RNA Levels and Activity of *FLOWERING LOCUS C* are Modified in Mixed Genetic Backgrounds of *Arabidopsis Thaliana*

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RNA LEVELS AND ACTIVITY OF FLOWERING LOCUS C ARE MODIFIED IN MIXED GENETIC BACKGROUNDS OF ARABIDOPSIS THALIANA

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Flowering time and FLOWERING LOCUS C (FLC) RNA levels were analyzed in different accessions of Arabidopsis thaliana and in mixed genetic backgrounds resulting from crosses between accessions. Dominant alleles of FRIGIDA (FRI) promote accumulation of FLC RNA, which in turn promotes late flowering. Although the coding regions of sequenced FLC alleles are identical, some accessions have genetically weak alleles that do not promote late flowering in the presence of FRI. In this study, a new weak allele of FLC with open reading frame identity to previously sequenced alleles was isolated from a Niederzenz (Nd) accession. The FLC-Nd allele accumulated less RNA in the presence of FRI than did the strong Columbia (Col) allele. The weak FLC-Nd allele was semidominant in the mixed Nd/Col genetic background containing FRI, and a linear correlation between the level of FLC RNA and lateness of flowering was observed. However, late-flowering transgressions with elevated levels of FLC RNA in the absence of FRI were also obtained from crosses between early-flowering accessions Col and Nd. Moreover, compared to Nd, the weak Landsberg erecta (Ler) allele of FLC was recessive and not semidominant in the mixed Ler/Col genetic background. However, very early-flowering transgressions lacking detectable FLC RNA were also obtained from crosses between FRI containing Col and Ler. The results indicate that modifier genes other than FRI influence the level and genetic activity of FLC RNA in different genetic backgrounds resulting from crosses between naturally occurring accessions of A. thaliana.

Keywords: flowering time, FLC, FRI, MADS domain, Niederzenz.

Introduction

The genetic characterization and recent molecular cloning of the two interacting flowering-time genes FRIGIDA (FRI) and FLOWERING LOCUS C (FLC) demonstrate that naturally occurring allelic variation between different accessions of Arabidopsis thaliana is a valuable resource for the analysis of genetic traits (Burn et al. 1993; Lee et al. 1993; Clarke and Dean 1994; Michaels and Amasino 1999; Sheldon et al. 1999; Alonso-Blanco and Koornneef 2000; Johanson et al. 2000; Reeves and Coupland 2000). FRI is responsible for most major flowering-time differences between accessions collected from the wild (Sanda et al. 1997). Functional alleles of FRI are dominantly late flowering and are considered to be wild type compared with mutant fri alleles, which are recessive and early flowering (Lee et al. 1993). This genetic interpretation was confirmed with the recent cloning of FRI, demonstrating that recessive alleles leading to early flowering are loss-of-function alleles (Simpson et al. 1999; Johanson et al. 2000).

As with FRI, allelic variation among different accessions of Arabidopsis has been described for the MADS box-containing gene FLC (Koornneef et al. 1994; Lee et al. 1994; Sanda and Amasino 1995). Classified by how they interact with FRI, most accessions, such as Columbia (Col), have strong or late-flowering alleles, whereas the Landsberg erecta (Ler) and C24 accessions contain weak or early-flowering alleles. Because the predicted protein sequences of strong and weak alleles analyzed thus far are identical, naturally occurring weak alleles of FLC are not loss-of-function alleles (Sheldon et al. 1999, 2000; this study). Strong FLC-Col and weak FLC-Ler alleles are semidominant in the presence of FRI when analyzed in the Ler genetic background (Lee et al. 1994).

FRIGIDA has complementary gene action with FLC to repress early flowering in Arabidopsis, most likely by activating a process resulting in an increased abundance of FLC RNA. Plants with induced loss-of-function alleles of FLC flower very early even in the presence of FRI, and conversely, increasing the copy number of strong FLC alleles in the presence of FRI or overexpressing its RNA levels in plants lacking FRI promotes late to very late flowering (Michaels and Amasino 1999; Sheldon et al. 1999, 2000). A consensus is thus emerging that Arabidopsis plants flower late in an FLC RNA dosage-dependent manner. These observations were best described by a rheostat model, stating that plants flower later with increased levels of FLC RNA (Michaels and Amasino 1999). It therefore appears that the MADS box gene FLC directly represses the transition from vegetative to reproductive development and that the principal role of FRI is to promote accumulation of FLC RNA (Michaels and Amasino 1999; Sheldon et al. 1999; Simpson et al. 1999). Repression of flowering by FLC is relieved when plants are vernalized, which results in a quantitative reduction of the abundance of FLC RNA and, in agreement with the rheostat model, in a proportional reduction in

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floral development (Michaels and Amasino 1999; Sheldon et al. 1999, 2000; Wilko and Schlappi 2000).

In this study, the identification of a new naturally occurring weak allele of FLC from an Nd accession is reported. The weak FLC-Nd allele was analyzed in different genetic backgrounds to correlate flowering time with the abundance of FLC RNA in the presence and absence of FRI. The strong FLC-Col and the weak FLC-Ler alleles were also analyzed in combination with the FLC-Nd allele and in different genetic backgrounds. Genetic evidence is discussed that indicates that modifier genes from different accessions influence both the level and biological activity of FLC RNA.

Material and Methods

Plant Material

Early-flowering Arabidopsis thaliana accession Landsberg erecta (Ler) (Laibach 1951) was kindly provided by T.-P. Sun (Duke University). Niederzenz (Nd) line 380-1-1 was kindly provided by D. Smith and N. Fedoroff (Pennsylvania State University) and was described previously as Nossen line 380-1-1 (Smith et al. 1996). A Columbia (Col) accession and lines Col-FRI-Sf2 and Ler-FRI-Sf2 containing the dominant San Felice-2 (Sf2) allele of FRIGIDA (FRI) introgressed into the Col and Ler backgrounds, respectively, were kindly provided by E. Himelblau and R. Amasino (University of Wisconsin—Madison) and were described previously (Lee et al. 1993, 1994). Late-flowering line Col/Nd-FRI-Sf2#13 was derived from crosses between Col-FRI-Sf2 and Nd line B22 containing a FRI-Sf2-linked T-DNA locus conferring kanamycin resistance (Osborne et al. 1995; described previously as Nossen line B22). Col/Nd-FRI-Sf2#13 had a mixed Col/Nd genetic background but was homozygous for FRI-Sf2, the B22 T-DNA locus, and FLC-Col and was kindly provided by E. Himelblau. Col/Nd-FRI-Sf2#114 was derived from an early-flowering F1 plant of a cross of Nd line 380-1-1 to Col/Nd-FRI-Sf2#13 and had a mixed Col/Nd background but was homozygous for FRI-Sf2, the B22 T-DNA locus, and FLC-Nd.

Growth Conditions

Per sterile petri dish (90-mm plate), ca. 100 surface-sterilized seeds were grown in vitro on 0.8% agar-solidified medium in a 90-mm dish and grown under cool fluorescent light for 12 d in long-day photoperiods and at a temperature of ca. 23°C. Seedlings from one dish were pooled and 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 (Yeh et al. 1990). Briefly, 100–500 mg of crushed tissue was resuspended in 1 mL of extraction buffer (7.5 M guanidine hydrochloride, 25 mM sodium citrate, 0.5% [w/v] sodium lauryl sarcosine, and 0.1 M 2-mercaptoethanol [all reagents from Sigma, St. Louis]) and spun for 10 min at maximum speed in an Eppendorf centrifuge. The supernatant was extracted three times with an equal volume of phenol : chloroform : isooamyl alcohol (24 : 24 : 1). Total RNA was precipitated at −20°C in an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (Sigma). The pellet was washed in 75% (v/v) cold ethanol, vacuum dried, and resuspended in 30–50 mL diethyl pyrocarbonate (DEPC)-treated water. Total RNA was quantified spectrophotometrically and stored in 25-μl aliquots in ethanol at −70°C.

RNA Gel Blot Analysis

Ca. 25 μg of total RNA was separated by electrophoresis in 1.2% formaldehyde gels containing ethidium bromide (Sambrook et al. 1989). RNA was transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, N.H.) and cross-linked for 2 h in a vacuum oven. Prehybridization and hybridization were performed at 65°C in 5 × Denhardt’s, 6 × SSC, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/mL denatured salmon sperm DNA. An Nd-FLC-specific cDNA fragment lacking most of the conserved MADS box domain was removed from pGEM-T with SalI and was labeled to a specific activity of ca. 0.5–1 × 106 cpm/μg DNA with [32P]dATP by the random primer method using the MegaPrime labeling kit, as recommended by the supplier (Amersham Pharmacia, Piscataway, N.J.), purified by G-50 spin columns, heat denatured, and hybridized at a concentration of 1 × 104 cpm/mL to nitrocellulose membranes in a Hybaid oven (Labnet International, Edison, N.J.) at 65°C for at least 16 h. Membranes were washed in 2 × SSC for 5 min at room temperature and then in 0.1 × SSC/0.2% SDS for at least 30 min at 65°C. Membranes were briefly rinsed in 2 × SSC before autoradiography. The relative intensities of hybridizing bands were analyzed by computing densitometry using the AMBIS Image Acquisition & Analysis system (San Diego, Calif.). RNA blots were sequentially probed or simultaneously probed with the
actin gene ACT8 (An et al. 1996) to normalize the relative intensities of FLC mRNA in each lane.

**SSLP Mapping and Reverse Transcription–Polymerase Chain Reaction**

FLC-Nd cDNA was isolated from line Col/Nd-FRI-Sf2#114 by reverse transcription–polymerase chain reaction (RT-PCR) with Superscript II (Gibco BRL, Grand Island, N.Y.) using primer 5’-GGCCACGCGTCGACTAC(T)17-3’ and Taq polymerase (Promega, Madison, Wis.) using primers 5’-GAATA GCAAGCGAATTGAGAAC-3’ and 5’-GGCCACGCGTCGAC TAC-3’. Reverse transcription–polymerase chain reaction products were cloned into pGEM-T (Promega), sequenced, and analyzed by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

The accession origin of FLC alleles in Col/Nd-FRI-Sf2#13 and Col/Nd-FRI-Sf2#114 was determined by simple sequence length polymorphism (SSLP) mapping using Tag polymerase and nga249 primers 5’-TACC GCAATTCTACGC 3’ and 5’-GGAT CCAAATCTGGGGAAT-3’. Reverse transcription–polymerase chain reaction products were cloned into pGEM-T (Promega), sequenced, and analyzed by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

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Results

**Some FRI-Containing Recombinant Columbia/Nd Lines of Arabidopsis thaliana Are Early Flowering**

The effect of the late-flowering gene FRI on flowering time and its interaction with FLC was analyzed in different genetic backgrounds of *A. thaliana*. To follow FRI more easily with a closely linked selectable marker, crosses were performed to recombine T-DNA locus B22 (Osborne et al. 1995), which contained a dominant kanamycin resistance gene, with a dominant allele of FRI. B22 and FRI were estimated to be 5–10 cM apart. Flowering times and genetic backgrounds of previously described lines and accessions and newly derived lines are shown in table 1. Col-FRI-Sf2, which contained the dominant Sf2 allele of FRI in the Col background (Lee et al. 1993), was crossed with pollen from the early-flowering Nd line B22 (Osborne et al. 1995). Pollen from F1 plants was then used for crosses with recessive fri-containing Col plants (Johanson et al. 2000), and kanamycin-resistant recombinants that were also late flowering were selected. Only ca. 6% of the kanamycin-resistant plants were also late flowering, confirming the estimated close linkage between FRI-Sf2 and B22. Selected plants were thus recombinants, by coupling, of dominant FRI-Sf2 with the dominant kanamycin-resistant locus B22 and were of a mixed Col/Nd genetic background. Plant Col/Nd-FRI-Sf2#13 was chosen for further studies because it had 100% late-flowering and kanamycin-resistant progeny and was, therefore, presumed to be homozygous for both FRI-Sf2 and B22.

To further introgress FRI-Sf2 into the Nd background, Col/Nd-FRI-Sf2#13 was backcrossed with Nd line 380-1-1. As shown in figure 1, the resulting F1 plants had a similar flowering-time distribution as the late Col/Nd-FRI-Sf2#13 parent, indicating that FRI-Sf2 was dominant in a genetic background containing more than 50% of the Nd genome. The same result was obtained for the F1 generation when Col/Nd-FRI-Sf2#13 was backcrossed with Col (fig. 1). However, F2 generations from the two backcrosses had significantly different flowering-time distributions. The backcross generation with Col clearly segregated 3 : 1 late- to early-flowering F2 plants (*P* > 0.5; fig. 1), which is the expected ratio for dominant epistasis of FRI-Sf2 (from Col/Nd-FRI-Sf2#13) over recessive fri-Col (from Col). By contrast, the F2 population from the backcross of Col/Nd-FRI-Sf2#13 with Nd showed a statistically significant (*P* < 0.05; fig. 1) segregation of 9 : 7 late- to early-flowering plants. This indicated epistasis with comple-
Fig. 1 A Niederzenz (Nd) accession of Arabidopsis has a semidominant modifier of FRIGIDA-Sf2 (FRI-Sf2). Shown are flowering-time distributions of F₁ and F₂ generations from crosses between Col/Nd-FRI-Sf2#13 and Columbia (Col) or Nd and between Col and Col/Nd-FRI-Sf2#114. Plants were grown in long-day photoperiods and at 20°C. Both Col/Nd-FRI-Sf2#13 and Col/Nd-FRI-Sf2#114 contained the functional FRI-Sf2 allele in a mixed Col/Nd genetic background. Frequency distribution of rosette leaf number represents the number of vegetative leaves produced at the time of flowering. Filled circles indicate the flowering-time means of parental, F₁, or reference lines, and the horizontal bars represent the range of rosette leaf number distributions. Statistical probability values P for the expected segregation ratio of two flowering-time loci with complementary gene action (9 : 7 late-flowering to seven early-flowering plants) or for a single flowering-time locus (3 : 1 late-flowering to early-flowering plant) are given. Arrows indicate the separation between early- and late-flowering plants.

The Nd Accession Contains a Weak Allele of FLC

Early- and late-flowering segregating F₁ plants presumed to contain FRI-Sf2 were selected from backcrosses of Col/Nd-FRI-Sf2#13 with Nd (fig. 1) to test the hypothesis that the Nd accession of Arabidopsis had a weak allele of FLC. F₁ plants were test crossed to Col with the premise that 50% (heterozygous FRI-Sf2) or 100% (homozygous FRI-Sf2) of the resulting F₂ plants were late flowering (Lee et al. 1994). This was indeed observed for four different F₁ plants such as Col/Nd-FRI-Sf2#114 (fig. 1). That is, 100% of the F₁ plants from the cross of Col with Col/Nd-FRI-Sf2#114 were later flowering than the early Col/Nd-FRI-Sf2#114 parent. Moreover, the F₁ generation had flowering-time means between Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13, indicating that the modifier of FRI-Sf2 in line Col/Nd-FRI-Sf2#114 was semidominant in this background.
The F₂ population from this cross segregated 9 : 7 late- to early-flowering plants (P > 0.1; fig. 1), indicating epistasis with complementary gene action between FRI-Sf2 and at least one modifier gene such as FLC. That many of the late-flowering plants were as late as Col/Nd-FRI-Sf2#114 indicated that Col/Nd-FRI-Sf2#114 indeed contained dominant FRI-Sf2 alleles.

To determine genetically whether Col/Nd-FRI-Sf2#114 had a weak allele of FLC, complementation test crosses were carried out with two Ler lines containing the well-characterized weak FLC-Ler allele (Koornneef et al. 1994; Lee et al. 1994). Ler flowered very early because it had both recessive fri-Ler and weak FLC-Ler alleles, whereas Ler-FRI-Sf2 had dominant FRI-Sf2 but weak FLC-Ler alleles and flowered slightly later than Col/Nd-FRI-Sf2#114 (table 1). No late-flowering plants were observed in F₁ generations of reciprocal crosses between Col/Nd-FRI-Sf2#114 and Ler, indicating that Col/Nd-FRI-Sf2#114 did not complement the weak FLC-Ler allele (data not shown). As shown in figure 2, a similar result was obtained when FRI-Sf2 was homozygous in the cross of Col/Nd-FRI-Sf2#114 to Ler-FRI-Sf2. In both the F₁ and the F₂ generations, flowering-time means were only slightly later than that of the Ler-FRI-Sf2 parent, again indicating lack of complementation. This indicated that as Ler, Col/Nd-FRI-Sf2#114 contained a weak or early allele of FLC, which was directly tested in backcrosses of Col/Nd-FRI-Sf2#114 with Nd. Flowering-time distributions of F₁ and F₂ plants were similar to those seen for crosses between Ler-FRI-Sf2 and Col/Nd-FRI-Sf2#114, and no significantly late-flowering plants were observed (fig. 2). This was in agreement with the conclusion that Nd had a weak FLC-Nd allele.

The accession origin of FLC in Col/Nd-FRI-Sf2#114, which was early flowering, was determined by PCR-based SSLP microsatellite analysis (Bell and Ecker 1994) using the tightly FLC-linked marker nga249 on chromosome 5 (Lee et al. 1994) and was compared to its parent Col/Nd-FRI-Sf2#13, which was late flowering. As shown in figure 3A, this analysis indicated that Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13 were homoygous for the FLC-Nd and FLC-Col alleles, respectively. Early-flowering F₂ plants lacking FRI-Sf2 that were homozygous for either FLC-Col (Col/Nd-FLC-Col) or FLC-Nd (Col/Nd-FLC-Nd) (fig. 3A) were then selected from the cross of Col with Col/Nd-FRI-Sf2#114 and used in backcrosses with Col/Nd-FRI-Sf2#114. As shown in figure 3B, F₁ plants from the cross of Col/Nd-FLC-Nd to Col/Nd-FRI-Sf2#114 flowered as early as the Col/Nd-FRI-Sf2#114 parent, and none of the F₂ plants flowered later than the latest F₁ plant. This showed that the mixed Col/Nd background containing FRI-Sf2 and homoygous FLC-Nd alleles remained early flowering. By contrast, F₁ plants from the cross of Col/Nd-FLC-Col to Col/Nd-FRI-Sf2#114 were semidominantly late, with flowering-time means between Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13. The F₂ generation segregated 9 : 7 late- to early-flowering plants (P > 0.5; fig. 3B), demonstrating the expected epistasis with complementary gene action between segregating FRI-Sf2 and weak FLC-Nd and strong FLC-Col alleles. Similar results were obtained in three independent experiments. Taken together, the genetic complementation experiments indicated that FLC-Nd interacted weakly with FRI-Sf2 in line Col/Nd-FRI-Sf2#114.

To determine whether other homozygous Nd regions correlated with the early-flowering phenotype of line Col/Nd-FRI-Sf2#114, SSLP mapping of all five chromosomes was done for Col/Nd-FRI-Sf2#114, Col/Nd-FRI-Sf2#13, and the two F₂ plants used for the above complementation experiments. This analysis indicated that only the top of chromosome 5 (fig. 3A) between markers ATHCTR1 and nga249 (Bell and Ecker 1994) contributed to the flowering-time differences between lines Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13. The whole region was Nd-derived in Col/Nd-FRI-Sf2#114 and Col/Nd-FLC-Nd but was Col-derived in Col/Nd-FRI-Sf2#13 and Col/Nd-FLC-Col.

**Correlation between Levels of FLC RNA and Flowering Time in Different Genetic Backgrounds**

To determine whether the open reading frame (ORF) of FLC-Nd was different from that of other alleles, FLC cDNA was cloned from Col/Nd-FRI-Sf2#114 by RT-PCR, sequenced, and compared to the published nucleotide sequence (Sheldon et al. 1999). This showed that the ORF of FLC-Nd was identical to FLC-Col, FLC-C24, and FLC-Ler (Sheldon et al. 1999, 2000). FLC-Nd was then used as a molecular probe to determine the abundance of FLC steady state mRNA in Col/Nd-FRI-Sf2#114 and several earlier- or later-flowering lines and accessions (table 1).

As shown in figure 4, the level of FLC RNA was always higher in unvernalized plants that contained FRI-Sf2. Ninety-two-day vernalized plants had no detectable amount of FLC RNA. However, the abundance of FLC RNA in Col/Nd-FRI-Sf2#114 was reduced two- to fourfold when compared to late-flowering Col/Nd-FRI-Sf2#13 or Col-FRI-Sf2. F₁ plants from the cross of Col/Nd-FRI-Sf2#114 to Col had FLC RNA levels and flowering-time means between those of Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13. FLC-Nd in Col/Nd-FRI-Sf2#114 was thus a hypomorph compared to FLC-Col in Col/Nd-FRI-Sf2#13, even though both lines had the same FRI-Sf2 alleles. The reduced level of FLC RNA was not because of mutations in its coding region nor was it attributable to detectable DNA insertions over a 6-kb region spanning the FLC gene (not shown). The reduction in FLC RNA expression from FLC-Nd allele was probably caused at the level of transcription or RNA processing.

The relative amount of FLC RNA was normalized with levels of constitutively expressed ACTIN-8 RNA (ACT8; An et al. 1996) in different preparations of total RNA (fig. 4A, 4B). This showed that Col/Nd-FRI-Sf2#13 and Col-FRI-Sf2 had the largest amounts of FLC RNA, whereas Col/Nd-FRI-Sf2#114 and Ler-FRI-Sf2 had only 18%–49% of the highest levels (fig. 4C). Although Col/Nd-FRI-Sf2#114 had higher levels of FLC RNA, it flowered slightly earlier than Ler-FRI-Sf2. Similarly, although both accessions had the same flowering time, Nd had consistently four- to fivefold higher levels of FLC RNA than Col. There was thus considerable "noise" in the trend for a linear correlation between flowering time and the relative abundance of FLC RNA (fig. 4D). A possible explanation for this might be that the downstream response to a particular amount of FLC RNA is influenced by modifier genes.
Fig. 2  Complementation test crosses of Col/Nd-FRI-Sf2#114 with Ler-FRI-Sf2 and Niederzenz (Nd). Shown are flowering-time distributions of F1 and F2 generations. Plants were grown in long-day photoperiods and at 20°C. Both Col/Nd-FRI-Sf2#114 and Ler-FRI-Sf2 had the functional 
FRIGIDA-Sf2 allele (FRI-Sf2). Col/Nd-FRI-Sf2#114 was of mixed Col/Nd genetic background. Ler-FRI-Sf2 was of the Landsberg erecta (Ler) genetic background and contained a naturally occurring weak Ler allele of FLOWERING LOCUS C (FLC-Ler). Frequency distribution of rosette leaf number represents the number of vegetative leaves produced at the time of flowering. Filled circles indicate the flowering-time means of parental, F1, or reference lines, and the horizontal bars represent the range of rosette leaf number distributions. Arrows indicate the separation between early- and late-flowering plants, and the star marks a group of very early–flowering transgressions.

Some Col/Nd Recombinant Lines Have Elevated Levels of FLC RNA in the Absence of FRI-Sf2

The F2 generation from the cross of Col/Nd-FRI-Sf2#13 to Nd produced fewer than expected early-flowering plants (fig. 1; P < 0.05 for 4/16 = 25% early-flowering plants with the Nd parental range). The same deficit of early-flowering plants was also observed in crosses between Col-FRI-Sf2 and Nd (not shown). An explanation for this was that the mixed Col/Nd genetic background produced late-flowering transgressions even in the absence of FRI-Sf2. To test this hypothesis, crosses between Col with Nd were made, and F1 and F2 populations were analyzed. As shown in figure 5, F1 plants from Col x Nd had a similar flowering-time distribution to the parents. However, the F2 population indeed produced a large amount of transgressions that flowered later than the latest parent. Three of the late F2 plants, Col/Nd#36, Col/Nd#39, and Col/Nd#47, were selfed and shown to maintain their later flowering phenotype, which correlated with elevated levels of FLC RNA (fig. 4B). This indicated that in crosses between two naturally occurring Arabidopsis accessions such as Col and Nd, FRI-Sf2 is not the only modifier of FLC RNA abundance.

FLC RNA Is Absent in Some FRI-Sf2-Containing Recombinant Col/Ler Lines

Ca. 1/16 (P > 0.1) of F2 plants from the cross of Ler-FRI-Sf2 with Col/Nd-FRI-Sf2#114 were transgressions that flowered earlier than the earliest parent (fig. 2). This indicated that a mixed Col/Nd/Ler genetic background can flower as early as Ler, even in the presence of homozygous FRI-Sf2. To determine whether some lines flowered as early as Ler in the less complex Col/Ler genetic background as well, crosses were made between Ler-FRI-Sf2 and Col-FRI-Sf2, and F1 and F2 populations were analyzed. F1 plants flowered as late as Col-FRI-Sf2 (fig. 5). This indicated that strong FLC-Col was dominant over weak FLC-Ler in this cross. However, again, 1/16
Fig. 3 Mapping of the early-flowering region in the Niederzenz (Nd) accession. A, Graphic representation of Arabidopsis chromosome 5 (V) with SSLP markers (Bell and Ecker 1994). The black and hatched segments represent Columbia (Col) and Nd regions, respectively. Both Col/Nd-FRI-Sf2#114 and Col/Nd-FRI-Sf2#13 had functional Sf2 alleles of FRIGIDA (FRI-Sf2) and were of mixed Col/Nd genetic background. Col/Nd-FLC-Col and Col/Nd-FLC-Nd were early-flowering F2 plants from crosses of Col with Col/Nd-FRI-Sf2#114. The Nd region cosegregating with early-flowering is shown. B, Flowering-time distributions of F2 generations from backcrosses between Col/Nd-FRI-Sf2#114 with Col/Nd-FLC-Col and Col/Nd-FLC-Nd. Plants were grown in long-day photoperiods and at 20°C temperature. Frequency distribution of rosette leaf number represents the number of vegetative leaves produced at the time of flowering. Filled circles indicate the flowering-time means of parental, F1, or reference lines, and the horizontal bars represent the range of rosette leaf number distributions. A statistical probability value $P$ for the expected segregation ratio of two flowering-time loci with complementary gene action (nine late-flowering to seven early-flowering plants) is given. An arrow indicates the separation between early- and late-flowering plants.

$(P > 0.5)$ of the F2 plants flowered as early as Ler. Selfed progeny from these transgressions maintained the early-flowering phenotype, but F1 plants from crosses with Col were very late flowering (not shown). This indicated that they indeed contained functional alleles of FRI-Sf2 and were not contaminants. Both a FRI-Sf2-containing transgression (Col/Ler-FRI-Sf2#1) and Ler had no detectable amount of FLC RNA (fig. 4B). This indicated that, as with 92-d vernalized Col-FRI-Sf2, the earliest-flowering phenotype in analyzed lines and accessions correlated with complete absence of FLC RNA.

The 1/16 ratio of very early flowering F2 plants indicated that two unlinked loci were responsible for the phenotype. SSLP mapping was done with 19 early-flowering F2 plants to map the Col and Ler components of each chromosome. This showed that, as expected, all 19 plants had the weak FLC-Ler allele on chromosome 5 (nga249). However, all plants had always the Col allele of nga8 on chromosome 4, whereas all other regions segregated randomly for Col and Ler markers. This indicated that, together with FLC-Ler, a Col-FRI-Sf2 derived modifier locus tightly linked to nga8 suppressed any de-
Fig. 4 Relationship between levels of FLOWERING LOCUS C (FLC) RNA and flowering time. A, RNA gel blot analysis of different Arabidopsis lines and accessions. Plants were grown for 12 d in long-day photoperiods and at 23°C on agar-solidified half-strength MS medium without sucrose before RNA isolation. Blots with 25 μg of total RNA were first probed with radiolabeled FLC cDNA and then reprobed with labeled ACTIN-8 (ACT8) cDNA. Nd, Niederzenz; Col, Columbia; Col-FRI-Sf2, Col background with the Sf2 allele of FRIGIDA (FRI-Sf2); Col-FRI-Sf2 vernalized, 92 d at 4°C; Ler-FRI-Sf2, Landsberg erecta (Ler) background with FRI-Sf2 and weak Ler-FLC; Col/Nd-FRI-Sf2#13, mixed Col/Nd background with the FRI-Sf2 and strong FLC-Col; Col/Nd-FRI-Sf2#114, mixed Col/Nd background with FRI-Sf2 and weak FLC-Nd; [Col × FRI-Sf2#114]F1, pooled F1 plants from the cross of Col with Col/Nd-FRI-Sf2#114. Rosette leaf number means for each population at the time of flowering (leaf number) are shown below each lane. B, RNA gel blots containing 25 μg of total RNA simultaneously probed with radiolabeled FLC and ACT8 cDNA. Col/Ler-FRI-Sf2#1, an early-flowering F2 transgression from the cross of Ler-FRI-Sf2 × Col-FRI-Sf2; Col/Nd#36–47, different late-flowering F2 transgressions from the cross of Col × Nd. C, Plot showing relationship between normalized levels of FLC RNA and flowering time for different lines and accessions. Intensities of FLC bands were normalized with intensities of ACT8 bands. D, Plot showing linear relationship between flowering-time means represented as the mean number of rosette leaves made at the time of bolting and the normalized amounts of FLC RNA. An r² value for the linear regression using the CA-Cricket Graph III program is shown.
Fig. 5 Early- and late-flowering transgressions from crosses between Arabidopsis accessions. Flowering-time distributions of F1 and F2 generations are shown. Plants were grown in long-day photoperiods and at 20°C. Col = Columbia, Nd = Niederzenz. Both Ler-FRI-Sf2 and Col-FRI-Sf2 contained the functional Sf2 allele of FRIGIDA (FRI-Sf2) in the Landsberg erecta or Col background, respectively. Frequency distribution of rosette leaf number represents the number of vegetative leaves produced at the time of flowering. Filled circles indicate the flowering-time means of parental and F1 plants, and the horizontal bars represent the range of rosette leaf number distributions. An arrow indicates the separation between early- and late-flowering plants, the diamond marks a group of late-flowering transgressions, and the star marks a group of very early-flowering transgressions.

Detectable FLC RNA, even in the presence of functional FRI-Sf2.

Discussion

An Nd Accession of Arabidopsis thaliana Has a Weak Allele of FLC

The Sf2 allele of FRI-Sf2 had previously been shown to be fully dominant when introgressed into the Col accession of Arabidopsis, resulting in line Col-FRI-Sf2 with a very late flowering phenotype (Lee et al. 1993). By contrast, it is shown here that early-flowering F2 plants from the cross of Nd to Col-FRI-Sf2 can be obtained, even in the presence of functional alleles of FRI-Sf2. For instance, line Col/Nd-FRI-Sf2#114 is homozygous for dominant FRI-Sf2 but flowers early. Genetic complementation experiments showed that Nd has an early or weak allele of FLC. Weak alleles were previously found in only two other accessions, Ler and C24 (Koornneef et al. 1994; Lee et al. 1994; Sanda and Amasino 1995). Nd is thus the third Arabidopsis accession that contains a naturally occurring weak allele of FLC.

The Quantitative Effect of Particular Amounts of FLC mRNA to Delay Flowering Depends on Genetic Background

Compared to early-flowering accessions, the abundance of FLC RNA is high in Arabidopsis plants containing FRI-Sf2. Functional alleles of FRI were previously shown to increase the level of FLC steady state mRNA, which proportionally represses the transition to flowering (Michaels and Amasino 1999; Sheldon et al. 1999, 2000). This is consistent with the results of this study. The earliest-flowering plants, such as Ler or vernalized Col-FRI-Sf2, which made only four to five rosette leaves at the time of flowering, had no detectable levels of FLC.
RNA. Late-flowering lines such as Col/Nd-FRI-Sf2#13 had higher levels of \( FLC \) RNA than early-flowering lines such as Col/Nd-FRI-Sf2#114. Moreover, levels of \( FLC \) RNA were elevated to intermediate levels in intermediate late-flowering plants. Thus, plants flower late in a \( FLC \) RNA dosage-dependent manner. This positive correlation between the amount of \( FLC \) RNA and lateness of flowering also provides a molecular explanation for the additive gene action observed in the cross of Col with Col/Nd-FRI-Sf2#114. That is, the intermediate late-flowering phenotype of heterozygous \( FLC \)-Col/FLC-Nd \( F_F \) plants (fig. 1) is the average between the corresponding homozygous parents because the level of \( FLC \) RNA is also the average of the level of \( FLC \) RNA between the corresponding homozygous parents.

This study, however, also presents cases in which the relationship between levels of \( FLC \) RNA and flowering time is less proportional (fig. 4). For example, the level of \( FLC \) RNA was higher in Col/Nd-FRI-Sf2#114 than in Ler-FRI-Sf2 plants, but Col/Nd-FRI-Sf2#114 flowered slightly earlier than Ler-FRI-Sf2. Similarly, both Col and Nd had the same flowering time, but Nd expressed more \( FLC \) RNA. Other examples can be found in the literature. For instance, as judged from published RNA gel blots, early-flowering accession C24 apparently contains higher levels of \( FLC \) RNA than the very late flowering accession Pitztal (Sheldon et al. 1999). Thus, accessions such as Col, Ler-FRI-Sf2, or Pitztal may react more sensitively to the same amount of \( FLC \) RNA than Nd, Col/Nd-FRI-Sf2#114, or C24. This indicates that although the amount of \( FLC \) RNA is generally a robust measure for the lateness of flowering in \textit{Arabidopsis}, as proposed by the rheostat model (Michaels and Amasino 1999), flowering time is also modulated downstream from the apparent level of \( FLC \) RNA, possibly at the translational or posttranslational level. Alternatively, modifier genes may modulate flowering time of different accessions by regulating the competence of their apical meristems to react to a particular amount of \( FLC \) RNA. Yet another explanation may be that different accessions or mixed genetic backgrounds have increased levels of flowering promoting genes that act downstream from \( FLC \), such as \textit{FLOWERING LOCUS T} (\( F_T \); Kardailisky et al. 1999; Kobayashi et al. 1999) or the recently identified and renamed \textit{SUPPRESSOR OF OVEREXPRESSION OF CO} (\( SOC1 \)) = \textit{AGAMOUS-LIKE 20} (\( AGL20 \); Lee et al. 2000; Samach et al. 2000).

\textit{Early- and Late-Flowering Transgressions from Crosses between Accessions}

Late-flowering transgressions can be obtained from crosses between the two early-flowering accessions Col and Nd, both of which lack dominant \( FRI-Sf2 \). Late-flowering \( F_F \) plants from such a cross have higher levels of \( FLC \) RNA than does either parent. This indicates that a combination of flowering-time modifiers from two early-flowering accessions can result in plants that flower even later than lines containing functional \( FRI-Sf2 \), such as Ler-FRI-Sf2 or Col/Nd-FRI-Sf2#114. This may also be the reason for the apparent deficit of early-flowering \( F_F \) plants from the cross between Col/Nd-FRI-Sf2#13 and Nd (fig. 1). Heterotic gene interactions were indicated previously for Nd in crosses with late-flowering accessions such as Pitztal and Innsbruck (Burn et al. 1993) but not for crosses between early-flowering accessions.

Extreme Col/Nd transgressions flower even later than some induced late-flowering mutants shown to have increased levels of \( FLC \) RNA (Koornneef et al. 1991, 1998; Michaels and Amasino 1999; Sheldon et al. 1999, 2000). Based on their late-flowering phenotype, it was proposed that wild-type alleles of such flowering-time genes repress \( FLC \) RNA accumulation in the absence of functional \( FRI \) (Simpson et al. 1999). It is thus possible that naturally occurring allelic variations in flowering-time genes such as \( FCA, FLD, FPA, FVE, \) or \( LD \) may account for the apparent upregulation of \( FLC \) RNA when brought together in crosses between Col and Nd. This hypothesis can be tested by determining whether the map positions of late-flowering modifiers in recombinant Col/Nd plants overlap with the position of previously mapped flowering-time genes.

Conversely, extreme early-flowering transgressions can be obtained in \( F_F \) plants of crosses between Col-FRI-Sf2 and Ler-FRI-Sf2. Such plants contain weak \( FLC-Ler \) but also a Col-FRI-Sf2 locus tightly linked to SSLP marker nga8 on chromosome 4. Further introgression of this locus into the Ler background will allow fine mapping and isolation of the Col-FRI-Sf2 gene responsible for suppressing RNA accumulation from \( FLC-Ler \).

\textit{Dominance of FLC Alleles Depends on Genetic Background}

The strong Col allele of \( FLC \) is semidominant in the \( F_F \) generation of the cross between Col and Col/Nd-FRI-Sf2#114 (fig. 1). Semidominance has also been reported when strong \( FLC-Sf2 \) and weak \( FLC-Ler \) alleles were heterozygous in the Ler-FRI-Sf2 genetic background (Lee et al. 1994). However, the Col allele of \( FLC \) appears fully dominant in \( F_F \) plants of crosses between Nd and Col/Nd-FRI-Sf2#13 or Col-FRI-Sf2 and Ler-FRI-Sf2. An explanation for this difference may be that modifier genes from different genetic backgrounds determine whether strong and weak alleles show additive gene action or epistasis. Because the FRI-Sf2 allele was identical in all crosses, additive gene action or epistasis between \( FLC \) alleles does not depend on FRI-Sf2 but rather more likely depends on interactions with modifier genes brought together in crosses between different accessions.

The phenomenon of weak \( FLC \) alleles is still poorly understood. A simple hypothesis is that the two types of alleles differ in their promoter elements (Sheldon et al. 2000). However, this does not explain how genetic background influences epistasis between strong and weak \( FLC \) alleles. Another explanation may be that \( FLC \)-linked enhancer or silencer elements regulate \( FRI \)-mediated \( FLC \) RNA accumulation from a distance. However, this would imply some communication between the regulatory elements of the two daughter chromosomes. A more likely explanation is that \( FLC \)-linked modifier genes influence the \( FRI \)-mediated regulation of \( FLC \) RNA accumulation. Mapping and isolation of these modifier genes will further help to unravel the genetic network regulating the
transition from vegetative to reproductive development in *A. thaliana*.

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