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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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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* (L*er*) allele of *FLC* was recessive and not semidominant in the mixed L*er*/Col genetic background. However, very early-flowering transgressions lacking detectable *FLC* RNA were also obtained from crosses between *FRI* containing Col and L*er*. 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* (L*er*) 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*-L*er* alleles are semidominant in the presence of *FRI* when analyzed in the L*er* 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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flowering time (Michaels and Amasino 1999; Sheldon et al. 1999, 2000; Wilkosz and Schläppi 2000).

In this study, the identification of a new naturally occurring weak allele of *FLC* from a 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*-L*er* 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* (L*er*) (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 L*er*-*FRI*-Sf2 containing the dominant San Feliu-2 (Sf2) allele of *FRIGIDA* (*FRI*) introgressed into the Col and L*er* 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 F_3 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 (Difco, Detroit, Mich.) containing half-strength Murashige and Skoog (MS, Gibco BRL, Grand Island, N.Y.) salts without sucrose (Murashige and Skoog 1962). Petri dishes were placed at 4°C for 2–3 d to break seed dormancy and were then grown under cool fluorescent light with a 16L : 8D long-day photoperiod and ca. 23°C day/night temperature. For vernalization, petri dishes were kept at 4°C for 92 d under indirect light and were then grown at ca. 23°C day/night temperature under cool fluorescent light and 16-h long-day photoperiod. After 10–14 d, plantlets were transferred from petri dishes to soil (2 : 1 : 1 mix of peatmoss : vermiculite : perlite) in 2-in pots (four plants per pot, 32 pots per flat) and were grown under cool fluorescent light with a 16L : 8D long-day photoperiod, 20° ± 1°C day/night temperature, and ca. 60%–70% relative humidity. Flats were watered three times per week and fertilized once per week with 2 g/L 15-16-17 Peters fertilizer (Grace-Sierra Horticultural Products, Milpitas, Calif.).

Analysis of Flowering Time

Flowering time of individual plants was measured as the number of rosette leaves produced by the main shoot when its floral bolt was 0.5–1 cm high. This measurement thus reflected the extent of the vegetative phase before the transition to reproductive development occurred.

RNA Isolation

RNA was isolated from *in vitro*–grown whole seedlings. Ca. 500 seeds were spread onto agar-solidified half-strength MS 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 N_2. 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 : isoamyl alcohol (24 : 24 : 1; FisherBiotech, Fair Lawn, N.J.), followed by one extraction with chloroform : isoamyl alcohol (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-μg 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 *Sal*I and was labeled to a specific activity of ca. $0.5 - 1 \times 10^9$ cpm/μg DNA with [^{32}P]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 1×10^6 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 reprobed or simultaneously probed with the

Table 1

Genotype and Rosette Leaf Number Range at Time of Flowering for Different *Arabidopsis thaliana* Lines and Accessions

Line or accession	*FRI*; *FLC* genotype	Rosette leaf number range
Landsberg *erecta* (L*er*)	*fri*-L*er*/*fri*-L*er*; *FLC*-L*er*/*FLC*-L*er*	4–5
Columbia (Col)	*fri*-Col/*fri*-Col; *FLC*-Col/*FLC*-Col	6–14
Niederzenz (Nd)	*fri*-Nd/*fri*-Nd; *FLC*-Nd/*FLC*-Nd	5–13
L*er*-*FRI*-Sf2	*FRI*-Sf2/*FRI*-Sf2; *FLC*-L*er*/*FLC*-L*er*	12–28
Col-*FRI*-Sf2	*FRI*-Sf2/*FRI*-Sf2; *FLC*-Col/*FLC*-Col	51–72
Col/L*er*-*FRI*-Sf2#1	*FRI*-Sf2/*FRI*-Sf2; *FLC*-L*er*/*FLC*-L*er*	3–5
Col/Nd-*FRI*-Sf2#114	*FRI*-Sf2/*FRI*-Sf2; *FLC*-Nd/*FLC*-Nd	12–22
Col/Nd-*FRI*-Sf2#13	*FRI*-Sf2/*FRI*-Sf2; *FLC*-Col/*FLC*-Col	42–88

Note. Plants were grown in long-day photoperiods and at 20°C. Flowering time is represented as the number of rosette leaves made when the flowering bolt was 0.5 cm high. L*er*-*FRI*-Sf2, Col-*FRI*-Sf2, and derived lines have the San Feliu-2 (Sf2) allele of *FRIGIDA* (*FRI*) introgressed in the L*er*, Col, and mixed genetic backgrounds, respectively (Lee et al. 1994). *FLC* = *FLOWERING LOCUS C*.

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′GAAATCAAGCGAATTGAGAAC-3′ and 5′-GGCCACGCGTCGACTAC-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 *Taq* polymerase and nga249 primers 5′-TACCGTCAATTTCATCGCC-3′ and 5′-GGATCCCTAACTGTAAAATCCC-3′ (Bell and Ecker 1994). The nga249 SSLP marker was shown previously to be linked by less than 1 cM to *FLC* (Lee et al. 1994). Polymerase chain reaction conditions were those recommended by Bell and Ecker (1994), and products were separated on a 2.5% agarose gel (Gibco BRL) in TAE buffer. Using 12 different SSLP markers, "Nossen" lines 380-1-1 and B22 were compared to ABRC (Ohio State University)-obtained Nossen accessions CS1394 and CS3081. The 12 SSLP markers matched the sizes published for Nd (Bell and Ecker 1994) but not those for Nossen. Lines 380-1-1 and B22 were thus reclassified as Nd lines (D. Greving and M. Schläppi, unpublished data). For instance, *FLC*-linked SSLP marker nga249 was 135 bp long in 380-1-1 and B22 but was 115 bp long in Nossen controls CS1394 and CS3081 (Bell and Ecker 1994).

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 F_1 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 F_1 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 F_1 generation when Col/Nd-*FRI*-Sf2#13 was backcrossed with Col (fig. 1). However, F_2 generations from the two backcrosses had significantly different flowering-time distributions. The backcross generation with Col clearly segregated 3 : 1 late- to early-flowering F_2 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 F_2 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-

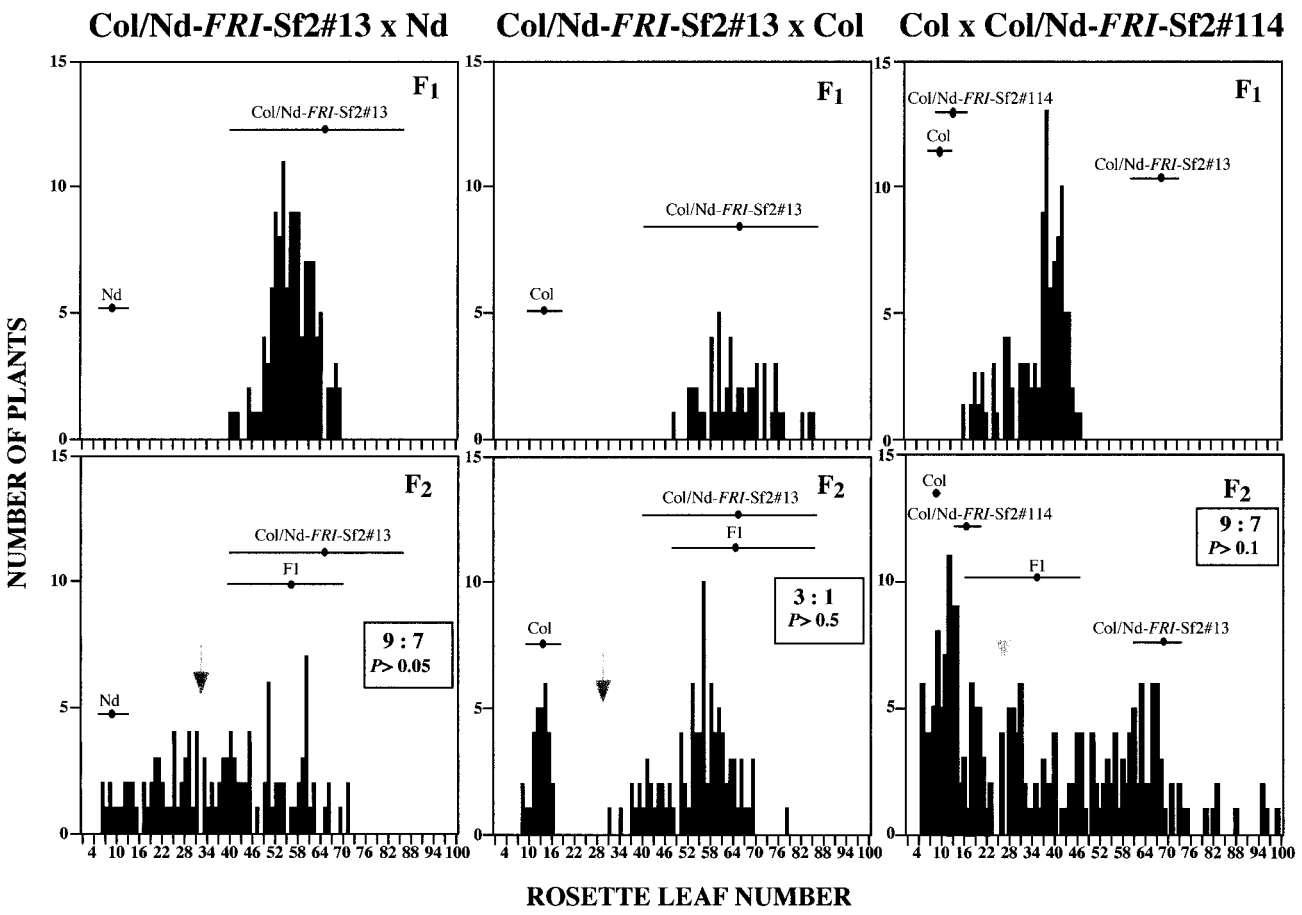

Fig. 1 A Niederzenz (Nd) accession of *Arabidopsis* has a semidominant modifier of *FRIGIDA*-Sf2 (*FRI*-Sf2). Shown are flowering-time distributions of F_1 and F_2 generations from crosses between Col/Nd-*FRI*-Sf2#13 and Columbia (Col) or Nd and between Col and Col/Nd-*FRI*-Sf2#114. Plants were grown 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, F_1, 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.

mentary gene action between *FRI*-Sf2 and a modifier gene from Nd.

When F_2 plants were positively selected for the presence of the *FRI*-Sf2-linked B22 locus, an unexpectedly large amount of plants, 19% (23/121), flowered relatively early. Among those, 5.8% (7/121) produced 100% very early flowering F_3 progenies and were likely to be recombinants between early *fri*-Nd and the B22 locus (from Col/Nd-*FRI*-Sf2#13). All remaining lines segregated again early- and late-flowering F_3 plants with a wide range of flowering times. It thus appeared that some F_2 plants were probably early flowering, even in the presence of dominant *FRI*-Sf2, indicating an interaction of *FRI*-Sf2 with modifier genes. A possible candidate for such a gene was *FLC* because it was shown previously that weak *FLC*-L*er* and *FLC*-C24 alleles from the L*er* and C24 backgrounds, respectively, had complementary gene action with *FRI*-Sf2 (Koornneef et al. 1994; Lee et al. 1994; Sanda and Amasino 1995).

The Nd Accession Contains a Weak Allele of FLC

Early- and late-flowering segregating F_3 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*. F_3 plants were test crossed to Col with the premise that 50% (heterozygous *FRI*-Sf2) or 100% (homozygous *FRI*-Sf2) of the resulting F_1 plants were late flowering. This was because the *FLC*-Col allele would interact strongly with *FRI*-Sf2 to confer late flowering (Lee et al. 1994). This was indeed observed for four different F_3 plants such as Col/Nd-*FRI*-Sf2#114 (fig. 1). That is, 100% of the F_1 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 F_1 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 F_2 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#13 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 L*er* lines containing the well-characterized weak *FLC*-L*er* allele (Koornneef et al. 1994; Lee et al. 1994). L*er* flowered very early because it had both recessive *fri*-L*er* and weak *FLC*-L*er* alleles, whereas L*er*-*FRI*-Sf2 had dominant *FRI*-Sf2 but weak *FLC*-L*er* alleles and flowered slightly later than Col/Nd-*FRI*-Sf2#114 (table 1). No late-flowering plants were observed in F_1 generations of reciprocal crosses between Col/Nd-*FRI*-Sf2#114 and L*er*, indicating that Col/Nd-*FRI*-Sf2#114 did not complement the weak *FLC*-L*er* 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 L*er*-*FRI*-Sf2. In both the F_1 and the F_2 generations, flowering-time means were only slightly later than that of the L*er*-*FRI*-Sf2 parent, again indicating lack of complementation. This indicated that as L*er*, 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 F_1 and F_2 plants were similar to those seen for crosses between L*er*-*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 3*A*, this analysis indicated that Col/Nd-*FRI*-Sf2#114 and Col/Nd-*FRI*-Sf2#13 were homozygous for the *FLC*-Nd and *FLC*-Col alleles, respectively. Early-flowering F_2 plants lacking *FRI*-Sf2 that were homozygous for either *FLC*-Col (Col/Nd-*FLC*-Col) or *FLC*-Nd (Col/Nd-*FLC*-Nd) (fig. 3*A*) 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 3*B*, F_1 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 F_2 plants flowered later than the latest F_1 plant. This showed that the mixed Col/Nd background containing *FRI*-Sf2 and homozygous *FLC*-Nd alleles remained early flowering. By contrast, F_1 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 F_2 generation segregated 9 : 7 late- to early-flowering plants ($P > 0.5$; fig. 3*B*), 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 F_2 plants used for the above complementation experiments. This analysis indicated that only the top of chromosome 5 (fig. 3*A*) 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*-L*er* (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. F_1 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. 4*A*, 4*B*). 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 L*er*-*FRI*-Sf2 had only 18%–49% of the highest levels (fig. 4*C*). Although Col/Nd-*FRI*-Sf2#114 had higher levels of *FLC* RNA, it flowered slightly earlier than L*er*-*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. 4*D*). A possible explanation for this might be that the downstream response to a particular amount of *FLC* RNA is influenced by modifier genes.

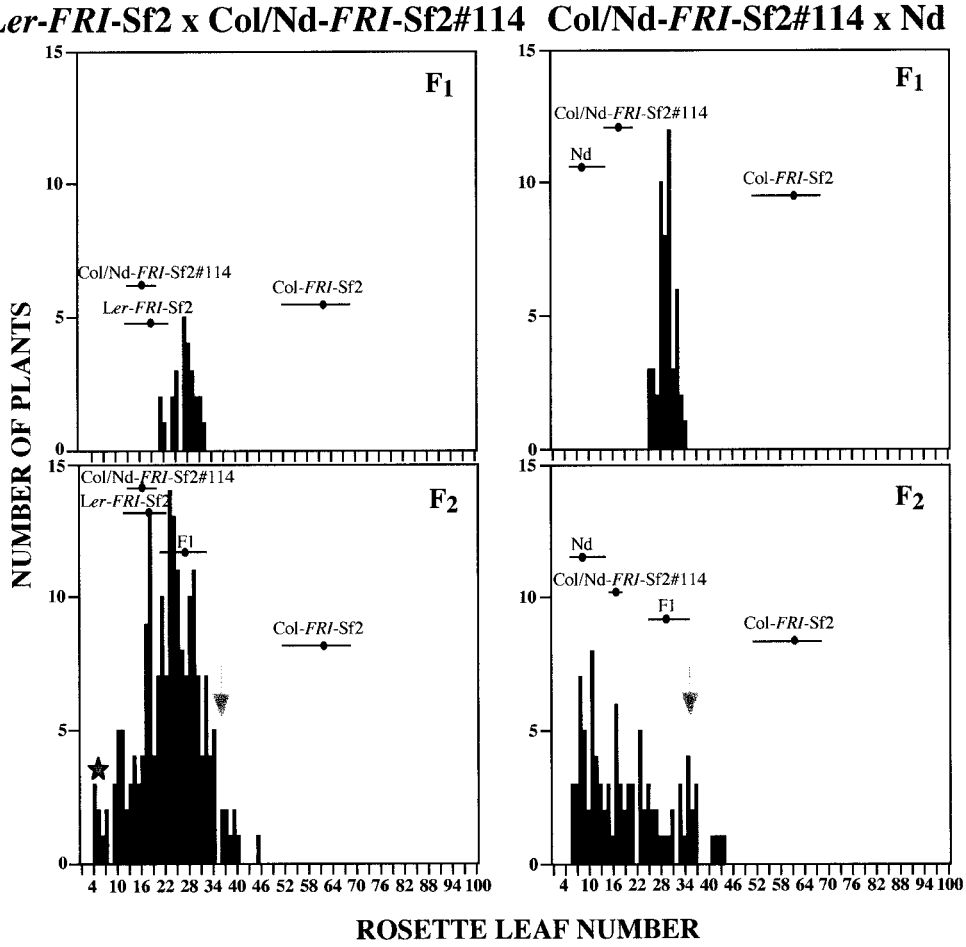

Fig. 2 Complementation test crosses of Col/Nd-*FRI*-Sf2#114 with L*er*-*FRI*-Sf2 and Niederzenz (Nd). Shown are flowering-time distributions of F_1 and F_2 generations. Plants were grown in long-day photoperiods and at 20°C. Both Col/Nd-*FRI*-Sf2#114 and L*er*-*FRI*-Sf2 had the functional *FRIGIDA*-Sf2 allele (*FRI*-Sf2). Col/Nd-*FRI*-Sf2#114 was of mixed Col/Nd genetic background. L*er*-*FRI*-Sf2 was of the Landsberg *erecta* (L*er*) genetic background and contained a naturally occurring weak L*er* allele of *FLOWERING LOCUS C* (*FLC*-L*er*). 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, F_1, 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 F_2 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 F_1 and F_2 populations were analyzed. As shown in figure 5, F_1 plants from Col × Nd had a similar flowering-time distribution to the parents. However, the F_2 population indeed produced a large amount of transgressions that flowered later than the latest parent. Three of the late F_2 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. 4*B*). 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/*L*er Lines*

Ca. 1/16 ($P > 0.1$) of F_2 plants from the cross of L*er*-*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/L*er* genetic background can flower as early as L*er*, even in the presence of homozygous *FRI*-Sf2. To determine whether some lines flowered as early as L*er* in the less complex Col/L*er* genetic background as well, crosses were made between L*er*-*FRI*-Sf2 and Col-*FRI*-Sf2, and F_1 and F_2 populations were analyzed. F_1 plants flowered as late as Col-*FRI*-Sf2 (fig. 5). This indicated that strong *FLC*-Col was dominant over weak *FLC*-L*er* in this cross. However, again, 1/16

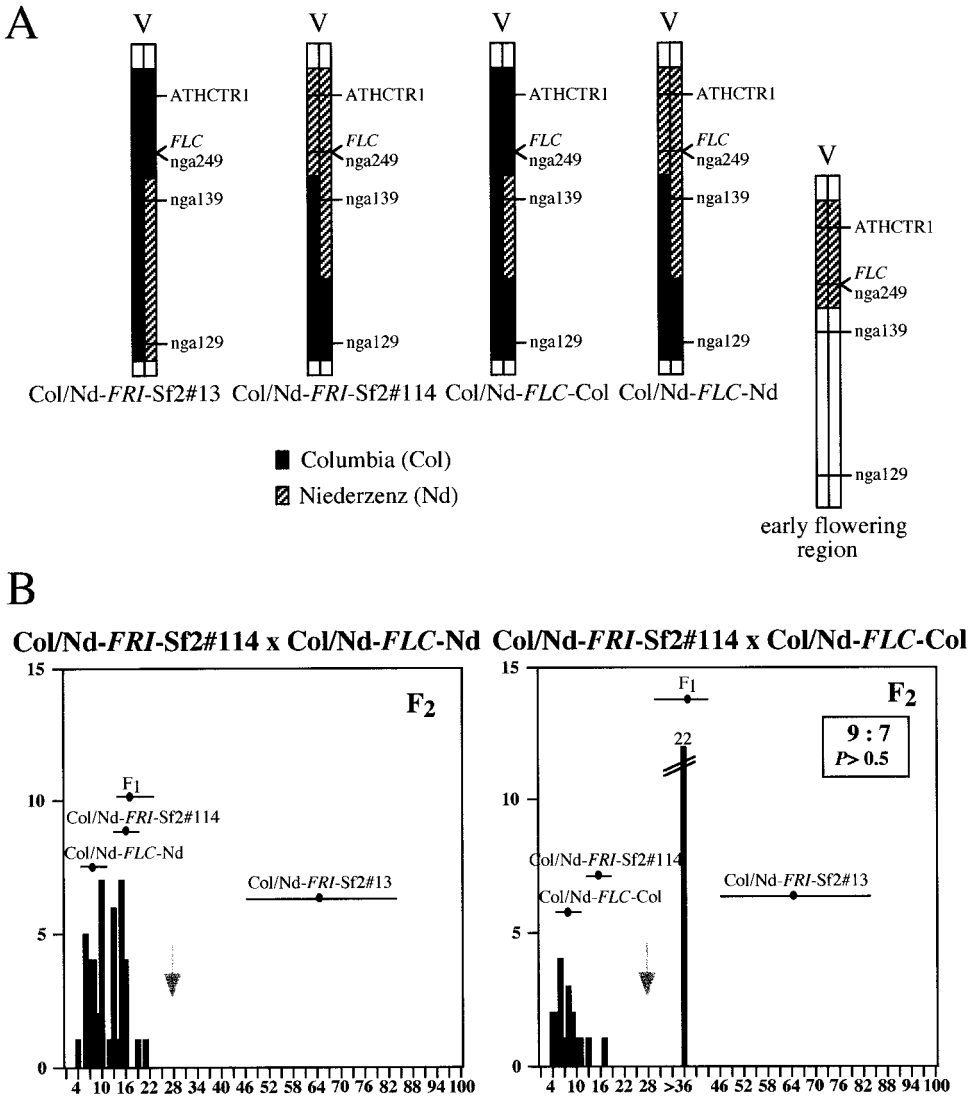

Fig. 3 Mapping of the early-flowering region in the Niederzenz (Nd) accession. *A*, Graphic representation of *Arabidopsis* chromosome 5 (V) with SSLP markers (Bell and Ecker 1994). The black and hatched segments represent Columbia (Col) and Nd regions, respectively. Both Col/Nd-*FRI*-Sf2#114 and Col/Nd-*FRI*-Sf2#13 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 F_2 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 F_2 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, F_1, 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 F_2 plants flowered as early as L*er*. Selfed progeny from these transgressions maintained the early-flowering phenotype, but F_1 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/L*er*-*FRI*-Sf2#1) and L*er* had no detectable amount of *FLC* RNA (fig. 4*B*). 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 F_2 plants indicated that two unlinked loci were responsible for the phenotype. SSLP mapping was done with 19 early-flowering F_2 plants to map the Col and L*er* components of each chromosome. This showed that, as expected, all 19 plants had the weak *FLC*-L*er* 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 L*er* markers. This indicated that, together with *FLC*-L*er*, a Col-*FRI*-Sf2 derived modifier locus tightly linked to nga8 suppressed any de-

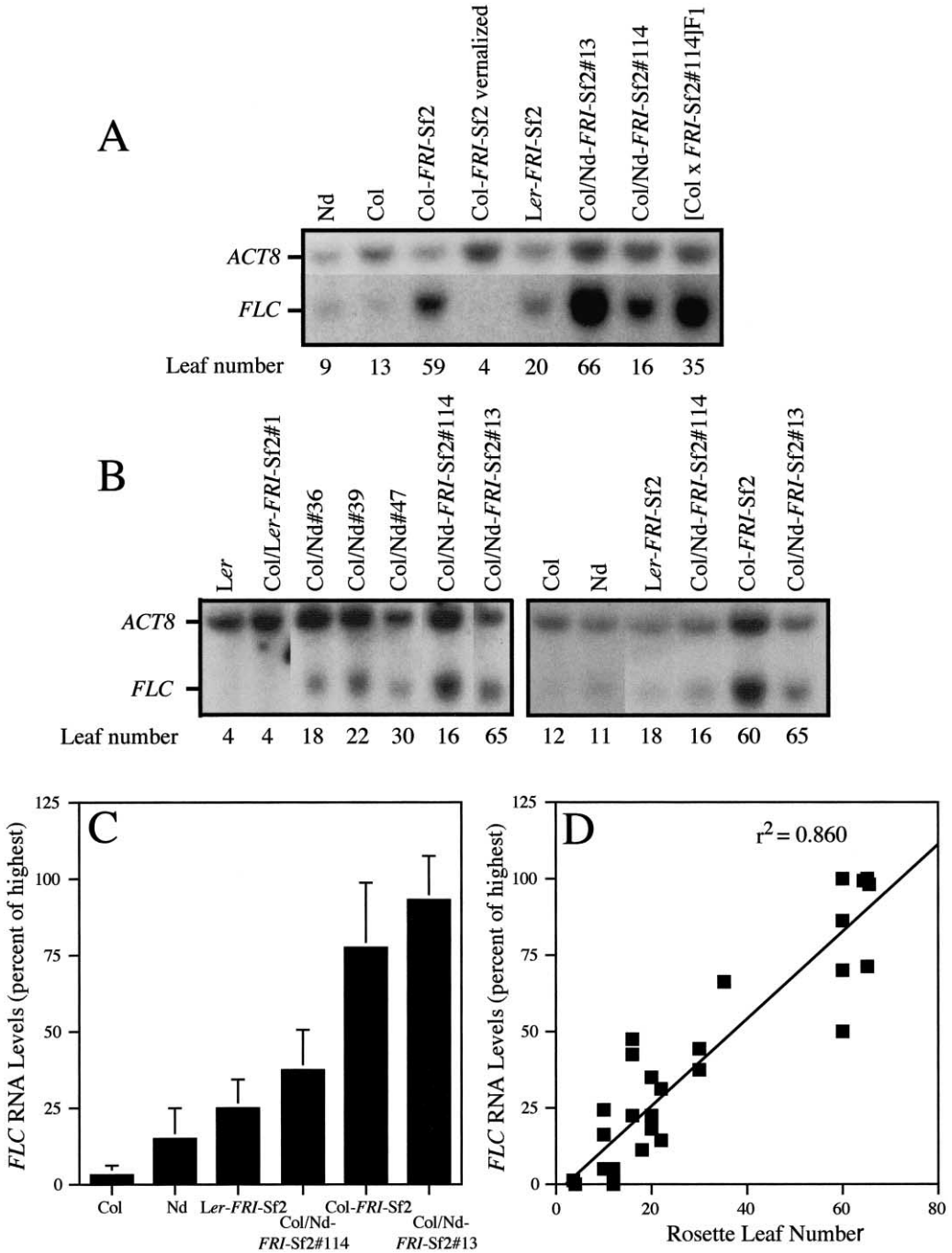

Fig. 4 Relationship between levels of *FLOWERING LOCUS C* (*FLC*) RNA and flowering time. *A*, RNA gel blot analysis of different *Arabidopsis* lines and accessions. Plants were grown for 12 d in long-day photoperiods and at 23°C on agar-solidified half-strength MS medium without sucrose before RNA isolation. 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; L*er*-*FRI*-Sf2, Landsberg *erecta* (L*er*) background with *FRI*-Sf2 and weak L*er*-*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]F_1, pooled F_1 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/L*er*-*FRI*-Sf2#1, an early-flowering F_2 transgression from the cross of L*er*-*FRI*-Sf2 × Col-*FRI*-Sf2; Col/Nd#36–47, different late-flowering F_2 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 r^2 value for the linear regression using the CA-Cricket Graph III program is shown.

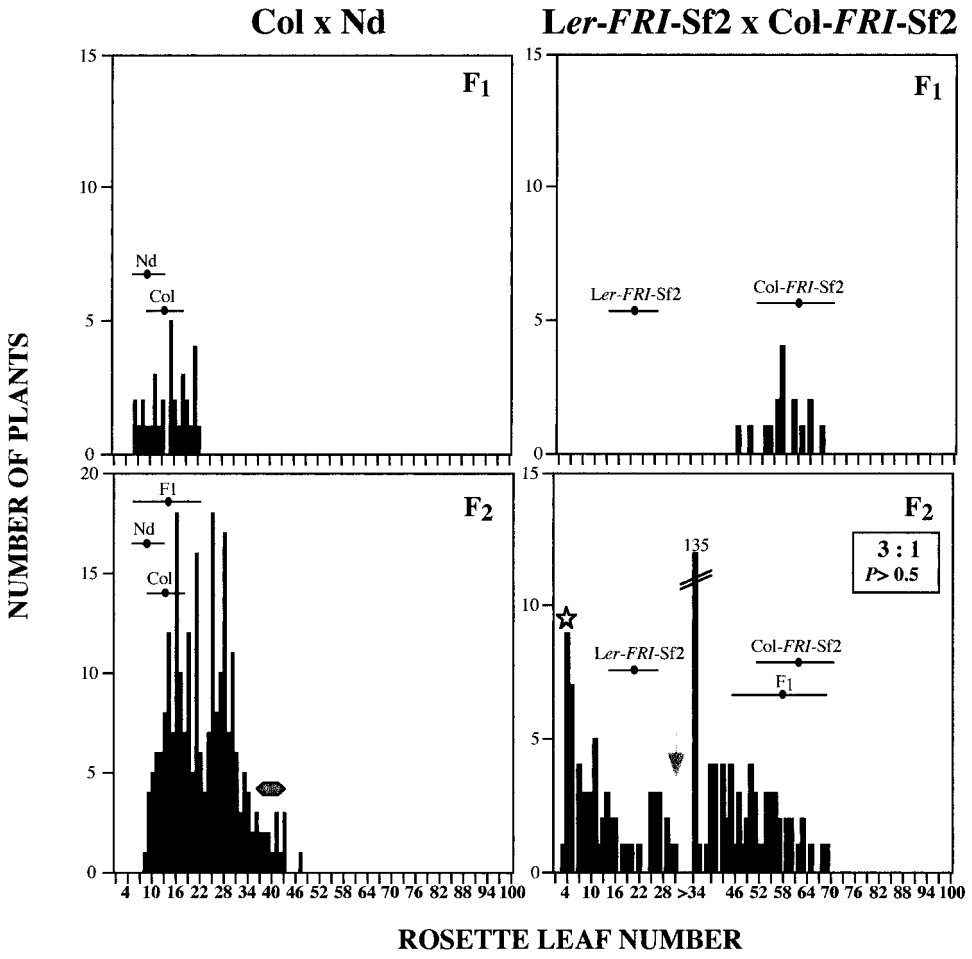

Fig. 5 Early- and late-flowering transgressions from crosses between *Arabidopsis* accessions. Flowering-time distributions of F_1 and F_2 generations are shown. Plants were grown in long-day photoperiods and at 20°C. *Col* = Columbia, *Nd* = Niederzenz. Both L*er-FRI*-Sf2 and Col-*FRI*-Sf2 contained 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 F_1 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.

tectable *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 F_2 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, L*er* 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 L*er* 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_1 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 L*er*-*FRI*-Sf2 plants, but Col/Nd-*FRI*-Sf2#114 flowered slightly earlier than L*er*-*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, L*er*-*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 *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 *FLOWERING LOCUS T* (*FT*; Kardailsky et al. 1999; Kobayashi et al. 1999) or the recently identified and renamed *SUPPRESSOR OF OVEREXPRESSION OF CO* (*SOC1*) = *AGAMOUS-LIKE 20* (*AGL20*; Lee et al. 2000; Samach et al. 2000).

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_2 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 L*er*-*FRI*-Sf2 or Col/Nd-*FRI*-Sf2#114. This may also be the reason for the apparent deficit of early-flowering F_2 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_2 plants of crosses between Col-*FRI*-Sf2 and L*er*-*FRI*-Sf2. Such plants contain weak *FLC*-L*er* but also a Col-*FRI*-Sf2 locus tightly linked to SSLP marker nga8 on chromosome 4. Further introgression of this locus into the L*er* background will allow fine mapping and isolation of the Col-*FRI*-Sf2 gene responsible for suppressing RNA accumulation from *FLC*-L*er*.

Dominance of FLC *Alleles Depends on Genetic Background*

The strong Col allele of *FLC* is semidominant in the F_1 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*-L*er* alleles were heterozygous in the L*er*-*FRI*-Sf2 genetic background (Lee et al. 1994). However, the Col allele of *FLC* appears fully dominant in F_1 plants of crosses between Nd and Col/Nd-*FRI*-Sf2#13 or Col-*FRI*-Sf2 and L*er*-*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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