Ler has two amino acid changes in the conceptual protein sequence. Plants homozygous for FRI-Sf2, FLC-Ler, FRL1-Ler, and FRL2-Col have no detectable FLC expression, resulting in an extremely early flowering phenotype. Transformation of a genomic fragment of FRL2-Ler, 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-Ler is the functional allele of FRL2. Taken together, these results suggest that in the two different Arabidopsis accessions Col and Ler, 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 plants 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; Johnson 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 (Johnson et al., 2000; Le Corre et al., 2002; 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 (Schlöppi, 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, LUMINDEPENDENS, 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. Schlöppi, 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 (Ler) accessions of Arabidopsis. In Col, FRL1 is functional, whereas in Ler, FRL2 is shown to be functional but not vice versa. This suggests 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 (Schlöppi, 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 Ler-FRI-Sf2 line (RLN range 12–28), both of which contained the active FRI-Sf2 allele in the Col or Ler background, respectively (Schlöppi, 2001). While F2 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 F2 plants (RLN range 3–5) were recovered from this cross. These early flowering Col/Ler-FRI-Sf2 F2 plants were considered as transgressions, because they flowered significantly earlier than the earliest Ler-FRI-Sf2 parent. Test crosses and mapping analyses showed that all early flowering F2 plants were homozygous for the active and dominant FRI-Sf2 gene, the weak and recessive FLC-Ler gene, and an unlinked recessive Col-specific suppressor gene of FRI-mediated late flowering (Schlöppi, 2001). The flowering time phenotype suggested that the naturally occurring dominant Ler variant of this suppressor gene was required for FRI-mediated late flowering in the Ler 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 Ler alleles of AFL, three randomly selected early flowering Col/Ler-FRI-Sf2 plants containing AFL-Col (lines #1, #3, and #5; RLN range 3–4) were backcrossed with AFL-Ler-containing Ler-FRI-Sf2 (RLN range 18–27). As shown in Figure 1A, the flowering time phenotypes of F1 plants (RLN range 5–18) from two of the three crosses were intermediate between that of the two parental lines, whereas F1 plants from the third cross (RLN range 16–28) flowered as late as the Ler-FRI-Sf2 parent. As shown in Figure 1B, F2 plants from F1 plants with an intermediate flowering F2 individual and simple sequence length polymorphism (SSLP) molecular markers (Supplemental Fig. S1). This analysis suggested that in this genetic background, AFL-Ler regulated FRI-mediated late flowering in a dosage-dependent manner.

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 F2 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-Ler was a functional allele of FRL2.

To test whether AFL-Ler was a functional allele of FRL2, a molecular-genetic approach was taken. Toward this end, a partial FRL2-Ler sequence was retrieved from the Monsanto Ler 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-Ler and FRL2-Col, one of which resulted in an AluI site in the Ler allele but not in the Col allele. As shown in Figure 2A, this AluI polymorphism was a useful cleaved amplified polymorphic sequence (CAPS) marker to distinguish between the Col and Ler alleles of FRL2. A prediction from the hypothesis that AFL is identical to FRL2 was that the very early flowering Col/Ler-FRI-Sf2 plants had the Col-specific suppressor allele of FRL2. Indeed, CAPS analysis of the 17 pooled very early flowering F2 plants from the cross of Col-FRI-Sf2 with Ler-FRI-Sf2 indicated that there was an apparent bias for FRL2-Col, the Col allele of FRL2 (Fig. 2B; see result for Col/Ler [#1–#17]). Lines Col/Ler-FRI-Sf2#1, #3, and #5 used for backcrosses with Ler-FRI-Sf2 were homozygous for FRL2-Col, as was early flowering F2 progeny (plants 1, 2, and 3; Fig. 2B) from the backcross shown in Figure 1B. Conversely, late-flowering F2 progeny (plants 40, 41, and 44; Fig. 2B) from the same backcross population was homozygous for FRL2-Ler, whereas intermediate to late-flowering F2 progeny (plants 14, 15, and 16; Fig. 2B).

Figure 1. Genetic analysis of very early flowering F2 plants Col/Ler-FRI-Sf2#1, #3, and #5 from the cross of Ler-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 S.Ds. A, Flowering time of F1 plants (black bars) from the cross of Col/Ler-FRI-Sf2#1, #3, and #5 with late-flowering Ler-FRI-Sf2. Flowering time of Ler and Col is presented for comparison. B, Frequency distribution of flowering time (RLN) for an F2 population from the cross of Col/Ler-FRI-Sf2#1 × Ler-FRI-Sf2. Flowering time ranges and means of parent plants and F1 hybrids are shown by horizontal lines and arrows, respectively.

Figure 2. A, Graphic representation of the AluI CAPS marker (Ala-132 site) for FRL2 and an example of how AluI cleaves the Ler-specific PCR fragment. B, CAPS marker-based analysis of FRL2 alleles in a pool of 17 plants and in three individual very early flowering Col/Ler-FRI-Sf2 F2 progeny from the cross of Ler-FRI-Sf2 × Col-FRI-Sf2 (#1–#17 and #1, #3, and #5, respectively) and in randomly selected very early flowering F2 progeny (plants 1, 2, 3, 40, 41, and 44) from the cross of Col/Ler-FRI-Sf2#1 × Ler-FRI-Sf2. C, RT-PCR analysis of FRL2 expression levels in Col, Col-FRI-Sf2, Ler, Ler-FRI-Sf2, and Col/Ler-FRI-Sf2. ACTIN2/8 (ACT) is shown as control for loading.
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-Ler, semiquantitative reverse transcription (RT)-PCR was performed on RNA isolated from Col, Ler, and FRL2-Col-containing, very early flowering Col/Ler-FRI-Sf2 F2 plants. As shown in Figure 2C, FRL2 transcript levels were comparable in the Ler and Col genetic backgrounds, indicating that FRL2-Col allele was not a hypomorph.

To directly show that FRL2-Ler was required for FRI-mediated late flowering, a genomic clone of FRL2-Ler was transformed into the very early flowering, recombinant inbred line (RIL) Col/Ler-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/Ler-FRI-Sf2#1, but earlier flowering than Ler-FRI-Sf2, the flowering time of 26 T1 plants was analyzed under less inductive 12-h photoperiods. As shown in Figure 3A, individual T1 plants had a range of flowering time phenotypes, from flowering as early as the Col/Ler-FRI-Sf2#1 parent to as late as the Ler-FRI-Sf2 control. Only six out of 26 T1 plants flowered as early as Col/Ler-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 T1 plants were recovered. All 14 plants flowered as early as the RIL control, producing only two plants or heterozygous (five plants) for KmR. This suggested that the late-flowering phenotype cosegregated with the single FRL2-Col transgene locus.

Moreover, as shown in Figure 3B, FRL2-Ler transgene-containing T2 progeny from T1 line 16 that flowered late under 12-h photoperiods was also late flowering under long-day photoperiods. Conversely, T2 progenies from T1 plants that were early flowering remained early flowering. The T2 flowering time distribution under long-day photoperiods of several transgenic plants is summarized in Table I. It is interesting to note that early flowering T2 lines generally had a range of kanamycin-resistance cosuppression phenotypes in the T2 generation, whereas selected T2 progenies from late-flowering T1 lines were fully kanamycin resistant.

Allelic Variation at FRL2 and FRL1

When a partial genomic FRL2-Ler 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-Ler fragment used to transform plants was sequenced for this study, only the following two polymorphisms were confirmed: the functional Ler allele has an Ala at position 132 (Ala-132;
To genotype the stop codon in FRL1-Ler, a derived CAPS (dCAPS) marker was generated that allows cleavage of FRL1-Ler, but not of FRL1-Col, by the restriction enzyme SpeI (acTAGt recognition site). As shown in Figure 6A, SpeI indeed cleaved the dCAPS site in FRL1-Ler, 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/Ler-FRI-Sf2 plants had the nonfunctional FRL1-Ler allele, as predicted from their early flowering phenotypes. As shown in Figure 6B, all early flowering Col/Ler-FRI-Sf2 plants assayed indeed had the FRL1-Ler nonsense allele. Taken together, these data suggest that in FRI-containing Ler, the functional FRL2-Ler allele is the major FRI-related gene required for FRI-mediated late flowering.

To determine whether the nonsense allele of FRL1-Ler was a result of mutagenesis in Ler, dCAPS analysis on Ler, the unmutagenized parent of Ler (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 AluI site observed in FRL2-Ler (Fig. 6C). This suggests that the premature stop codon in FRL1 and the AluI polymorphism in FRL2 are naturally occurring in Ler and not the result of mutagenesis.

**FRL2-Ler Promotes FRI-Mediated Activation of FLC**

To determine whether introducing the genomic copy of FRL2-Ler into the early flowering line Col/Ler-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 T1 plants and untransformed control plants was done. As shown in Figure 7, the level of FLC transcript was very low in line Col/Ler-FRI-Sf2#1, comparable to Ler controls lacking active FRI-Sf2. This was consistent with previous results from RNA gel-blot analyses (Schläppi, 2001). By contrast, compared to Col/Ler-FRI-Sf2#1, three individual T1 plants that were late flowering after transformation with FRL2-Ler (Fig. 3; Table I) had higher levels of FLC transcript. This demonstrated that the late-flowering phenotype, after introducing a genomic copy of FRL2-Ler into Col/Ler-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-Ler is a functional FRI-related gene and required for FRI-mediated up-regulation of FLC transcripts in Ler.

**DISCUSSION**

The focus of this study was to characterize AFL, a gene required for FRI-mediated late flowering and FLC up-regulation in the Ler genetic background of Arabidopsis. The activity of AFL was discovered, because 1/16th of F2 plants from the cross of Col-FRI-Sf2 with
Ler-FRI-Sf2 were very early flowering, even in the presence of active FRI-Sf2 (Schläppi, 2001). Based on phenotype and FLC expression, these F₂ plants were determined to be transgressions, because they flowered significantly earlier than the earliest Ler-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 Ler, recombined in those F₂ plants, which resulted both in suppression of FRI-mediated late flowering and FLC up-regulation.

The Ler 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 Ler-specific component was the weak FLC-Ler allele and that the Col-specific suppressor of FRI-mediated up-regulation of FLC-Ler was located near UFO on chromosome 1. A candidate gene for AFL was 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 Ler-specific genomic fragment of FRL2 was genetically transformed into Col/Ler-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 T₁ plants (Figs. 3 and 7). This suggested that AFL is identical to the Ler allele of FRL2 and was thus renamed FRL2-Ler. By contrast, transformation of a Col-specific genomic fragment of FRL2 did not restore late flowering in the Col/Ler-FRI-Sf2#1 RIL, indicating that the Ler allele of FRL2 is functional and that the Col

### Table: CLUSTAL alignment of FRL2 and FRI

| FRL2-Col | 1 | TAAAAIEASIAINQIDER | 1 |
|----------|---|---------------------|---|
| FRL2-Ler | 1 | TAAAAIEASIAINQIDER | 1 |
| FRI-Sf2  | 1 | MNPYyPTVAATTNRPPR | 1 |
| FRL2-Col | 10 | QAKAKDPDLLQARKSLC | 10 |
| FRL2-Ler | 10 | QAKAKDPDLLQARKSLC | 10 |
| FRI-Sf2  | 10 | QAKAKDPDLLQARKSLC | 10 |
| FRL2-Col | 61 | ELSLAVAETRPQDPDCDKIESIAEIDKLESNGKEAAANRFHPPLSPPPNNW | 61 |
| FRL2-Ler | 61 | ELSLAVAETRPQDPDCDKIESIAEIDKLESNGKEAAANRFHPPLSPPPNNW | 61 |
| FRI-Sf2  | 61 | ELSLAVAETRPQDPDCDKIESIAEIDKLESNGKEAAANRFHPPLSPPPNNW | 61 |

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).
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 Ler genetic background and required for FRI-mediated late flowering.

The reason why FRL2-Ler, 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-Ler 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 might define a region or regions critical for the function of FRI-related proteins and potentially for FRI itself.

Figure 5. CLUSTAL alignment of FRL1 and FRL. Polymorphisms in the amino acid sequence of the FRL1 proteins are shown with the # sign. The stop codon in FRL1-Ler is indicated by an asterisk. The underlined sequences above and below the alignment indicate the predicted coiled-coil domains of FRL1 and FRL, respectively.
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-Ler indicates that it is a nonsense allele in the Ler genetic background and suggests that it is nonfunctional. It is interesting to note, however, that FRL1-Ler 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-Ler. It is possible that this polymorphism leads to a nonfunctional protein even in the absence of the premature stop codon 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-Ler 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-Ler 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-Ler has a premature stop codon at position 279 (Figs. 5 and 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 Ler, and, conversely, that FRL1, but not FRL2, is active in Col. It is important to note that both the Ler-type nonsense allele of FRL1 and the AluI polymorphism of functional FRL2-Ler were also found in the Ler accession (Fig. 6), the unmutagenized parent of Ler (Rédei, 1962). This indicates that the described variations at FRL1 and FRL2 in Ler are naturally occurring and not the result of mutagenesis.
That FRL1 is nonfunctional in Ler is most likely the reason why about 1/16th of the F2 progeny from the cross of Col-FRI-Sf2 with Ler-FRI-Sf2 were very early flowering, because nonfunctional FRL1-Ler is closely linked to weak FLC-Ler. Thus, early flowering transgressions homozygous for FRL2-Col are probably not only produced in the presence of a weak FLC-Ler, as assumed before (Schläppi, 2001), but rather because FRL1 is linked to FLC on chromosome 5. The most likely scenario, therefore, is that FRL1-Ler and FRL2-Col are the two recessive genes with complementary gene action necessary for an early flowering transgression phenotype. However, FLC-Ler always cosegregated with FRL1-Ler 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 later-flowering transgressions with recombination events between FLC-Col and FRL1-Ler should be identified when larger populations of early flowering transgressions are analyzed in future studies.

The observation that neither Col nor Ler have fully active alleles of both FRL1 and FRL2 may also explain, at least in part, why the FLC-Col allele appeared dominant in F1 plants from the cross of Col-FRI-SF2 with Ler-FRI-Sf2 but semidominant in other crosses (Lee et al., 1994; Schläppi, 2001). The reason for this dominance may be that F1 plants from the Col × Ler cross have active alleles of both FRL1 and FRL2, which together might effect stronger up-regulation of either FLC-Col, FLC-Ler, or possibly both, thus compensating for the weak FLC-Ler copy in the F1 hybrid. This question can be addressed in future experiments designed to determine whether FRL1-Col alone or a combination of FRL1-Col and FRL2-Ler enhances FRI-mediated up-regulation of weak FLC-Ler 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 F1 plants from backcrosses between very early flowering transgressions and the late-flowering Ler-FRI-SF2 tester had flowering times between the two parents, whereas other F1 plants were as late as Ler-FRI-Sf2 (Figs. 1 and 2). One explanation for this observation is that FRL2-Ler may be semidominant or that the nonfunctional FRL2-Col protein somehow interferes with full FRL2-Ler activity in some crosses. This does not explain, however, why in other crosses FRL2-Ler 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 Ler, 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 such as FLG on chromosome 5 or AD.121C on chromosome 1, which were identified in crosses of Ler 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 Ler 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 × Ler 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 F2 plants from the Ler × Col cross where earliness was linked to the recessive ms1 allele of Ler, 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 Ler 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 Ler-FRI-Sf2 containing the dominant FRI-Sf2 were kindly provided by R. Amasino and were described previously (Lee et al., 1993, 1994). Col/Ler-FRI-Sf2#1, #3, and #5 lines were homozygous for FRI-Sf2 and FLC-Ler and were derived from very early flowering F2 plants of crosses between Col-FRI-Sf2 and Ler-FRI-Sf2. Col/Ler-FRI-Sf2#1 was described previously (Schläppi, 2001). A RIL was generated with Col/Ler-FRI-Sf2#1 by selfing the plant and single seed propagation for five consecutive generations (Supplemental Table S2).

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 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 mix of peat moss/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 RN P 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 Ler or Col DNA using the ExTag DNA polymerase (Takara) and primers 5'-'AAGAAAGCTG- 
CATCTGCCTGCT-3' (KpnI site underlined) and 5'-'ATGGGCTTTATGC- 
CATCCGTTATG-3' (BarnHI 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). Modified FRL2 DNA was sequenced using the M13 sequencing service (MGW-Biotech). The FRL2-Ler genomic fragment was removed as a KpnI-BamHI fragment from pGEM-T and ligated into the binary vector pZIP211 (Hajdukiewicz et al., 1994), cloned in Agrobacterium tumefaciens strain AGL10011 (Hajdukiewicz et al., 1994), and introduced into the genetic background (up to flowering line Col/Ler) of Arabidopsis thaliana by Agrobacterium-mediated transformation (Agrobacterium tumefaciens strain AGL10011, a donor of the FRL2-Ler genomic fragment was isolated from Ler DNA using primers 5'-'ACGCCAAAGAATCTCTAGAGATC-3' and 5'-'TAAAGCTTTATGCAGAGATC-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 N2. 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'-'GGCCACCGTGCTACACTGTG-3' and Superscript II reverse transcriptase according to the manufacturer's instructions. FLC was amplified using intron-spanning primers 5'-'AGCTAAACGAAGATCCTCTAGAGATC-3' and 5'-'TAAAGCTTTATGCAGAGAT-3'; and 5'-'TCACTGTCCTGAGAGATC-3'. ACT2 was amplified using the generic-specific primer 5'-'TACCGCCCACCGTATGAT-3' and Superscript II reverse transcriptase according to the manufacturer's instructions. Intron II. FLC was amplified using intron-spanning primers 5'-'AGCAGTGGATGAGATCAGAGATC-3' and 5'-'TCCAGTGGATGAGATCAGAGATC-3'; and 5'-'TCACTGTCCTGAGAGATC-3'. FRL2 was amplified using the generic-specific primer 5'-'TACCGCCCACCGTATGAT-3' and Superscript II reverse transcriptase according to the manufacturer's instructions.

SSLP Mapping and CAPS/dCAPS Analysis

A total of 18 SSLP markers (Bell and Eckel, 1994; Lukowicz et al., 2000) were used to map AFL-Col to the top of chromosome I, about 2 CM south of LFO (Supplemental Fig. 1; Supplemental Table S1). For CAPS analysis of FRL2 alleles, primers 5'-'AGCTAAACGAAGATCCTCTAGAGATC-3' and 5'-'ACGCCAAAGAATCTCTAGAGATC-3' were used to amplify a 136-bp product spanning a Lr-specific Alul site. After digestion with Alul (New England Biolabs), the Lr-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 analysis (Neef et al., 1998) analysis of FRL1 alleles, dCAPS forward primer 5'-'GCTCTGCAGCAGCTATGAGATC-3' (mismatch underlined) and regular reverse primer 5'-'TCTTATGAGCTTCTGTC-3' were used to amplify a 161-bp fragment. The C nucleotide at the end of the dCAPS primer introduced a SpeI site spanning a stop codon in FRL1-Ler (ACTAGT) but not in FRL1-Col (ACGAGT). After digestion with SpeI, the Lr-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.

Supplemental Data

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

Supplemental Figure S1. SSLP mapping of AFL-Col.

Supplemental Table S1. Recombination frequencies.

Supplemental Table S2. RIL characterization.

ACKNOWLEDGMENTS

I thank R. Amasino and S. Michaels for encouragement and insightful discussions and Y. Pan for expert advice and help with the RT-PCR experiments.

Received June 30, 2006; accepted October 12, 2006; published October 20, 2006.

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Plant Physiol. Vol. 142, 2006

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