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FRIGIDA-Independent Variation in Flowering Time of Natural *Arabidopsis thaliana* Accessions

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ABSTRACT

FRIGIDA (*FRI*) and *FLOWERING LOCUS C* (*FLC*) are two genes that, unless plants are vernalized, greatly delay flowering time in *Arabidopsis thaliana*. Natural loss-of-function mutations in *FRI* cause the early flowering growth habits of many *A. thaliana* accessions. To quantify the variation among wild accessions due to *FRI*, and to identify additional genetic loci in wild accessions that influence flowering time, we surveyed the flowering times of 145 accessions in long-day photoperiods, with and without a 30-day vernalization treatment, and genotyped them for two common natural lesions in *FRI*. *FRI* is disrupted in at least 84 of the accessions, accounting for only ~40% of the flowering-time variation in long days. During efforts to dissect the causes for variation that are independent of known dysfunctional *FRI* alleles, we found new loss-of-function alleles in *FLC*, as well as late-flowering alleles that do not map to *FRI* or *FLC*. An *FLC* nonsense mutation was found in the early flowering Van-0 accession, which has otherwise functional *FRI*. In contrast, Lz-0 flowers late because of high levels of *FLC* expression, even though it has a deletion in *FRI*. Finally, eXtreme array mapping identified genomic regions linked to the vernalization-independent, late-flowering habit of Bur-0, which has an alternatively spliced *FLC* allele that behaves as a null allele.

A plant's decision to initiate reproductive development is an important event that is controlled by the intersection of an endogenous program with environmental factors such as temperature and light. Biotic and abiotic factors affecting plant growth and survival are variable across habitats, requiring plants to specialize in sensing environmental stimuli and adapting their development accordingly. Presumably, natural selection has optimized this program to time the transition to flowering to provide optimal fitness in a given environment. Indeed the existence of latitudinal clines in many species, including *Arabidopsis thaliana*, is strongly indicative of adaptive variation in flowering (Mikola 1982; Hurme *et al.* 1997; Van Dijk *et al.* 1997; Stinchcombe *et al.* 2004) or light response (Maloof *et al.* 2001).

The study of floral induction in *A. thaliana* has long been approached through the isolation and characterization of mutants with altered flowering times. A large number of flowering-time genes have been identified and a scaffold of the underlying molecular network has been constructed (for review, see Mouradov *et al.* 2002; Simpson and Dean 2002; Searle and Coupland 2004; Sung and Amasino 2004). As may be expected, this network is complex. On the basis of an extensive body of physiological, genetic, and molecular studies, four main pathways that regulate the key floral identity genes have been outlined: photoperiod, vernalization, autonomous, and gibberellin. The photoperiod pathway utilizes photoreceptors in conjunction with the circadian clock to strongly accelerate flowering in the presence of long-day photoperiods. Flowering is also accelerated when plants with high levels of the floral inhibitor *FLC* are exposed to an extended period of winter-like temperatures. This process, called vernalization, causes epigenetic silencing of the *FLC* locus and thereby relieves repression of flowering. Independently of vernalization, *FLC* is also negatively regulated by the autonomous pathway, which was originally thought to function independently of the environment. Recently, however, it has been found that this pathway may also mediate response to ambient growth temperature (Blázquez *et al.* 2003).

Sequences data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY781906–AY785055, AY849982–AY850002, AY854371, AY854372, and AH014566.

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Finally, hormones of the gibberellin class promote flowering in the absence of positive cues from the photoperiod pathway.

The MADS-domain transcription factor encoded by *FLC* plays a central role in establishing the annual winter habit of many *A. thaliana* accessions. When active alleles of a second gene, *FRI*, are present, high levels of *FLC* potently inhibit the floral transition in a dosage-dependent manner until these plants have been vernalized (Michaels and Amasino 1999; Sheldon *et al.* 1999). In the wild, this combination of factors has been presumed to prevent germinated seedlings from flowering in the fall.

Conversion of winter annual to summer annual strains can result from natural variation at either *FRI* or *FLC*, as loss-of-function mutations in either gene eliminate the late-flowering phenotype. In accessions collected from the wild, variation at the *FRI* locus is common; to date, at least nine different lesions at *FRI* have been reported (Johanson *et al.* 2000; Le Corre *et al.* 2002; Gazzani *et al.* 2003). Two deletions first detected in the Columbia (Col) and Landsberg *erecta* (L*er*) laboratory strains are frequently found in other accessions, leading to the conclusion that *FRI* is the predominant locus controlling flowering-time variation in native *A. thaliana* (Johanson *et al.* 2000; Le Corre *et al.* 2002). In addition, a smaller fraction of accessions seems to flower early because of weak alleles at the *FLC* locus (Gazzani *et al.* 2003; Michaels *et al.* 2003).

From the current framework, it appears that wild accessions of *A. thaliana* should fall into two distinct classes. The first group, which lacks *FLC* activity either because of a lesion in *FRI* or because of a weak *FLC* allele, should flower relatively early. The second group, possessing high levels of *FLC* activity, should flower very late even in long days, unless vernalized. Here, we have sought to estimate the amount of variation among wild accessions that is independent of *FRI* and to identify additional loci in wild accessions that influence flowering time. To this end, we have surveyed the flowering times of 145 single-seed descent accessions in long-day photoperiods, with and without a 30-day vernalization treatment, and genotyped these accessions for the Col-type and L*er*-type *FRI* deletions.

We report substantial variation in flowering time that appears to be independent of *FRI* in accessions both with and without functional *FRI* alleles. While a large majority of the late-flowering accessions respond to vernalization, suggesting that much of the variation seen in this survey acts through *FLC*, there is evidence for late-flowering accessions that were not due to *FLC* expression. Furthermore, we characterized several accessions with interesting flowering-time phenotypes, given their *FRI* status to determine the genetic and molecular origins of their flowering-time phenotypes. Through genetic mapping and sequencing or genotyping loci known to affect flowering time, we identified novel loss-of-function *FRI* and *FLC* alleles as well as mapped late-flowering loci that are not allelic to *FRI* or *FLC*.

MATERIALS AND METHODS

Plant material: A list of accessions and their phenotypes can be found at http://naturalvariation.org/werner. Most accessions were obtained from the Arabidopsis Biological Resource Center or Lehle Seeds. The L*er*K and CviK accessions are parents of the L*er*/Cvi recombinant inbred line set in which the *EDI* allele of *CRY2* was identified (El-Assal *et al.* 2001) and were kindly provided by Maarten Koornneef (Wageningen, The Netherlands). Combinations of functional and nonfunctional *FRI* and *FLC* alleles in Col (Lee *et al.* 1994b; Michaels and Amasino 2001) were kindly provided by Rick Amasino (Madison, WI). All lines were propagated prior to genotypic and phenotypic analysis.

Growth conditions: Seeds were suspended in 1 ml of 0.1% phytagar (Invitrogen, San Diego) and imbibed at 4° in darkness for 4 days. Seeds were then sown onto presoaked Sunshine Mix no. 5 (McConkey, Garden Grove, CA) and thinned after 5 days. Plants were grown in a growth room at 22° under 16 hr of light provided by a 3:1 mixture of Cool-white and Gro-Lux (Sylvania) fluorescent bulbs, followed by 8 hr of darkness. For the long-day surveys, six plants for each line were raised in one pot, except for several control genotypes grown in three blocks of six plants. Over the course of the experiment, the pots were randomized across all flats several times and flats were rotated across and between shelves on a daily basis. Vernalization treatments were performed at the seedling stage, when 5-day-old plants were transferred from the growth room to incubators (Percival, Boone, IA) and kept under noninductive short-day photoperiods, consisting of 9 hr of light followed by 15 hr of darkness at 4°. After 30 days, plants were returned to the growth rooms. A replicate experiment with three blocks of six plants was performed for 31 accessions using newly harvested seeds. Plants used for RNA extractions were grown similarly, except at densities of 12–15 plants/pot.

For all F_1, F_2, and BC_1 populations, seeds suspended in 0.1% phytagar were sown onto soil, allowed to germinate, and thinned (in an unbiased manner) to six seedlings per pot. Flats were rotated across and between shelves on a daily basis and pots were randomized frequently.

Flowering-time measurements: Flowering time was measured both as total leaf number (TLN) and as days to flowering (DTF). Rosette leaf number and cauline leaf number on the main shoot were determined independently and added to yield TLN prior to formation of the first flower. DTF was recorded daily as the time from the date of sowing (and minus 30 days for vernalization treatment) until the first appearance of floral buds in the apex as seen by the unaided eye. Within a given genotype, we found a high correlation between TLN and DTF. Consequently, for some F_1 and F_2 experiments, only DTF was recorded to simplify data collection and allow better seed set for later analysis.

DNA sequencing: For all genes, at least three independent PCR reactions with genomic DNA or cDNA as a template were pooled, gel purified, and used as templates for direct sequencing. The sequenced regions are indicated in Table 1.

RNA analysis: RNA from 14-day-old seedlings grown in long-day periods was isolated using the RNeasy plant mini kit (QIAGEN, Chatsworth, CA). Ten micrograms of total RNA was separated in a 1% agarose gel and blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech). The membrane was hybridized with a random-labeled probe covering 38205 to 38535 of the *FLC* gene (GenBank accession no. AL356332) and hybridization signal was determined with a phosphorim-

TABLE 1

Sequenced regions in flowering-time genes

Gene (At ID)	Genomic/cDNA	GenBank accession no.	Region (nt)
FCA (At4g16280)	Genomic	ATZ82992	1485–2454, 4317–8841
FVE (At2g19520)	Genomic	AF498101	2337–5590
FPA (At2g43410)	Genomic	AT2G43410.1	1–4593
LD (At4g02560)	Genomic	AT4G02560	123–4828
FRI (At4g00640)	Genomic	AF228499	40–3356
FLD (At3g10390)	Genomic	AC011560	115506–118107
		AC009400	1517–2236
FLK (At3g04610)	Genomic	AC011437	28984–33405
FLC (At5g10140)	cDNA	AF116527	29–802

ager (Molecular Dynamics, Sunnyvale, CA). ImageQuant software (Molecular Dynamics) was used to quantify the signal strength. The blot was then stripped and rehybridized with a probe to 25S rRNA in a similar manner.

First-strand cDNAs were synthesized using the Reverse Transcription System (Promega, Madison, WI) starting with ~1 μg of total RNA. RT-PCR for the *FRI* locus used primers JW159 (5′-AGG GCG TAG AGC ATT TAC-3′) and JW22 (5′-CTT GTG AGT CTC CAT ACA CTG-3′).

DNA genotyping: A list of all PCR-based markers can be found at http://naturalvariation.org/werner.

eXtreme array mapping: A total of 561 F_2 individuals from the cross of Lz-0 with Col were grown in long-day periods and their flowering-time phenotypes were measured as DTF. Two leaves of similar size were collected from the 50 earliest and the 50 latest plants, yielding duplicate early and late pools. Similar amounts of tissue were collected from the parental lines. All tissue was flash frozen in liquid nitrogen and ground to a fine powder, and DNA was extracted according to a modified version of the standard CTAB protocol scaled up to 15 ml of 2× CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM TrisHCl, pH 8.0), followed by three rounds of phenol/chloroform extraction and ethanol precipitation. Three random-priming labeling reactions were prepared for each sample using the Bioprime labeling kit (Invitrogen) starting with ~400 ng of genomic DNA and labeling overnight at room temperature. For each sample, the three reactions were pooled, ethanol precipitated, and ~20 μg of DNA was hybridized to an Affymetrix ATH1 array as outlined in standard Affymetrix protocols for RNA. In total, 10 samples were prepared, hybridized, and analyzed: three replicates of Lz-0, three replicates of Col, and two replicates each for the early and late-flowering pools. Analysis of the hybridization data was similar to that described previously (Wolyn *et al.* 2004). For the Bur-0 × *flc*-3 F_2 population (n = 330), the 65 earliest and the 65 latest plants, as measured by days to flowering, were pooled and processed similarly. A total of eight samples were prepared and hybridized: three replicates of Bur-0 and *flc*-3 and one sample each for the pools.

Lz-0 × L*er* QTL mapping: A total of 178 F_2 plants from the cross of Lz-0 to L*er* were grown in long days and genotyped for 32 markers distributed across all five chromosomes with an average distance of 15 cM, and a genetic map was determined using MapMaker/EXP 3.0 (Lander *et al.* 1987). Marker order was as expected from the physical locations of the markers in the Col reference sequence.

For QTL analysis presented in the text, composite interval mapping was performed with QTL Cartographer 1.16c (Basten *et al.* 2002) using model 6 with a window size of 4.00 cM and a 1.00-cM walking speed. Markers ciw1, nga172, *FLC*, and F5O24 were selected as background markers. The significance threshold, 3.06, was calculated as the 95th percentile of top LOD scores for 1000 permutations of the phenotypic data. Additional QTL mapping was done utilizing the bqtl package (http://hacuna.ucsd.edu; Borevitz *et al.* 2002) developed for the statistical package R (http://www.R-project.org; Ihaka and Gentleman 1996). Results from these analyses are available at http://naturalvariation.org/werner.

Bur-0 × *flc-3* QTL mapping: A random sample of 288 F_2 plants from the same population that was analyzed by eXtreme array mapping (XAM) was genotyped for 16 markers located at sites identified by XAM. Ninty-six of the plants were also genotyped for two additional markers. As with the Lz/L*er* population, these genotypes were used to construct a genetic map and QTL were identified using bqtl. The results of this analysis are available at http://naturalvariation.org/werner.

Estimates of QTL effects: QTL effects for the Van-0 × L*er* F_2, Lz-0 × L*er* F_2, and Bur-0 × *flc-3* (Col) F_2 populations were calculated using the bqtl package. For the Van-0 × L*er* F_2 population, the final QTL model included additive and dominance main-effect terms for the markers FRI, HU13 (near *FLC*), and nga139 (near FLG; Alonso-Blanco *et al.* 1998) and the interaction terms FRI:HU13 and nga139:HU13. In the case of Lz-0 × L*er* F_2, we included additive and dominance main terms for ciw1, nga172, FLC, and F5O24, and the interaction terms *FLC*:F5O24 and ciw1:F5O24. Finally, the QTL model for the Bur-0 cross included additive and dominance main-effect terms for FKF1, FCA3, and BurFLC and an interaction term, FKF1:BurFLC.

RESULTS

Association of *FRI* deletions with flowering-time effects: We measured the flowering times of 145 single-seed descent accessions in long-day photoperiods and genotyped them for the Col and Ler *FRI* deletions. As observed in previous surveys (Johanson *et al.* 2000; Le Corre *et al.* 2002; Stinchcombe *et al.* 2004), the *FRI* deletions first described in the Col and L*er* laboratory strains occur with considerable frequency among accessions, with 34 having the L*er*-type and 46 the Col-type deletion (Figure 1). No accession has both deletions, as expected due to their very tight linkage (253 bp). The effect of these deletions on flowering was obvious,

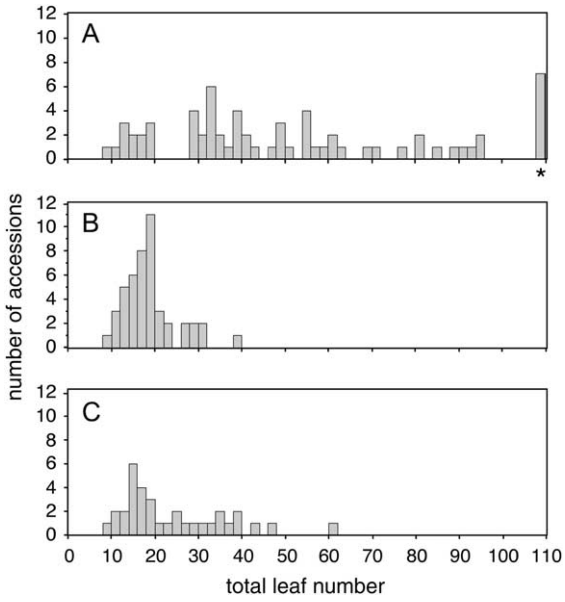

FIGURE 1.—Frequency distributions of flowering-time means for 145 accessions grown in long days. (A) Sixty-five accessions lacking Col- and L*er*-type *FRI* deletions. Seven lines did not flower during the course of the experiment (asterisk). (B) Forty-six accessions having the Col-type *FRI* deletion. (C) Thirty-four accessions having the L*er*-type *FRI* deletion.

with the mean flowering times of the L*er*-type lines and Col-type lines being 21.1 ± 3.7 (standard error) leaves ($P < 7 \times 10^{-8}$) and 26.5 ± 3.4 (standard error) leaves ($P < 2 \times 10^{-12}$) earlier, respectively, than that of the remaining 58 accessions that flowered under our growth conditions. The presence or absence of these deletions, along with three other rare potential loss-of-function alleles in *FRI* (described below), account for 40% of the variation in flowering time among our accessions.

Early flowering in lines lacking *FRI* deletions: Even though the 65 accessions lacking the Col- and L*er*-type deletions in *FRI* flowered on average much later than those with these deletions, these accessions still showed a considerable range of flowering times (Figure 1A). Eleven accessions flowered with <20 leaves, while 7 had still not flowered after 115 days, when these accessions all had >90 leaves.

Given the substantial number of different *FRI* lesions discovered from natural populations to date (GAZZANI *et al.* 2003; JOHANSON *et al.* 2000; LE CORRE *et al.* 2002), a likely cause for the early flowering is that at least some of the 11 early lines harbored alternate mutations in *FRI*. Indeed, two of these lines are Cvi accessions, which have recently been shown to contain a premature stop codon in *FRI* (GAZZANI *et al.* 2003). In the remaining 9 early lines, we sequenced 3.3 kb of the *FRI* gene encompassing the entire coding region, plus 534 bp upstream and 471 bp downstream of it. An apparent disruption in the *FRI* coding sequence is evident in only two lines. Accession An-1 has a 99-bp deletion combined with a 31-bp insertion at the start of exon 3. This indel is likely identical to "Indel2" found in the BUI accession (LE CORRE *et al.* 2002). Accession Or-0 was found to have a novel 1-bp deletion in exon 2 of *FRI*, causing a frameshift in the coding sequence that leads to a premature stop codon.

While we found variation at the nucleotide and amino acid level in the remaining seven early accessions [Da(1)-12, Dra-0, Est, Shahdara, Van-0, Wa-1, Wil-1], none had an obvious disruption of the *FRI* coding sequence. Furthermore, we could detect *FRI* mRNA by RT-PCR in the six accessions that we examined (data not shown; Wil-1 not tested), suggesting that they may have subtle defects in *FRI* expression or may carry alleles at other loci that suppress *FRI* activity.

A new *FLC* loss-of-function allele in Van-0: Since *FRI* acts entirely through *FLC* to control flowering (MICHAELS and AMASINO 2001), accessions with a functional *FRI* allele could be early flowering because of defects in *FLC*. In fact, two of these seven early flowering accessions, Da(1)-12 and Shahdara, have *FLC* alleles that show attenuated responses to *FRI* (GAZZANI *et al.* 2003; MICHAELS *et al.* 2003).

One of the early accessions that lacked an obvious *FRI* lesion, and which we analyzed in more detail, is Van-0. The F_1 progeny derived from a cross of Van-0 to L*er*, which has nonfunctional *FRI* and a weak *FLC* allele, flowered later than either parent, suggesting an interaction between at least two loci that delay flowering. The transgression observed in the F_1 progeny is even greater when Van-0 is crossed to Col (J. BOREVITZ, unpublished data), which also lacks functional *FRI*. Since the *FLC* allele from Col is notably stronger than that of L*er* (KOORNNEEF *et al.* 1994; LEE *et al.* 1994b), we reasoned that variation at *FLC*, along with the observed polymorphism at *FRI*, may be partially responsible for the late flowering of F_1 hybrids derived from Van-0. Another potential locus is the *FLG* QTL that interacts with the *FLF* QTL, which is very likely allelic to *FLC*, first identified in the Cvi × L*er* recombinant inbred lines (RILs) (ALONSO-BLANCO *et al.* 1998).

An F_2 population from the cross of Van-0 to L*er* was genotyped for three markers, the L*er* deletion at *FRI*, a marker tightly linked to *FLC*, and another 15 cM south on chromosome 5, near *FLG*. The L*er* allele at *FLC* had a major additive effect (10 leaves increase) that interacted with both Van-0 alleles at *FRI* and *FLG* to increase the phenotype, together explaining 81% of the variance in flowering time (supplementary material at http://naturalvariation.org/werner). Figure 2 shows that all plants homozygous for the Van-0 *FLC* region were early flowering, while other genotypes were quite variable. Backcross analysis of F_1 plants crossed to L*er* revealed *FRI* variation dependent on *FLC* copy number controlling flowering time, while a backcross to Van-0 revealed that only the *FLC*-linked marker had a significant effect (supplementary material at http://naturalvariation.org/

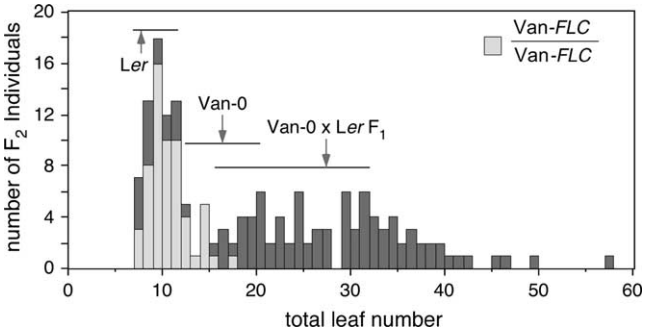

FIGURE 2.—Frequency distribution of total leaf number for an F_2 population derived from a cross of Van-0 with L*er*, grown in long days. Shaded bars indicate individuals homozygous for the Van-0 allele of HU13, a marker close to *FLC*. Flowering-time ranges and means of the parents and F_1 hybrid are denoted by arrows and horizontal lines, respectively.

werner). This suggests that the Van-0 allele of *FLC* is even less active than the weak L*er* allele and may be a null.

Sequencing of the *FLC* gene from Van-0 revealed a nucleotide substitution in exon 6, creating a premature stop codon at position 158 of the open reading frame. Termination of translation at this site would produce a protein containing the MADS box, I-box, and K-box, but lacking the final 39 amino acids. Reverse transcription followed by PCR showed that Van-0 produces, in approximately equal proportions, a normal length *FLC* transcript with the nonsense mutation, as well as an alternatively spliced transcript lacking exon 6, which is apparently the consequence of nonsense-associated altered splicing (WANG *et al.* 2002). As exon 6 is 42 nucleotides long, translation of this transcript is predicted to yield an FLC protein with an internal deletion of amino acids 150–163. This nonsense mutation in *FLC* and the behavior of the QTL linked to *FLC* make *FLC* a strong candidate gene for causing the early flowering of Van-0.

Late flowering in lines with *FRI* deletions: There was also significant variation in the accessions having nonfunctional *FRI* alleles (Figure 1, B and C). Of the 80 lines harboring a L*er*- or Col-type deletion in *FRI*, under our conditions 24 flowered with significantly more than 20 total leaves, with several flowering as late as *FRI*-Sf2 *FLC*-Col, a line with the late-flowering *FRI* allele from Sf-2 in the Columbia background (LEE *et al.* 1994b). For 19 of these late-flowering accessions and for 10 early flowering accessions lacking the Col and L*er* deletions, we confirmed the flowering phenotypes in a second experiment containing more individuals per line ($r = 0.923$ with first experiment).

To evaluate both the dominance of the late-flowering phenotypes and the effect of adding functional *FRI* and *FLC* alleles in F_1 hybrid backgrounds, three of these accessions were crossed to four lines containing different homozygous combinations of active and inactive *FRI* and *FLC* alleles in the Columbia background. Inactive alleles were the fast-neutron *flc*-3 allele and the *FRI* deletion allele from Col (*fri*-Col). Active alleles were *FLC* from Col (*FLC*-Col) and *FRI* introgressed from the accession Sf-2 (*FRI*-Sf2) (LEE *et al.* 1994b; MICHAELS and AMASINO 1999; MICHAELS and AMASINO 2001). Early flowering plants were obtained when the three accessions, Er-0, Rak-2, Wu-0, were crossed to *fri*-Col *flc*-3 and *fri*-Col *FLC*-Col, revealing that their late-flowering phenotypes are largely recessive (Table 2). In contrast, adding a functional copy of *FRI*, through crossing to *FRI*-Sf2 *flc-3*, resulted in late flowering, with the F_1 progeny from the cross to *FRI-Sf2 FLC-Col* being even later flowering, proving the effect of the *FRI* deletions and the absence of dominant suppressors. In addition, since introduction of functional *FRI* alone in the *FRI*-Sf2 *flc-3* hybrids caused late flowering, the accessions in question have functional *FLC* alleles, perhaps of varying strength.

It is plausible that lines lacking *FRI* activity may still have significant *FLC* expression due to the presence of alternate activators of *FLC* such as *FLG* or *ART1* (GRBIC and BLEECKER 1996; ALONSO-BLANCO *et al.* 1998; PODUSKA *et al.* 2003). Consequently, we examined *FLC* expression in 15 of these accessions by RNA blot analysis (Figure 3). These late-flowering lines showed considerable variation in *FLC* levels, with modest correlation between *FLC* levels and flowering time ($r = 0.55$). Be-0, Oy-0, Pi-0, and Wt-1 had very little or no detectable *FLC* expression, while Er-0, Na-1, Ge-1, Gie-0, Lz-0, Ob-0, and Rak-2 had substantial *FLC* levels, despite the presence of nonfunctional *FRI* alleles.

Flowering-time QTL in Lz-0: Lz-0 was the latest flowering of all accessions with a *FRI* deletion (62 leaves) and had high levels of *FLC* RNA (Figure 3). This lateness was largely eliminated by vernalization (25 leaves after a 30-day vernalization treatment), suggesting that the late flowering in absence of vernalization is due, at least in part, to *FLC* activation. In agreement with this notion, *FLC* RNA levels in Lz-0 were much lower after 30 days of vernalization (data not shown). When Lz-0 was crossed to Col and L*er*, the resulting F_1 hybrids were early (data not shown), indicating that activation of *FLC* in Lz-0 is suppressed by dominant alleles present in Col and L*er*. This behavior is very much reminiscent of loss-of-function mutations in autonomous pathway genes such as *FCA*, *FLD*, *FLK*, *FPA*, *FVE*, *FY*, or *LUMINIDEPENDENS* (*LD*) (KOORNNEEF *et al.* 1991; LEE *et al.* 1994a; SANDA and AMASINO 1996; NELSON *et al.* 2000; LIM *et al.* 2004; MOCKLER *et al.* 2004). To examine whether the Lz-0 behavior is associated with a major mutation in any of these genes, we sequenced the entire coding regions including small introns for *FCA*, *FLD*, *FPA*, *FVE*, *FLK*, and *LD*. Although nonsynonymous changes were found, there were no obvious loss-of-function mutations.

To identify loci responsible for the *FRI*-independent activation of *FLC* in Lz-0, we next examined 178 F_2 plants derived from the cross of Lz-0 to L*er*. These plants showed a wide range of flowering times, with many individuals flowering earlier or later than either parent (Figure

TABLE 2

Flowering-time analysis of late-flowering accessions

Accession	Flowering time[a] (mean ± SE)				
	Parent	× *flc-3* Col F_1	× Col F_1	× *FRI*-Sf2 *flc-3* Col F_1	× *FRI*-Sf2 *FLC*-Col F_1
Wu-0	23.6 ± 0.43	18.5 ± 0.60	18.8 ± 0.31	29.0 ± 0.85	57.1 ± 3.74
Er-0	30.7 ± 0.68	20.6 ± 0.96	19.9 ± 0.13	36.6 ± 0.94	>90[b]
Rak-2	36.6 ± 1.29	19.3 ± 0.31	21.3 ± 0.61	57.3 ± 3.41	>90[b]
Bur-0	38.9 ± 0.23	24.1 ± 0.13	>67[b]	22.1 ± 0.35	74.8 ± 4.18

$n = 7$ or 8 for all genotypes. Comparisons of parents with the corresponding F_1 populations are significant at $P < 0.05$ (*t*-test).

[a] Flowering time was measured as days to flowering in long days without vernalization.

[b] Some or all of the plants had not flowered after 90 days.

4A). A QTL analysis using 32 markers spaced at ~15-cM intervals throughout the genome failed to detect any major QTL near the autonomous pathway genes *FVE*, *FPA*, *FLK*, *FLD*, *LD*, or *FCA*. Suggestive peaks were found at the bottom of chromosome 1, near marker ciw1, and at the top of chromosome 3, in the vicinity of *FLK* and *FLD*, respectively (Figure 4B).

The likelihood of QTL was significant for much of chromosome 5. Background markers placed at *FLC* (4.4 cM) and 11.5 cM south of *FLC* (F5O24) could resolve two peaks. One QTL within this region is very likely to be *FLC*, given the known weakness of the L*er FLC* allele and the high levels of *FLC* RNA present in Lz-0. The peak south of *FLC* is in the same region as known natural alleles that interact with *FLC*, such as *ART1, FLG*, and the QTL that we detected in the Van/L*er* cross (Alonso-Blanco *et al.* 1998; Poduska *et al.* 2003). Genome-wide testing for epistasis suggested interactions that further delayed flowering between Lz-0 alleles at markers near ciw1 and F5O24 as well as between Lz-0 alleles at markers near F5O24 and *FLC* (supplementary data, QTL effect table at http://naturalvariation.org/werner).

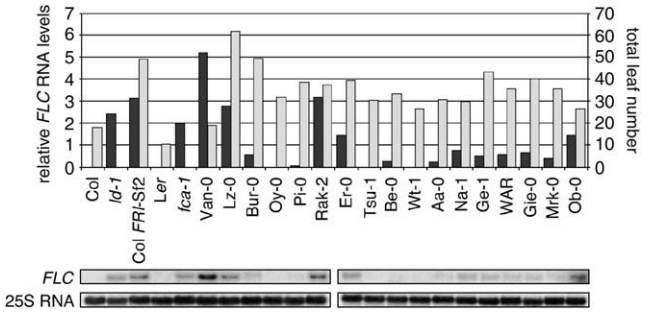

Figure 3.—(Top) Comparison of relative *FLC* RNA levels (solid bars) with flowering times (shaded bars) in late-flowering accessions with deletions in *FRI*. The five leftmost strains are controls; flowering times were not determined for *ld*-1 (in Col background) and *fca*-1 (in L*er* background), which are late-flowering mutants known to have elevated *FLC* levels. Also shown is Bur-0, which does not have a deletion in *FRI*. (Bottom) RNA blots. *FLC* levels are expressed relative to 25S rRNA.

XAM on an F_2 population derived from the cross of Lz-0 with Col grown in long days (Figure 4C) provided a complementary analysis of the genomic regions responsible for the lateness of Lz-0 (Wolyn *et al.* 2004). As a first step, labeled Lz-0 and Col genomic DNA was hybridized to Affymetrix ATH1 arrays (Borevitz *et al.* 2003), and 15,000 single-feature polymorphisms (SFPs) were identified as differences in hybridization intensity between the two strains [20% false discovery rate (FDR) by permutations and ~30% FDR by sequence analysis]. Clusters of SFPs revealed 286 potential deletions. When probes prepared from DNA pools of the 50 earliest and the 50 latest segregating F_2 plants (earliest and latest 8.9% each) were hybridized independently to ATH1 arrays, allele frequencies for each SFP could be estimated. Allele frequencies of SFPs linked to QTL will deviate in opposite directions between early and late pools while unlinked loci will have intermediate allele frequencies, *i.e.*, equal contributions of parental alleles in both pools.

Significant allele frequency differences caused by QTL were detected in three regions of the genome: the bottom of chromosome 1, a large part of chromosome 4, and the top of chromosome 5 (Figure 4D). In the late-flowering pool, Lz-0 alleles were enriched at the bottom of chromosome 1 at ~19 Mb, colocalizing with a suggestive QTL peak seen in the cross with L*er*. A similar overlap with the L*er* QTL results was seen for QTL on chromosome 5. However, unlike the results from the L*er* cross, allelic variation was observed at the top of chromosome 4. Here, Lz-0 alleles were also enriched in the late-flowering pool. Although *FRI* is located in this region, both Lz-0 and Columbia contain lesions at the *FRI* locus, suggesting that this QTL corresponds to another gene(s).

Variation in vernalization response: As exemplified in mutants with defects in the photoperiod pathway, *A. thaliana* has the potential for a late-flowering, vernalization-insensitive growth habit. To date, however, most late-flowering accessions have been found to be vernalization responsive and therefore are assumed to have high levels of *FLC* causing the late flowering in the absence of vernalization. To determine whether there are exceptions to this general rule, we measured the flowering

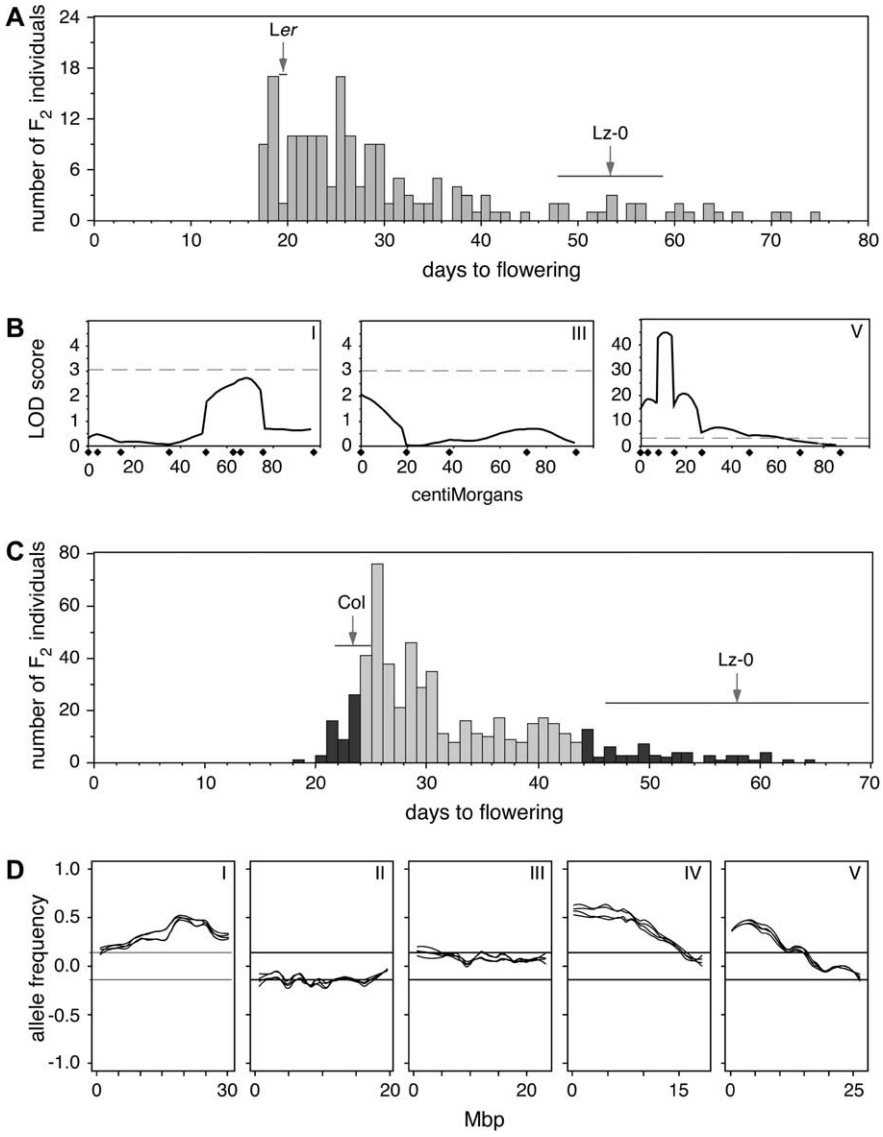

FIGURE 4.—Flowering-time QTL in crosses of Lz-0 with L*er* and Col. (A) Flowering-time distribution in an F_2 population derived from a cross of Lz-0 with L*er*, grown in long days. Parental means and ranges are denoted by arrows and horizontal lines, respectively. (B) Flowering-time QTL maps for chromosomes 1, 3, and 5 on the same population genotyped for 32 markers spanning the genome. QTL likelihoods were calculated with QTL Cartographer (see MATERIALS AND METHODS). The dashed line corresponds to a $P = 0.05$ threshold set by permutations (LOD 3.06). No evidence for QTL was apparent on chromosomes 2 and 4. Solid diamonds indicate marker locations. (C) Flowering times of 561 F_2 individuals derived from a cross of Lz-0 with Columbia, grown in long days. Tissue from the 50 earliest and the 50 latest individuals (solid bars) was used for XAM. Ranges and mean values of parental lines are indicated by horizontal lines and arrows, respectively. (D) XAM results for four comparisons between two independent replicates from the earliest 50 plants and two independent replicates from the latest 50 plants. Positive allele frequency indicates bias to Col alleles, negative toward Lz-0 alleles. Roman numerals indicate chromosomes.

times of 78 late-flowering accessions grown in long days after a 30-day vernalization treatment, which is considered a nearly saturating vernalization treatment for most late-flowering, vernalization-responsive genotypes (LEE and AMASINO 1995).

For the vast majority of lines that flowered with >22 leaves without vernalization or did not flower at all during the course of the experiment, the 30-day treatment substantially reduced the number of leaves produced prior to flowering. However, there was considerable variation in vernalization sensitivity, ranging from no effect to >70% reduction in total leaf number (Figure 5). Specifically, four accessions, Aa-0, Be-0, Bur-0, Tsu-1, that flowered with >30 total leaves showed little response to vernalization, with less than a 10% decrease in leaf number. In a previous study, Tsu-0, another accession from Tsu (Japan), showed a similar behavior (NORDBORG and BERGELSON 1999).

Flowering-time QTL in Bur-0: The most dramatic behavior among the late-flowering, vernalization-insensitive group was shown by Bur-0, which flowered with ~50 leaves regardless of vernalization. Unlike strong photoperiod pathway mutants, such as *co* and *gi* (KOORNNEEF *et al.* 1991), Bur-0 flowered later in short days than in long days, demonstrating that it can still respond to day length (data not shown). When crossed to *fri*-Col *flc*-3 and *FRI*-Sf2 *flc*-3, the F_1 hybrids were early flowering in long days (Table 2). In contrast, very-late-flowering plants were obtained from the cross of Bur-0 with Col, which contains an active *FLC* allele. Taken together, these results indicate that Bur-0 has an *FLC* allele that does not respond to *FRI* and that its recessive late-flowering phenotype may be independent of *FLC*.

FLC transcript in Bur-0 was detected by both RNA gel-blot analysis (Figure 3) and RT-PCR (data not shown), although the transcript size appeared larger than that found in other accessions. When we cloned the *FLC* cDNA from Bur-0, we found that it contained 64 bp of

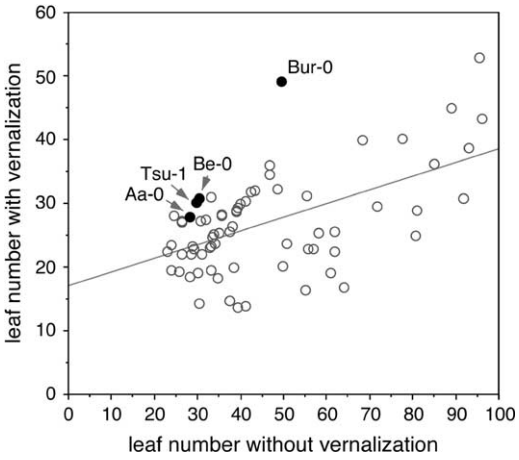

FIGURE 5.—Response of late-flowering accessions to 30-day vernalization treatment. Only the 70 accessions having >22 total leaves and flowering in long days are included. Bur-0 stands out as displaying no response to vernalization.

intron sequence immediately upstream of exon 7. The use of an alternate 3′ splice site appeared to be the result of a mutation of an invariant G in the final position of intron 6. The inserted sequence causes a frameshift, leading to the addition of four new codons followed by a premature stop codon. The result is an *FLC* open reading frame that, similar to that of Van-0, would encode the MADS-, I-, and K-boxes while lacking the C-terminal 33 amino acid residues. On the basis of the failure of the Bur-0 *FLC* allele to respond to *FRI* and the similarity among the proteins encoded by the Van-0 and Bur-0 *FLC* transcripts, we reason that the Bur-0 *FLC* allele may also be a null.

To identify QTL affecting flowering time in Bur-0 aside from *FLC*, we again turned to XAM to analyze a population of 330 F_2 plants from the cross of Bur-0 with *flc*-3 (Col background) grown in long days (Figure 6A), where any QTL that we detect are likely to be *FLC* independent. A total of 15,000 SFPs were identified between Bur and Col (7% FDR by permutations and 30% FDR by sequence analysis) that could be clustered into 253 potential deletions (BOREVITZ *et al.* 2003). There was severe segregation distortion on the bottom of chromosome 1, when the average allele frequency of pools was compared to the midparent genotype (see http://naturalvariation.org/werner). However, because this segregation distortion was seen in both late and early pools, it does not affect the mapping results.

As shown in Figure 6B, XAM suggests the presence of long-day flowering time loci on the bottom of chromosome 1 and on the top of chromosome 5. The calculated peak for the QTL on chromosome 1 was at ~21 Mb, while the QTL on chromosome 5 peaked at 4.6 Mb. Sites with less drastic deviations from neutral allele frequency appeared at the top of chromosome 2 and the middle of chromosome 4. We also individually genotyped a large number of plants from the entire F_2 population used for XAM, using molecular markers surrounding the predicted QTL. This analysis confirmed the position of QTL predicted by XAM. As seen with XAM, an extreme deficiency in Col homozygotes (7% observed, 25% expected, χ^2 test, $P < 1 \times 10^{-16}$) was observed on the bottom of chromosome 1.

The flowering time QTL on chromosome 1 and chromosome 5 were semidominant, with single Bur-0 alleles delaying flowering by ~6 and 10 days, respectively, relative to single Col alleles. Interestingly, these loci showed a significant interaction with both Bur-0 alleles, further delaying flowering. Furthermore, even the chromosomal regions showing only slight biases in allele frequency proved to significantly affect flowering time in this population. The Bur-0 allele at the chromosome 4 locus was estimated to delay flowering by 4 days/allele, while Col homozygotes at the chromosome 2 locus flowered ~3 days later than did Bur-0 homozygotes.

DISCUSSION

Previous studies of natural variation in flowering time in *A. thaliana* either have focused on a small number of accessions (*e.g.*, GAZZANI *et al.* 2003) or did not include genetic analyses (*e.g.*, STINCHCOMBE *et al.* 2004). Here, we examined variation of flowering time in a large collection of single-seed descent accessions available from stock centers. We have confirmed and extended previous observations regarding the frequency and magnitude of effect of two natural *FRI* deletions. Furthermore, we not only have estimated the extent of variation likely to be due to genetic loci other than *FRI*, but also have carried out initial genetic and molecular analyses of accessions that have interesting flowering phenotypes with respect to their genotype at *FRI*.

With the identification of the Or-0 *fri* allele, there are now at least 10 different mutations known to disrupt the *FRI* coding region (JOHANSON *et al.* 2000; LE CORRE *et al.* 2002 ; GAZZANI *et al.* 2003). At least 84 of the 145 accessions that we surveyed carry *FRI* loss-of-function alleles, with the Col and L*er* deletions being, by far, the most prevalent. At a minimum, the presence or absence of natural *fri* alleles are responsible for 40% of the variation in long-day flowering time seen in our experiment.

Some of the *FRI*-independent flowering-time variation is likely due to allelic variation at *FLC* itself. L*er*, C24, Da(1)-12, and Shahdara all have *FLC* alleles often described as "weak" due to their attenuated responses to active *FRI* alleles (KOORNNEEF *et al.* 1994; LEE *et al.* 1994b; SANDA and AMASINO 1996; GAZZANI *et al.* 2003; MICHAELS *et al.* 2003). In the case of L*er*, the reduced sensitivity to *FRI* is the result of a transposon insertion in the first intron, leading to decreased steady-state levels of *FLC* mRNA. Similarly, a larger and unrelated insertion was found in intron 1 of the Da(1)-12 *FLC* allele and may be responsible for its behavior (MICHAELS *et al.* 2003).

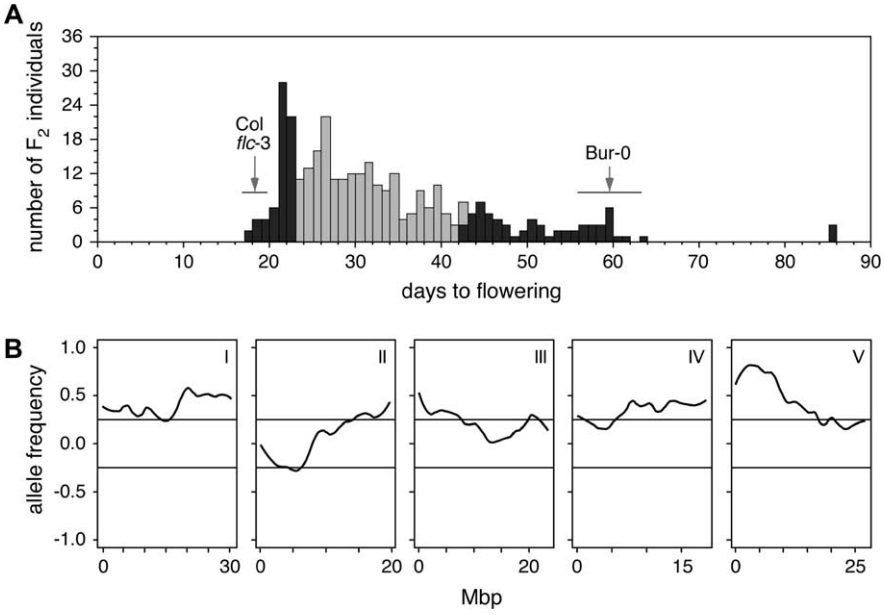

FIGURE 6.—Mapping of genomic regions that delay flowering in Bur-0. (A) Flowering times of 330 F_2 individuals derived from a cross of Bur-0 with *flc*-3 (Col background), grown in long days. Tissue from the 65 earliest and the 65 latest individuals (solid bars) was used for eXtreme array mapping. Ranges and mean values of parental lines are indicated by horizontal lines and arrows, respectively. (B) XAM results for earliest 65 plants. Positive allele frequency indicates bias to Col *flc*-3 alleles, negative toward Bur-0 alleles. Roman numerals indicate chromosomes.

In our work, we identified additional early flowering accessions, Dra-0, Est, Wa-1, and Wil-1, that appear to have intact *FRI* coding sequences and, except for Wil-1 (not tested), express the *FRI* transcript, making them good candidates for having defects in *FLC*. GAZZANI *et al.* (2003) have analyzed another accession from Wilna (Lithuania), Wil-2, and found that its early flowering may be the result of either a nonfunctional *FRI* allele caused by changes outside the coding region or a closely linked dominant suppressor of *FRI* activity. Wil-1 may be early flowering for the same reason.

In contrast to other natural *FLC* alleles that have been characterized as weak, the Van-0 and Bur-0 *FLC* alleles affect the coding region and appear to be incapable of delaying flowering. All *FLC* transcripts that we found in Van-0 and Bur-0 were expressed at reasonably high levels and code for FLC proteins with disrupted C termini, suggesting that these amino acids are essential for FLC function. In Van-0, the defect in *FLC* is very likely the source of its early flowering phenotype, while the effect of the inactive *FLC* allele in Bur-0 is masked by the presence of other late-flowering loci.

Another possible source of flowering-time variation in nonvernalized plants is through *FRI*-independent modulation of *FLC* expression levels. To search for alternate regulators of *FLC* in natural populations, we sought accessions that flowered late and were vernalization responsive, but lacked *FRI* activity. Principal among the accessions that we uncovered with this phenotype was Lz-0.

QTL mapping in the Lz/L*er* population identified at least two linked QTL on the top of chromosome 5, one at *FLC* and one south of *FLC*, near marker F5O24. A flowering-time QTL not only is expected at *FLC*, due to the known phenotype of the L*er* allele, but also is consistent with *FLC* mediating the vernalization-sensitive late-flowering phenotype of Lz-0. Other late-flowering loci from Lz-0 detected in the cross would then be expected to interact with the Lz-0 *FLC* allele. Indeed, we found interaction effects between Lz-0 alleles for QTL at marker F5O24 and *FLC*. However, the statistical support for this interaction was weak, which may well be a consequence of the limited recombination between these linked QTL. The QTL linked to F5O24 is near *ART1* and *FLG*, known or suspected natural modifiers of *FLC* (ALONSO-BLANCO *et al.* 1998; PODUSKA *et al.* 2003). We also identified an *FLC*-interacting locus in this region in the cross between Van-0 and L*er*. However, *ART1* and *FLG*, as well as the Van-0 allele in this region, seem to act in a dominant or semidominant fashion compared to those of L*er*, while the Lz-0 gene appears to be recessive.

XAM identified several additional regions, near markers ciw1 and F5O24, as well as *FLC* as associated with late flowering in the cross of Lz-0 with Col. Relative to L*er*, Col has a strong *FLC* allele capable of responding to *FRI* and to mutations in autonomous pathway genes. Consequently, if allelic variation at *FLC* underlies the effect of this QTL at *FLC*, it would suggest that the Lz-0 *FLC* allele may be stronger than the Col allele or may show a particular interaction with other loci. Alternatively, enrichment for Lz-0 alleles at *FLC* could be a consequence of linkage between *FLC* and loci from Lz-0 that activate its expression or that delay flowering independently of *FLC*.

Contrary to the ample natural variation acting through *FLC*, little variation has been assigned to the genes of the photoperiod pathway, with the notable exception of the *EDI* amino acid substitution in *CRY2* (EL-ASSAL *et al.* 2001), which has been found only in accessions originating from the Cape Verde Islands. It is possible that variation in this pathway does exist in the wild, but

that the effects are more subtle and therefore generally masked by the dramatic variation conferred by the *FRI/FLC* pathway. Consistent with this, several small-effect QTL in the Cvi/L*er* RIL population were detected as significant only after vernalization, although it is possible that these QTL may not have effects in the absence of vernalization (Alonso-Blanco *et al.* 1998).

We took an alternative approach to examining natural variation in the photoperiod pathway by identifying late-flowering accessions with limited *FLC* activity and little response to vernalization. One such accession is Bur-0. Since we found that Bur-0 has a strong loss-of-function allele at *FLC*, we used a mapping cross with a Col line that carries a null allele, *flc*-3. Thus, any flowering-time loci that we detect should be *FLC* independent. XAM identified four regions of the genome as responsible for flowering-time differences between Bur-0 and Col *flc*-3. Despite substantial segregation distortion, the effects of all four regions could be confirmed by genotyping the population with individual molecular markers.

Interestingly, Bur-0 has a short circadian period (Michael *et al.* 2003), which may be causally related to its flowering-time phenotype. The confidence interval for the QTL on chromosome 1 includes the genomic location of the clock gene and the putative blue-light photoreceptor *FKF1* (Nelson *et al.* 2000; Imaizumi *et al.* 2003), while the XAM QTL on chromosome 5 peaks at 4.6 Mb, which is ~1.4 Mb south of *FLC*, near *CONSTANS* (*CO*), the major output of the photoperiod pathway (Putterill *et al.* 1995). Flowering-time QTL at the bottom of chromosome 1 or at the top of chromosome 5, but distinct from *FLC*, also have been found in other mapping crosses (Clarke *et al.* 1995).

Early flowering has arisen independently many times in natural *A. thaliana* populations, demonstrated first by numerous lesions in *FRI* and now by a growing collection of weak and possibly null *FLC* alleles. However, in contrast to the Col- and L*er*-type deletions in *FRI*, these disruptions in *FLC* are relatively rare. The Bur- and L*er*-type *FLC* alleles were found in only one and four additional strains, respectively, while the *FLC*-Van allele was unique among the 145 accessions that we surveyed here. For the other loci mapped in this work, determining the prevalence and effect among accessions awaits their molecular identification.

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