Role of *FRIGIDA* and *FLOWERING LOCUS C* in Determining Variation in Flowering Time of Arabidopsis[1[w]]

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Arabidopsis (*Arabidopsis thaliana*) accessions provide an excellent resource to dissect the molecular basis of adaptation. We have selected 192 Arabidopsis accessions collected to represent worldwide and local variation and analyzed two adaptively important traits, flowering time and vernalization response. There was huge variation in the flowering habit of the different accessions, with no simple relationship to latitude of collection site and considerable diversity occurring within local regions. We explored the contribution to this variation from the two genes *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), previously shown to be important determinants in natural variation of flowering time. A correlation of *FLC* expression with flowering time and vernalization was observed, but it was not as strong as anticipated due to many late-flowering/vernalization-requiring accessions being associated with low *FLC* expression and early-flowering accessions with high *FLC* expression. Sequence analysis of *FRI* revealed which accessions were likely to carry functional alleles, and, from comparison of flowering time with allelic type, we estimate that approximately 70% of flowering time variation can be accounted for by allelic variation of *FRI*. The maintenance and propagation of 20 independent nonfunctional *FRI* haplotypes suggest that the loss-of-function mutations can confer a strong selective advantage. Accessions with a common *FRI* haplotype were, in some cases, associated with very different *FLC* levels and wide variation in flowering time, suggesting additional variation at *FLC* itself or other genes regulating *FLC*. These data reveal how useful these Arabidopsis accessions will be in dissecting the complex molecular variation that has led to the adaptive phenotypic variation in flowering time.

A central aim in ecology and evolutionary biology is to understand the molecular genetic basis for variation in important life history traits. In plants, this variation has underpinned the successful adaptation to different environmental niches. The timing of the transition from vegetative to reproductive development is a critical adaptive trait because it is essential for plants to complete flower development, pollination, and seed production in favorable conditions. Environmental conditions change depending on geographical location as well as season, so plants have developed mechanisms to perceive environmental cues like light, temperature, and water availability in order to alter their flowering time in response to these signals.

Since the 1850s, many attempts have been made to elucidate the mechanisms mediating light and temperature responses in the control of flowering (Garner and Allard, 1920; Salisbury and Marinos, 1985). More recently, the genetic control of flowering has been studied intensively in Arabidopsis (*Arabidopsis thaliana* L. Heynh.). Since Napp-Zinn published his first report (Napp-Zinn, 1955), a large effort focused on genetic and mutational analyses has identified an integrated network of pathways that promote or repress flowering in response to different environmental and endogenous cues (Coupland, 1995; Levy and Dean, 1998; Simpson and Dean, 2002). Vernalization—the acceleration of flowering by a long period of cold temperature, generally below 10°C but above 0°C—is one such pathway.

Three key pathways affect the induction of flowering in response to long periods of cold; the *FRIGIDA* (*FRI*) repression and the autonomous promotion pathways confer dominant and recessive vernalization requirements, respectively, whereas the vernalization promotion pathway confers the ability to respond to cold temperature (Henderson et al., 2003). These pathways interact through quantitative, antagonistic effects on the expression of at least one common target, the floral repressor *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 1999; Sheldon et al., 1999).

Arabidopsis has a near-worldwide distribution, so it must have adapted to growth in a wide range of climatic conditions. This phenotypic variation is attracting an increasing amount of attention by ecologists and evolutionary biologists (for review, see

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Pigliucci, 2003). However, some commonly used accessions, like Columbia (Col) and Landsberg *erecta* (L*er*), are inappropriate materials for such analyses because they represent only a limited amount of the phenotypic variation present in the species. Extensive collections of Arabidopsis accessions obtained from throughout the northern hemisphere reveal a wide range of variation, and these have been the focus of a number of biogeographical and evolutionary studies (Nordborg and Bergelson, 1999; Alonso-Blanco and Koornneef, 2000; Koornneef et al., 2004). Furthermore, the availability of the complete genome sequence has enabled the development of different technologies based on single-nucleotide polymorphism (SNP) information to identify naturally occurring molecular variation within these varied Arabidopsis accessions (Borevitz and Nordborg, 2003; Maloof, 2003; Torjek et al., 2003).

Many tools are therefore in place to assess the role of allelic variation at known loci in flowering-time diversity. These resources may also enable the discovery of novel genes that are important for controlling flowering-time variation in the wild. Initial work focused on the role of *FRI* alleles in the evolution of rapid-cycling Arabidopsis variants from late-flowering ancestral accessions (Johanson et al., 2000). *FRI* represses flowering by promoting the expression of the floral repressor *FLC*. Vernalization accelerates flowering by down-regulating *FLC* expression and thus antagonizes the effect of *FRI*. Molecular analysis of *FRI* revealed that nine different loss-of-function mutations at this locus have provided the basis for the independent evolution of many rapid-cycling types, including Col and L*er* (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003). Natural allelic variation at the *FLC* locus has also been identified, suggesting that rapid-cycling types have been derived from late-flowering ancestral accessions through loss-of-function mutations of *FRI* and/or *FLC* (Gazzani et al., 2003; Michaels et al., 2003). From an ecological point of view, rapid cycling corresponds to a summer-annual habit (or perhaps even multiple generations per year), whereas functional alleles of both *FRI* and *FLC* confer a winter-annual habit. A nonrandom geographical distribution of accessions with active or null *FRI* alleles is consistent with a selective advantage for the presence or absence of *FRI* function in certain niches (Johanson et al., 2000; Stinchcombe et al., 2004).

It is noteworthy that *FRI* and *FLC* appear to contribute so much to natural variation in flowering time when more than 80 genes regulating flowering time have been identified (Levy and Dean, 1998). Early flowering can arise from late-flowering progenitors through mutagenesis of many genes (Boss et al., 2004). Furthermore, early flowering does not always require loss-of-function mutations. Overexpression of one of the genes of the autonomous promotion pathway, *FCA* (Macknight et al., 1997), can suppress the *FRI*-induced floral repression (Quesada et al., 2003). This is explained at the molecular level by a quantitative interaction between *FRI* and *FCA* function in the regulation of *FLC* levels. Therefore, obvious questions are whether loss of function of any of these other characterized genes, or overexpression of *FCA*, a count for earliness in natural accessions.

We have continued to exploit the natural variation in Arabidopsis flowering time to study the mechanism of vernalization. We have utilized a set of 192 Arabidopsis accessions from the northern hemisphere, which includes populations from diverse habitats in Sweden (ranging from mountainsides close to the Arctic Circle to agricultural fields in the far south of the country) and populations from the United States, where Arabidopsis was recently introduced. Detailed descriptions of the collection sites are available for most accessions, and this may facilitate future ecological analysis with this material. In addition, our sample of 192 Arabidopsis accessions includes 96 accessions for which genome-wide polymorphism data have been generated (Nordborg et al., 2005). Variation in flowering time with and without an 8-week cold pretreatment was analyzed together with *FLC* RNA levels and *FRI* sequence in this set of 192 accessions. These data provide a detailed picture of the reproductive strategy adopted by Arabidopsis accessions over a wide range of latitudes and gives an overview of the extent to which different *FRI* and *FLC* alleles contribute to that phenotypic variation.

RESULTS

Flowering-Time Variation in a Collection of Arabidopsis Accessions

The 192 Arabidopsis accessions analyzed are from diverse environments, mainly from Europe, Scandinavia, Asia, and North America (Table I). For each accession, the final total leaf number (FLN; rosette plus cauline leaves) was counted for plants grown in greenhouse conditions with (+V) and without (−V) 8 weeks of cold treatment. FLN has previously been found to be a reliable measure of flowering time (Koornneef et al., 1991). FLN was then compared to latitude of collection site (Fig. 1, A and B). For those accessions that had not flowered within 5 months, it became increasingly difficult to determine leaf number due to loss of apical dominance and the appearance of secondary leaves (Fig. 1C). These plants were scored as did not flower (DNF); all had a minimum of 120 leaves.

This analysis revealed wide variation in flowering time among the accessions. One might expect that FLN (−V) would correlate with latitude of collection site. However, while a relationship between latitude of collection site and FLN was evident on a gross scale, there was no simple correlation (Fig. 1A). All of the accessions collected above 62°N (11 accessions) were categorized into the DNF group, and there were no DNF accessions below 45°N (42 accessions). Very-early-flowering accessions (FLN $<$ 20) were

Table I. *Accessions used in this project*

Latitude °N	Countries	Accessions
15–20	Cape Verde Islands	Cvi-0
20–25		
25–30	Canary Islands	Can-0
30–35	Libya, Japan, India	Mt-0, Tsu-1, Kas-1
35–40	U.S. (California), Italy, Tajikistan, Portugal	NC-6, Santa Clara, Ct-1, Yo-0, Pa-1, Col-0, Kondara, Shahdara, Sorbo, Dem-4, Co, C24
40–45	Portugal, Spain, U.S. (Midwest), Croatia, Italy, France	Alc-0, Fei-0, Knox-10 and 18, Sf-1, Se-0, Ts-1 and 5, Bla-1, RRS-7 and 10, Pla-0, Ll-0, RMX-A02 and A180, PNA-10 and 17, Pu2-7, 8, and 23, Pro-0, PHW230,002, Mir-0, Mr-0, Kin-0, Ag-0, Tu-0
45–50	France, Switzerland, Austria, U.S. (Washington), Czech Republic, Germany, Canada, Poland, Kazakhstan	Lz-0, Ra-0, Ge-0, Ka-0, Pi-0, Gr-1, Di-0, Wei-0, N6187, Bg-2, Bs-1, In-0, Na-1, Lm-2, Uod-1, 2, and 7, Blh-1, N6034, Ren-1 and 11, Br-0, Gy-0, Bay-0, Mrk-0, Cen-0, Rubezhnoe-1, Rak-2, Zdr-1 and 6, Bor-1 and 4, DraII-1, DraIII-1, LP2-2 and 6, Van-0, Jm-0, H55, Duk, Hod, HSm, Sap-0, Kz-1, 9, and 13, Ta-0, En-1, Lip-0
50–55	Germany, Belgium, England, Ukraine, Netherlands, Poland, Russia, Ireland	Ei-2, Gu-0, Ga-0, Mz-0, Ang-0, Is-0, Rd-0, Nw-0, Bu-0, Köln, Kent, HR-5 and 10, NFA-8 and 10, SQ-1 and 8, CIBC-5 and 17, An-1, L*er*-1, GOT-7 and 22, Ws-0, Wt-5, Nok-3, Wa-1, Ws-2, Hi-0, Hs-0, Stw-0, Gd-1, Bur-0
55–60	Lithuania, S. Sweden, Scotland, Russia, Estland, Finland	Wil-2, San-2, Var-2–1 and 6, Dra-3–1, Tottarp-2, Kin-1, Lund, Rev-1, Fly-2–2, Kävlinge-1, Lu-1, Boo-2–1, Liarum, Ms-0, Edi-0, Stu-1–1, Lis-1, 2, and 5, Kulturen-1, Lillö-1, Hov-2–1 and 4–1, Lom-1–1, Hovdala-2 and 6, Ull-2–5, and 3, Ull-1–1, Omo-2–1 and 3, Brö-1–6, App-1–4, Bå-1–2, 3–3, 4–1, and 5–1, Gul-1–2, Rsch-4, Spr-1–2 and 6, Algutsrum, Ör-1, Vimmerby, Lc-0, Per-1, Est-1, St-0, Sr:5, Petergof, TAMM-2 and 27
60–65	Norway, N. Sweden, Russia	Oy-0, Ost-0, CS22491, Eds-1, Sanna-2, Lov-1 and 5, Eden-1 and 2, Fab-2 and 4, Nyl-2, Bil-5 and 7

found at all latitudes below 60°N, but the majority was found within the latitudinal range of 45°N to 55°N. The widest range of flowering time was found at around 55°N.

Focusing on the accessions collected in close proximity to each other revealed patterns in FLN (−V) on a finer scale. There was considerable variation in flowering time in southern Sweden (of a total of 36 accessions, eight were very early flowering and 13 DNF). Genome-wide polymorphism data (Nordborg et al., 2005) show that the southern Swedish accessions are not the product of very recent admixture, suggesting that this diversity in flowering time may have evolved locally. In contrast to the phenotypic variation observed in southern Sweden, all 11 accessions from northern Sweden were extremely late flowering (DNF). Genome-wide polymorphism data indicate that northern Swedish accessions are genetically quite distinct from other accessions. It is thus plausible that these accessions share common late-flowering alleles; however, as we discuss further below, variation in flowering time in response to a period of cold temperature exists even within this group.

It is also of interest to ask whether the DNF accessions from northern and southern Sweden may share late-flowering alleles. The data of Nordborg et al. (2005) can be used to shed light on this question. Of the three southern Swedish DNF accessions for which data were available, two (Var-2-1 and Var-2-6) show clear similarity with northern Swedish accessions, whereas the third, Ull-2-5, is genetically quite distinct (Supplemental Fig. 1). This suggests that the DNF phenotype of Ull-2-5 is due to different genetic loci or alleles than the other Swedish DNF accessions.

FLN (+V) was measured in order to calculate the vernalization response (i.e. the decrease in FLN after 8 weeks of cold treatment). Comparable levels of variation were observed in FLN (+V) as described above for FLN (−V), and a similar relationship with latitude was found (Fig. 1B). Accessions generally showed an acceleration of flowering after an 8-week cold treatment (Fig. 1D). Very-early-flowering accessions (FLN < 20) showed the lowest reduction in FLN after vernalization, whereas the DNF and very-late-flowering accessions (FLN > 70) showed the greatest reduction. Thus, the later the flowering in our assays, the larger the vernalization response. More interestingly, within the late (FLN > 70) and DNF accessions, there was a wide range of FLN after vernalization, suggesting that vernalization response differs between these accessions.

Among the accessions with FLN (−V) > 20, it was possible to identify some with an enhanced vernalization response, including a number of accessions from around 40°N in Spain, Portugal, and Tajikistan (Pla-0, Sorbo, Kondara, Se-0, Ts-1, Ts-5, Ll-0, and C24). These accessions flowered relatively late without vernalization but much earlier after vernalization than accessions with a similar FLN (−V) from higher latitudes (Fig. 1, A and B, white diamonds). In addition,

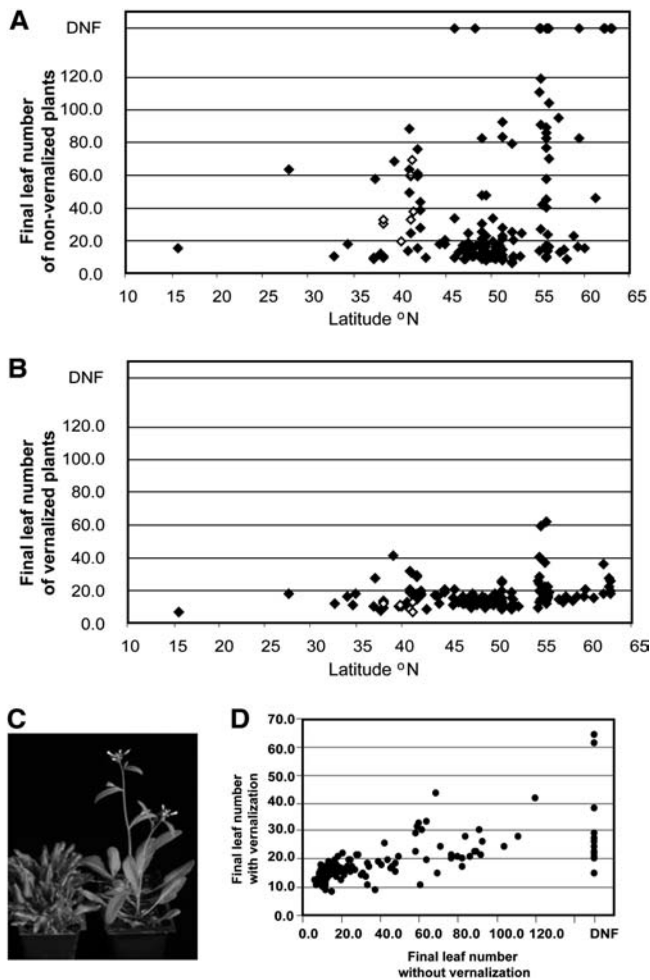

Figure 1. Variation of flowering time among Arabidopsis accessions. Flowering time was assayed by the FLN. A, The relationship between FLN of nonvernalized plants and latitude of collection site. B, FLN of vernalized plants. For white diamonds, see details in the main text. C, Plants grown in long-day conditions without vernalization. A very-late-flowering accession from Sweden (left) compared to an early-flowering Col-0 (right). D, FLN without vernalization plotted against FLN with vernalization to show variation in vernalization response. DNF represents accessions that did not flower without vernalization.

there were accessions with apparently reduced response to vernalization. For example, six accessions collected in the United States from about 40°N (Yo-0, Dem-4, Knox-10, RRS-10, RMX-A02, and PNA-10) flowered with FLN > 30 even after an 8-week cold treatment.

Relationship of *FLC* RNA Levels with Vernalization Requirement and Response

Previous work using a limited number of genotypes suggested that there is a tight correlation between the level of *FLC* expression and FLN (−V; Sheldon et al., 2000). Therefore, the level of *FLC* mRNA in nonvernalized plants for all the accessions was examined to see whether this relationship holds true in nature. There was a wide range in *FLC* transcript abundance (Fig. 2A). Alternative *FLC* transcripts were not observed. As expected, flowering time of nonvernalized plants did correlate significantly with *FLC* level ($P < 0.001$; Fig. 2B). However, this correlation was not as tight as expected as there was a considerable range in

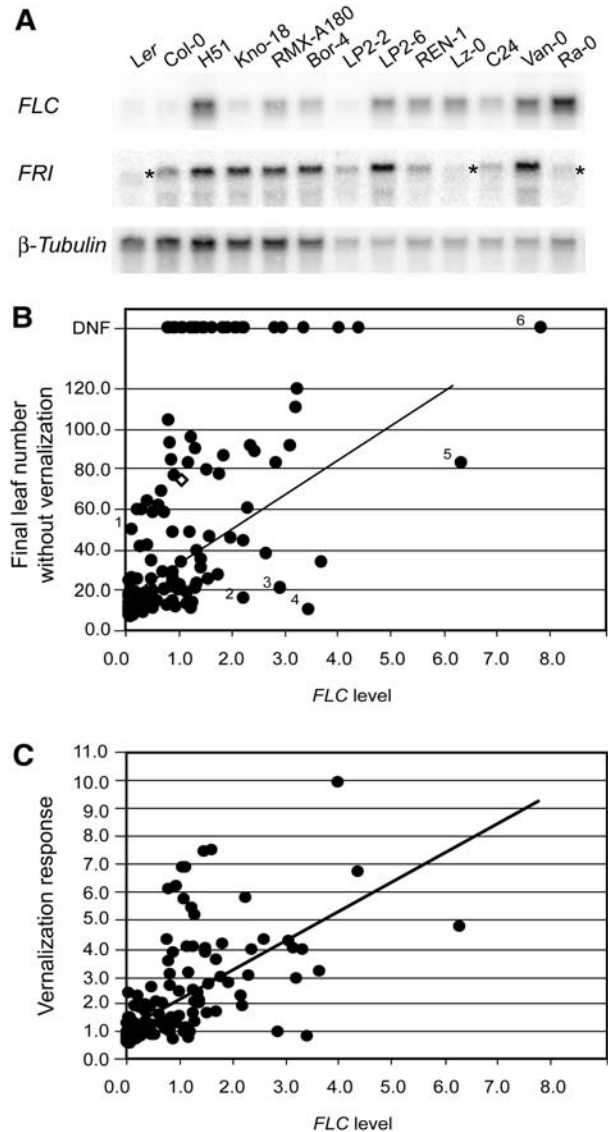

Figure 2. A, Expression levels of *FLC* and *FRI* genes detected by northern hybridization in total RNA from nonvernalized plants. Expression levels were shown as relative to our standard accession H51. *, Very low level of *FRI* transcripts in the accessions that carry the L*er*-type deletion, as reported by Johanson et al. (2000). B, Relationship between *FLC* expression level and flowering time of nonvernalized plants. The regression line was calculated without DNF accessions ($y = 16.233x + 17.628$; $R^2 = 0.331$; $P < 0.001$). The white diamond shows the value of the control accession H51. 1, Kno-18; 2, Cvi-0; 3, Ag-0; 4, Ra-0; 5, Br-0; 6, N6034. C, Relationship between vernalization response and *FLC* expression level in nonvernalized plants. Vernalization response was measured as the ratio of flowering time without and with an 8-week cold treatment. For DNF plants, we calculated the value by assigning FLN = 150.0. The regression line was $y = 1.0662x + 1.0864$; $R^2 = 0.3771$; $P < 0.001$.

FLC level for a given FLN (−V). For example, Knox-18, originating from the United States, was relatively late flowering (49.7 leaves), although its *FLC* level was very low (0.068; Fig. 2B, datapoint 1). By contrast, Cvi-0, Ag-0, and Ra-0 flowered very early but had relatively high *FLC* expression (Fig. 2B, datapoints 2–4). Interestingly, there seems to be a threshold effect because no very-late-flowering or DNF accessions had *FLC* levels below 0.7.

To some extent, variation in *FLC* expression appears to be related to the site of collection (Supplemental Fig. 2). For example, a subgroup of U.S. accessions flowered relatively late but had low *FLC* expression (<0.7). Three of these accessions (RRS-10, Knox-10, and PNA-10) are very closely related based on genome-wide polymorphism data (Nordborg et al., 2005) and are thus likely to have a similar phenotype because of shared alleles. However, Knox-18, which was discussed above, appears to be genetically distinct from these accessions. Accessions from France showed great variation in relationship between FLN and *FLC* (Supplemental Fig. 2), suggesting that the molecular mechanisms controlling *FLC* expression and its effect might be widely divergent among French accessions.

The vernalization response (measured as the ratio of FLN without and with vernalization) is also significantly correlated with *FLC* expression (Fig. 2C; $P < 0.001$). However, dividing the data based on FLN (−V) into early, late, and DNF (Supplemental Fig. 3) revealed a stronger association between *FLC* expression and vernalization response in earlier flowering (<50 FLN) accessions than in later flowering (>50 FLN) accessions ($P < 0.001$ and $P = 0.003$, respectively). The DNF class showed no linear correlation between *FLC* levels and vernalization response, which may be an artifact of using an FLN (−V) of 150 for this calculation.

Sequence Variation in *FRI*

To pursue the role of *FRI* allelic variation further, the *FRI* gene from all 192 accessions used in this study was sequenced. This covered approximately 550 bp of the 5′-untranslated region, the entire coding region, including introns, and approximately 220 bp of the 3′-untranslated region. Across all accessions, 93% of the target sequence was obtained, and it was possible to assign a *FRI* haplotype in almost all cases. Previous reports have shown that allelic variation at *FRI* has a major effect on flowering time in natural accessions (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003; Stinchcombe et al., 2004). Given that the major function of *FRI* is to up-regulate *FLC*, we determined whether each *FRI* allele was likely to be functional by analysis of *FLC* expression levels, together with prediction of the effect of specific mutations in *FRI* (Fig. 3, A and B).

A total of 20 haplotypes were identified that are predicted to cause nonfunctional *FRI* alleles. These included the deletions observed in L*er* and Col-0, previously reported by Johanson et al. (2000; Fig. 3A, b–e and l, respectively). In the L*er* haplotype, a 375-bp deletion over the promoter region of *FRI* and a 31-bp insertion were observed, and *FRI* transcripts were barely detectable in northern hybridization experiments (Fig. 2A). Among the accessions, four groups of L*er* haplotypes were identified; each group contains the typical L*er* insertion/deletion, together with a further nucleotide change diagnostic for each group. In addition to the Col and L*er* types, other haplotypes had single-nucleotide changes that gave in-frame translation stop codons and/or small insertions or deletions that caused frameshifts that led to premature translation termination. Le Corre et al. (2002) and Gazzani et al. (2003) have reported some of the same mutations and polymorphisms, but an additional nine mutations that are likely to give nonfunctional *FRI* alleles have been identified here. In these putatively nonfunctional haplotypes, *FRI* transcripts of the same size as the intact *FRI* were detected; however, expression levels were highly variable (data not shown).

Accessions with nonfunctional *FRI* haplotypes are typically expected to have low *FLC* levels. Some haplotypes, including the Col-type (Fig. 3B, white squares), caused consistently low *FLC* expression. However, accessions with the L*er* haplotypes showed a wide range of *FLC* levels, three of which were particularly high (Ka-0, Lz-0, and Ra-0; Fig. 3B, white/black circles). Other accessions with nonfunctional *FRI* haplotypes also had high *FLC* levels. These data implicate variation in other genes causing up-regulation of *FLC*. Interestingly, not all the accessions with high levels of *FLC* flowered late, suggesting that their *FLC* transcript may be nonfunctional or there may be variation in other loci that affect FLC action.

In addition to the clear loss-of-function mutations, changes leading to amino acid polymorphisms in the *FRI* sequence were frequently observed (Fig. 3A, 1–31). The majority of these polymorphisms were clustered within exon 1 (1 change/50 bp), confirming that this region is hypervariable, as previously reported (Le Corre et al., 2002). In particular, four amino acid changes (nos. 5–8) were identified in the first coiled-coil region of the FRI protein. Computational analysis of the protein sequence suggested that changes numbered 6 and 7 might lower the probability of the coiled-coil structure (data not shown).

A haplotype tree was constructed based on the association of both synonymous and nonsynonymous amino acid changes and the loss-of-function mutations. In conjunction with the flowering-time and *FLC* expression data, this tree was used to generate hypotheses about the likelihood of specific amino acid changes modifying or causing the loss of function of the FRI protein (Supplemental Fig. 4, A and B). For example, 20 accessions, which only carry the number 16 and 6 polymorphisms, showed a wide range in *FLC* levels, including accessions with high transcript abundance. This suggested that these amino acid changes do not disrupt FRI function (although it should be noted that *FLC* levels are also highly variable

Figure 3. A, Polymorphisms in the amino acid sequence of the FRI protein. The protein sequence of each accession is compared to that of H51. The letters (a–t) above the sequence show the major mutations that disrupt protein function. Single substitutions of amino acids within the sequence are indicated with numbers (1–31). The underlined sequences indicate the two coiled-coil domains. ↓, Amino acid substitutions; ▲ and ▼, insertion and deletion of nucleotides, respectively; and STOP, the premature stop codons. Accessions containing each polymorphism are shown in Supplemental Figure 4, A and B. B, Variation in *FLC* expression level among accessions with putative nonfunctional *FRI* haplotypes. ○, Accessions with L*er*-type deletion (b–e in A); □, accessions with the Col-type deletion (l in A); △, accessions with the other nonfunctional haplotypes. Black symbols indicate the very-late-flowering accessions.

among accessions carrying loss-of-function mutations). Similarly, high levels of *FLC* variation in other *FRI* haplotypes suggest that, in many cases, low *FLC* expression might be caused by variation in *FLC* itself or loci other than *FRI*.

Three accessions (Wil-2, Wa-1, and Tottarp-2) carrying *FRI* with polymorphism numbers 6, 16, and 21 all showed low *FLC* expression. F_1 plants, generated from crosses between these accessions and Col-0, flowered very early, indicating that they carry a nonfunctional or weak *FRI* allele or possibly a dominant suppressor of *FRI* (Supplemental Table I). Since only amino acid change number 21 was specific to them, this polymorphism may define a functionally important domain within the FRI protein.

Relationship between *FRI* Alleles and Variation in *FLC* Level and Flowering Time

Overall, for 176 accessions, complete data for *FRI* haplotype, flowering time, and *FLC* level were obtained. Of these, 104 accessions were predicted to carry *FRI* alleles that are likely to be functional (i.e. do not have obvious loss-of-function mutations), whereas 72 carried potentially nonfunctional *FRI* alleles (Fig. 4). Among accessions with functional *FRI* alleles, flowering time and *FLC* level varied widely and to a greater extent than in accessions with nonfunctional *FRI* alleles. Some accessions with functional *FRI* alleles (30 out of 104) behaved like rapid-cycling accessions, such as Col-0 and L*er*-1, i.e. they flowered very early (<20 FLN), showed little vernalization response, and/or showed very low levels of *FLC* expression (<0.3; Supplemental Fig. 5). These accessions may carry a weak *FLC* allele or have nonfunctional *FLC* transcripts. As mentioned above, in each analysis there were some accessions with an exceptional flowering phenotype or unexpected *FLC* expression levels. However, most accessions with highly expressed *FLC* carried functional *FRI* alleles. Indeed, we estimate that 74 out of 89 (approximately 83%) of the late-flowering accessions in this study had a functional *FRI* allele. Similarly, most early-flowering accessions have predicted nonfunctional *FRI* alleles and low *FLC* levels (47 out of 87; approximately 54%). Thus, we conclude that allelic variation at *FRI* is a major determinant of flowering-time variation.

As discussed above, there was no simple latitudinal cline in FLN (−V and +V) when all the accessions were used in this analysis (Fig. 1, A and B). It was interesting to determine whether taking into account both *FRI* function and *FLC* allelic type would reveal a correlation between FLN and latitude, as has been reported previously (Caicedo et al., 2004; Stinchcombe et al., 2004). First, late-flowering accessions with a functional *FRI* allele were analyzed (very early accessions were excluded as they are predicted to carry a weak *FLC* allele or nonfunctional transcripts). Figure 5, A and C, shows FLN (−V) and (+V) of these accessions plotted against the latitude of their collection sites. Second, the same analysis was carried out for the accessions carrying nonfunctional *FRI* alleles and those carrying putatively weak *FLC* alleles (Fig. 5, B and C). There was no significant correlation between FLN and latitude in either case.

DISCUSSION

Flowering time has a critical role in determining reproductive success, and, therefore, it has been featured in many ecological and evolutionary studies. In this research, we focused on the natural variation in the flowering time of Arabidopsis and the role of two key genes that control the flowering transition, *FRI* and *FLC*. A high level of variation among the accessions has been described here for both flowering time (with and without vernalization) and in degree of vernalization response (reduction in FLN caused by extended cold treatment). Furthermore, analysis of the *FRI* sequence has revealed considerable genetic variation at this locus, and this has been shown to make an important contribution to natural variation in flowering time.

Previous studies have shown that early-flowering rapid-cycling accessions have evolved on multiple

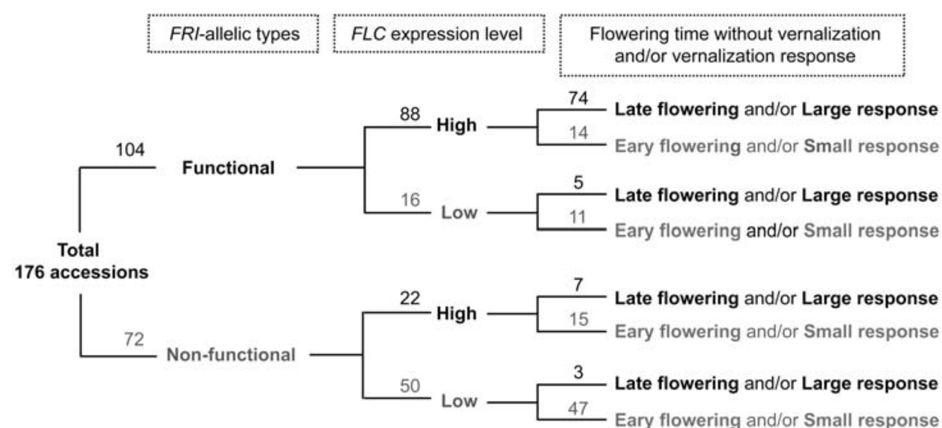

Figure 4. Number of accessions with functional or nonfunctional *FRI* and their *FLC* expression level and flowering time.

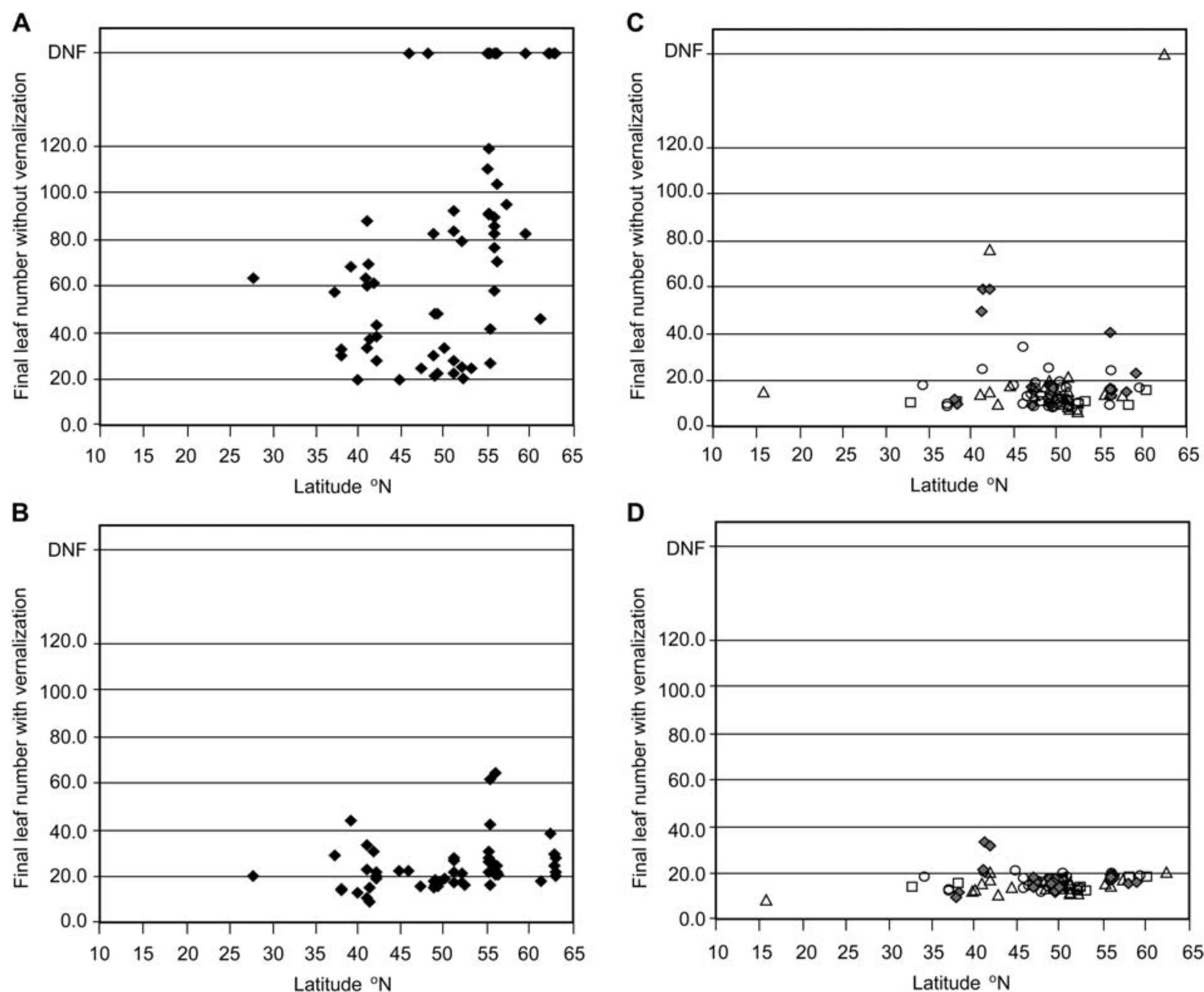

Figure 5. The effect of the *FRI* and *FLC* allelic types on the relationship between flowering time and latitude. A and B, Variation of flowering time in nonvernalized and vernalized accessions carrying the functional *FRI* and *FLC* alleles. C and D, Variation in flowering time in nonvernalized and vernalized accessions with nonfunctional *FRI* alleles or weak *FLC* alleles. ♦, Accessions with functional *FRI*/*FLC* alleles; ○, with L*er*-type *FRI* alleles; □, with Col-type *FRI* allele; △, with other nonfunctional *FRI* alleles; gray diamonds, with the putative weak *FLC* allele.

independent occasions through loss of *FRI* function (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003). Consistent with these results, this study revealed that evolution of early-flowering types has been caused by multiple loss-of-function mutations in the *FRI* gene, and nine loss-of-function *FRI* alleles have been described here.

It was interesting to analyze the geographical distribution of the different nonfunctional *FRI* alleles because this could provide a model to understand the factors leading to their maintenance in the population. The L*er*-type deletion was the most common nonfunctional *FRI* allele; it was found in four different *FRI* haplotypes in accessions from western Europe, Russia, southern Sweden, and the United States (Fig. 6). The Col-type deletion, which was the next most frequent nonfunctional *FRI* allele, was mainly found in northern Germany. By contrast, the other nonfunctional *FRI* alleles were found in a single or a few accessions collected from more peripheral European countries, such as those in the Iberian Peninsula. These rarer, nonfunctional *FRI* alleles are likely to have arisen more recently than the abundant alleles. It is noteworthy that no L*er*- or Col-type alleles were detected in accessions in the Iberian Peninsula, but that there were four accessions with rare nonfunctional *FRI* alleles from this region.

In addition to the *FRI* gene, its target *FLC* also plays an important role in natural variation of flowering time. Natural variation of this gene has been described in several early-flowering accessions/genotypes. For example, the *FLC* locus of L*er*-1 and Da (1)-12 contains a transposon insertion in the first intron that perturbs function (Gazzani et al., 2003; Michaels et al., 2003). Although we did not examine the molecular variation per se in *FLC* in this study, many accessions with functional *FRI* alleles were predicted to carry the putative weak *FLC* alleles because they were early

Figure 6. The geographical distribution of the nonfunctional *FRI* and weak *FLC* alleles. ○, Accessions with L*er*-type deletion; □, accessions with the Col-type deletion; △, accessions with other nonfunctional *FRI* alleles; gray diamonds, accessions with putative weak *FLC* alleles.

flowering and/or had very low *FLC* levels. Accessions with these putative weak *FLC* alleles were found frequently in central Asia, eastern Europe, southern Sweden, and the United States (Fig. 6). It should be noted that it is also possible that some early-flowering accessions carry weak *FLC* alleles as well as a nonfunctional *FRI* allele (as has been previously described for L*er*-1). In central Asia, there are no accessions with a nonfunctional *FRI* (based on our sequence analysis), suggesting that evolution of early flowering in this region had been caused exclusively by variation at *FLC* or other loci. Interestingly, the weak *FLC* alleles in accessions from eastern Europe are likely to have originated independently of the Asian accessions because only the eastern European accessions contain an insertion in the first intron of *FLC* (M. Nordborg, unpublished data). Therefore, like *FRI*, earliness due to allelic variation in *FLC* is likely to have occurred independently multiple times.

It is striking that these independent loss-of-function *FRI* mutations and weak *FLC* alleles have been maintained and propagated during the divergence of Arabidopsis. This implies that these mutations confer a selective advantage by enabling a rapid-cycling strategy that increases reproductive fitness in certain environmental conditions. In this study, early-flowering accessions were found to predominate in central and northern Europe and very-late-flowering accessions were nonrandomly distributed in certain regions of the Scandinavian Peninsula. A future challenge will be to elucidate the selection pressures that have led to the evolution of rapid-cycling ecotypes and to understand the environmental variables that determine whether a rapid-cycling or winter-annual habit is best adapted.

Latitude provides a crude measure of environmental conditions, but, in this study, no significant latitudinal cline was found in flowering time. This is in direct contrast to recent reports of a latitudinal cline dependent on *FRI* allelic type (Stinchcombe et al., 2004). This conflict in results could be attributed to differences in accession sets or experimental design. Arguably, the lack of a strong latitudinal cline is not surprising given that environmental conditions will vary considerably at a given latitude and plants are most likely to adapt to local habitats.

Previous studies have tried to find associations between flowering time and more specific environmental variables. For example, McKay et al. (2003) reported a highly significant positive correlation between dehydration avoidance and flowering time. In our study, no strong relationship was found between flowering time and minimum winter temperature, average length of winter (weeks under 5°C), or precipitation in January and July (data not shown). In the future, more detailed ecological experiments may shed light on the selective advantage that underlies the different flowering strategies.

Consistent with previous findings, many early-flowering accessions had loss-of-function *FRI* mutations and low *FLC* levels, and many late-flowering types had active *FRI* alleles and high *FLC* levels. However, this study revealed considerable variation in the relationship between *FRI* functionality, *FLC* level, and flowering time. For example, *FLC* expression varied widely in all accessions with nonfunctional *FRI* alleles (with the interesting exception of those carrying the Col-type allele that all had low *FLC* levels). This could reflect recent variation in genes that control *FLC* expression, which has arisen since the loss-of-function mutation in *FRI*. Loss-of-function mutations in *FLC* repressors found in the autonomous pathway (*FCA*, *FVE*, *FY*, *FPA*, *LD*) might have caused this *FRI*-independent up-regulation of *FLC*.

Exceptional accessions, including early-flowering types with high *FLC* levels or late-flowering accessions with low *FLC* levels, also indicate that there is wide variation in flowering-time genes such as *FRI* suppressors, genes that act like *FLC* to repress flowering, genes downstream of *FLC*, and other modifier loci. For example, two closely related French accessions,

Lz-0 and Ra-0, had exceptional flowering-time characteristics. Lz-0 has a L*er*-type deletion at *FRI*, but high *FLC* levels, and flowered relatively late, whereas Ra-0 has the same *FRI* deletion and high *FLC* levels, but flowered early. It is possible that they have a mutation that causes *FRI*-independent up-regulation of *FLC*, but that Ra-0 has a defective *FLC* transcript or gene(s) acting downstream of *FLC*. Further molecular analysis will be required to understand this complicated exception observed in two accessions with a similar genetic structure.

Another outstanding phenotype was found in several North American accessions that flowered late despite low *FLC* expression levels. These accessions may therefore have a gene that functions like *FLC* to cause late flowering. The genome-wide SNP data suggest that the accessions from North America are closely related to accessions in the United Kingdom, France, and Germany and are therefore likely to be recent introductions. Interestingly, none of the accessions that were analyzed from these countries were found with this late-flowering/low-*FLC* phenotype, and only two accessions of this type were found in southern Sweden (Lis-1 and Hovdara-6). It is possible that a very rare accession with this late-flowering/low-*FLC* phenotype was introduced into the New World and then propagated due to a bottleneck effect.

A major aim of future research will be to identify the loci that are contributing to the variation in the relationships between flowering time, *FLC* level, and *FRI* function. A good starting point to characterize this genetic variation is the collection of accessions from Sweden with diverse flowering behaviors. The many different loss-of-function *FRI* and *FLC* alleles present in these accessions suggest that early-flowering variants had accumulated in this geographical region either through migration of accessions from northern Europe already containing nonfunctional/weak alleles or by the accumulation of new mutations after colonization. The considerable range of flowering-time variation in southern Swedish accessions suggests that Arabidopsis colonization of this region originated from a number of different sources. The recent report by Stenøien et al. (2005) with Norwegian accessions supports this idea. Interestingly, the early-flowering and very-late-flowering accessions were often found in close proximity to each other. This could be due to genetic drift, selection for flowering variants in different microclimates, or the result of balancing selection maintaining both phenotypes because of differing climates from year to year.

This study has revealed a vast amount of natural variation in flowering strategies of Arabidopsis. We have shown the importance of variation in two key genes, *FLC* and *FRI*, on flowering time in the wild. In the future, quantitative trait loci mapping will be a powerful approach to uncover the other loci that contribute to naturally occurring flowering-time variation. New molecular data, including genome-wide SNP information, and more detailed ecological studies will enable us to look more widely at the molecular basis of natural diversity in flowering time and to see how this diversity has evolved.

MATERIALS AND METHODS

Plant Materials

The 192 Arabidopsis (*Arabidopsis thaliana*) accessions used were coordinated by M. Nordborg. The first set of 96 accessions was derived both from the stock centers or originally collected by J. Bergelson, M. Kreitman, and M. Nordborg. Their genome-wide SNP data, which has been produced by the National Science Foundation Arabidopsis 2010 project "A genomic survey of polymorphism," are available at the Web site http://walnut.usc.edu/2010.html. For determination of *FRI* functionality, certain accessions were reciprocally crossed with Col-0.

Plant Growth

Seeds were sown on soil in plastic pots (7 cm × 7 cm) and stratified in the dark at 4°C for 3 d. For vernalization, 7-d-old seedlings were grown in the cold room (4°C, constant humidity, 8-h light). After the 8-week vernalization treatment, plants were moved to the greenhouse with supplemental lighting to give a 16-h photoperiod. Seeds for nonvernalized plants were sown in the same way as described above for vernalized plants, but 10 d before the end of the vernalization period, so that both vernalized and nonvernalized plants were grown in the greenhouse simultaneously. Young seedlings were transferred to trays with 40 cells of 2 cm × 2 cm. The trays were moved regularly to random positions to prevent any positional effects on plant growth. Flowering time was measured by counting FLN, which was scored as the number of rosette leaves plus cauline leaves.

RNA Extraction

Total RNA was extracted from young leaves after 4 weeks of growth in the greenhouse. RNA extraction was performed using the TRIzol Reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Approximately 10 μg of total RNA were fractionated on 1.2% (w/v) formaldehyde-agarose gels and blotted onto Hybond N^+ nylon filters (Amersham Biosciences, Buckinghamshire, UK). The RNA gel blot was probed with a ^{32}P-ATP-labeled FLC probe (lacking the MADS box domain). After stripping in boiling 0.5% (w/v) SDS, the same blot was rehybridized with a *FRI* cDNA probe containing exon 1 only. Blots were exposed to PhosphoImager screens (Molecular Dynamics, Sunnyvale, CA). To normalize the relative intensities of *FLC* and *FRI* mRNA, stripped blots were rehybridized and probed with the β-*TUBULIN* coding region. The normalized intensities of each accession were divided by those of our standard accession H51 for analysis.

Statistical Analysis

Flowering time, which was measured by counting FLN, was calculated as the average of 20 plants. Correlation coefficients were calculated using Microsoft Excel or Genstat (Genstat 5 Committee, 1993).

Sequencing *FRI* Genes and Data Analysis

Genomic DNA of 192 accessions was extracted from young leaves using the DNeasy plant mini kit (Qiagen, Valencia, CA). The *FRI* gene (including 544 bp upstream of ATG and 219 bp downstream of the stop codon) was sequenced, in both directions, on Beckman CEQ sequencers (Fullerton, CA). The published *FRI* sequence of the ecotype H51 (GenBank accession no. AF228499; Johanson et al., 2000) was used for haplotype analysis. The analyzed region was located between nucleotide positions 30 and 3,104 of H51 (AF228499).

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