Analysis of Natural Allelic Variation at Flowering Time Loci in the Landsberg erecta and Cape Verde Islands Ecotypes of Arabidopsis thaliana

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ABSTRACT

We have analyzed the flowering behavior of two Arabidopsis ecotypes: the laboratory strain Landsberg erecta (Ler) and an ecotype from the tropical Cape Verde Islands (Cvi). They differ little in their flowering phenotypes and in their responses to photoperiod length changes and to vernalization treatment. However, segregating populations derived from crosses between them showed a much larger variation. An approach of quantitative trait locus (QTL) mapping in recombinant inbred lines (RILs) grown under three environments differing in day-length and/or vernalization treatment has been used to detect and locate flowering loci. Four main QTLs were identified, designated early-day-length insensitive (EDI), flowering F, G, and H (FLF, FLG, and FLH, respectively), to which most of the flowering behavior differences could be attributed. To further characterize the individual loci, near isogenic lines were constructed by introgressing Cvi early alleles of EDI and FLH into the Ler genetic background. EDI-Cvi alleles produce earliness under both long- and short-day photoperiods, rendering Ler plants almost day-length neutral. In addition, RILs were selected to analyze FLF and FLG. These loci interact epistatically and RILs carrying late alleles at FLF and FLG were very responsive to vernalization and showed an increased response to photoperiod length changes. The possible role of these loci for the control of flowering is discussed in the context of the current Arabidopsis model.

To reproduce successfully, plants must flower under favorable environmental conditions, and therefore the time of flowering is likely to have an important adaptative significance (Murfet 1977). The transition from the vegetative to the reproductive phase is influenced by environmental factors such as photoperiod length and temperature, indicating that plants detect fluctuations in these parameters. The model plant Arabidopsis thaliana is being extensively used to dissect this developmental process genetically (reviewed in Martínez-Zapater et al. 1994; Coupland 1995; Asamino 1996; Koornneef et al. 1998b). A large number of mutations affecting flowering initiation, mostly in a quantitative manner, have been artificially generated. The genetic and physiological characterization of these mutations has shown that the regulation of this developmental switch in meristem fuction is complex. Several elements controlling the perception and transduction of light quality and day-length, such as the phytochromes A and B (Goto et al. 1991; Whiteman and Harberd 1997), the cryptochromes (Bagnall et al. 1996; Guo et al. 1998), and components of the circadian clock, like the ELF3 and LHY genes (Hicks et al. 1996; Carré et al. 1997), have been identified. Other genes, like the VRN loci, seem to control the cold signaling involved in the flowering response to vernalization (Chandler et al. 1996). The environmental factors are thought to modulate the action of several endogenous signaling components such as gibberellins (Bagnall 1992; Wilson et al. 1992) and sucrose (Roldan et al. 1997). Furthermore, several loci that might be involved in the signal transduction pathways to flowering have been identified. Some of these have already been cloned and encode putative transcription factors such as LD (Lee et al. 1994b) and CO (Puttrill et al. 1995) or an RNA binding protein like FCA (MacKnight et al. 1997), indicating that the regulation of flowering involves the sequential activation of genes.

In addition to induced mutations, genetic variation for flowering time has been found among natural populations (ecotypes) of Arabidopsis (Laibach 1951; reviewed in Napp-Zinn 1969, 1987). Arabidopsis has a wide distribution throughout the Northern hemisphere (Rédei 1970) and differences found among ecotypes grown under the same environmental conditions are considered to reflect adaptations to different natural environments. Karlsson et al. (1993) analyzed 32 ecotypes in several environments with different photoperiod length and vernalization treatments, and they have shown that genotype by environment (G × E) interactions are very significant, which illustrates the diversity of responses found in nature. The identification of the loci responsible for this natural variation has been at-

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tempted for over 40 years (Napp-Zinn 1957; Van der Veen 1965). The advent of molecular markers and the development of genetic maps has facilitated the localization and characterization of some of the large effect alleles. Thus, the flowering behavior difference between very late ecotypes that respond to vernalization and the early ecotypes [classified under long-day (LD) light conditions] has been shown to involve two epistatic loci: the FRI locus mapped on the top of chromosome 4 (Clarke and Dean 1994; Lee et al. 1993; Brun et al. 1993) and FLC located on chromosome 5 (Koornneef et al. 1994; Lee et al. 1994a). Dominant alleles at both loci confer the lateness and vernalization requirement of late ecotypes. Moreover, these late alleles respond strongly to photoperiod changes, causing facultative LD strains to behave as “obligate” LD when they are not vernalized (Lee and Amasino 1995).

The identification of natural allelic variation of smaller effect has required the combination of genetic maps with statistical methods to locate quantitative trait loci (QTLs). Flowering QTL analyses have been performed in crosses between late and early ecotypes (Clarke et al. 1995; Kuittinen et al. 1997) as well as between early ones (Kowalski et al. 1994; Jansen et al. 1995; Mitchell-Olds 1996). The distinct number of QTLs detected in different crosses, varying between 2 and 12, does not fairly reflect the different number of segregating loci, but rather differences in the QTL detection power through the coverage of the corresponding molecular maps, the type and size of mapping population and the statistical approach. The combination of recombinant inbred line (RIL) populations and statistical methods that take into account the effect of multiple QTLs is particularly powerful [multiple QTL model (MQM) mapping, Jansen and Stam 1994; or composite interval mapping (CIM), Zeng 1994], and allows the separation of linked flowering loci (Jansen et al. 1995; Kuittinen et al. 1997).

The analysis of QTL by environment (QTL × E) interactions in these populations enables the detection of loci causing the G × E interactions (Clarke et al. 1995; Jansen et al. 1995). Furthermore, epistasis has been detected among some QTLs (Clarke et al. 1995; Kuittinen et al. 1997). All of these studies have shown the wealth and complexity of the natural genetic variation that is available, but most of them were restricted to determine the number and approximate location of segregating loci. With the exception of the FRI and FLC loci no further analysis of this allelic variation has been reported. The genetical and physiological characterization of QTLs requires the introgression of the new alleles in a genetic background similar to the laboratory strains used to generate artificial mutations. By constructing near isogenic lines (NILs) comparisons of allelic effects, allelism tests and fine mapping can be performed. Consequently, the loci at which the natural variation occurs might be determined, and eventually their characterization at the molecular level will be achieved.

In the present study we have analyzed the allelic variation affecting flowering time in two early ecotypes: the laboratory strain Landsberg erecta (Ler) and an ecotype originating from the Cape Verde Islands (Cvi). A QTL mapping approach in RILs has been used to identify and locate the loci responsible for the flowering variation in three environments differing in photoperiod length and/or vernalization treatment. The four largest effect QTLs have been further characterized genetically and physiologically in relation to the flowering responses to day-length and vernalization. For that, NILs containing Cvi early alleles in a Ler genetic background and several selected RILs carrying Cvi late alleles have been analyzed. The possible role of these loci for the control of flowering is discussed in the context of the current Arabidopsis model.

MATERIALS AND METHODS

Plant material: A set of 162 recombinant inbred lines (RILs) derived from crosses between the laboratory strain Landsberg erecta (Ler) originating from Northern Europe (Rédei 1992) and the ecotype Cvi, from the tropical Cape Verde Islands (Lobin 1983) was used to identify flowering QTLs. These lines have been previously characterized for amplified fragment polymorphism (AFLP) and cleaved amplified polymorphic sequence (CAPS) markers (Alonso-Blanco et al. 1998).

Selected RILs were crossed with the following late flowering genotypes, in a predominantly Ler genetic background: (i) the FRI M73 introgression line containing the FRI locus from the genotype M73 (Koornneef et al. 1994) and (ii) the ld introgression line with the ld-1 mutation originally generated in Columbia (Col) background (Koornneef et al. 1994). All crosses were performed using the Ler background plants as female parents.

Construction of NILs: As a first step to constructing near isogenic lines (NILs), early flowering Cvi alleles were introgressed into Ler genetic background by phenotypic selection under LD light conditions. Selection was basically performed to introgress nonrecessive Cvi alleles with relatively large effect. Three early flowering inbred lines were obtained with four backcross generations, and three final selfing generations. These lines were genotyped using 370 AFLP and CAPS markers. One line, referred to as S10, appeared to be completely Ler for chromosomes 2, 3, and 4, and contained Cvi introgressions at three genomic regions: top and bottom of chromosome 1 (genetic segments of ~25 and 20 cM, respectively), and bottom of chromosome 5 (~10 cM). This line was backcrossed to Ler and an F2 was genotyped for CAPS markers in the segregating regions. Two different F2 plants for each of the three different homozygous introgression genotypes were selected as the final NILs. These lines are designated EDI-Cvi, FLH-Cvi, and EDI-Cvi,FLH-Cvi, because they contain Cvi alleles at the loci EDI and/or FLH, respectively. Lines containing Cvi alleles at the bottom of chromosome 1 were constructed but were removed from the analysis because no significant effect on flowering could be detected.

Growth conditions: In experiments without vernalization treatment, seeds were sown in petri dishes on water-soaked filter paper and incubated for 3 days in a growth chamber at 24°C with 16 hr light (for LD light conditions) or 8 hr light per day (for short-day (SD) light conditions). The vernalization
treatment was given as described in Koornneef et al. (1994). For that, seeds were sown on Murashige-Skoog medium supplemented with 1% sucrose (MS-10). Subsequently, petri dishes were incubated in a cold room at 4°C for 3 wk and then transferred to a climate chamber (24°C, with 8 or 16 hr light per day) for 2 days before planting. LD experiments were performed in an air-conditioned green house supplemented with additional light from middle September until the beginning of April, providing a day-length of at least 14 hr. SD experiments were carried out in a single climate chamber with 8 hr light as described by Koornneef et al. (1995).

RIL evaluations: The complete set of RILs, parental lines, and reciprocal F1 hybrids were evaluated for flowering under three different environmental conditions: LDs with and without vernalization treatment, and SD photoperiod conditions without vernalization. RILs were grown under both LD conditions, with and without vernalization treatment, in the same experiment and therefore the nonvernalized seeds were also sown on MS-10 medium. Twelve plants for each RIL and 24 other RIL population (out vernalization treatment, and SD photoperiod conditions LOD score. A LOD score of 2.4 was used as the significance level for the QTL analyses. Every trait was checked for normality of the distributions and the values of 10 plants per RIL were used to calculate the line means for each of the four traits (FT, TLN, RLN and CLN) and the three environmental conditions (LD, SD, and LD with vernalization). The line means were used to perform the QTL analyses. For FT and TLN a search for interactions between QTLs across environments was performed according to Alonso-Blanco et al. (1997). Two-way interactions were searched among all pairwise combinations of the 99 markers using as significance threshold a log-likelihood ratio equivalent to \( P < 0.005 \). Ten thousand trials were used in the Monte Carlo simulations performed with Epistat to establish the statistical significance of the log-likelihood ratios of the interactions detected (Chase et al. 1997).

The overall G × E interaction was tested for each trait by a two-factor ANOVA using genotypes (RILs) and environments as classifying factors. For each trait and for each putative QTL, QTL × E interaction was tested by repeated measures ANOVA using the corresponding marker and the environment (repeated measurements of the RILs) as classifying factors (\( P < 0.005 \)). The General Linear Model module of the statistical package SPSS version 7.5 was used for the ANOVAs and for the variance component analyses from the Type III sum of squares ANOVA.

Molecular markers: The introgression lines containing early flowering Cvi alleles were genotyped using AFLP marker analysis, which was performed according to Vos et al. (1995). About 350 polymorphic bands amplified with the 14 primer combinations used previously to build the Cvi molecular map were scored for absence and presence. The genetic location of AFLP bands was therefore known previously and covered most of the genetic map. CAPS and microsatellite markers previously mapped in the region containing flowering loci, in the introgression lines and in the backcrosslike and F1 populations. CAPS markers were ana-
conditions with and without vernalization treatment provided an estimate of the vernalization response. Both ecotypes flower at rather similar times under LD conditions and can be considered as early flowering. The later flowering time of Ler under SD indicates that Cvi responds less than Ler to photoperiod length changes. In contrast, Cvi shows a more pronounced response to the vernalization treatment. The F1 hybrids flower earlier or similar to the earliest parent (Table 1), although the FT means of the nonvernalized reciprocal F1s grown under LD conditions were significantly different (P < 0.001; which was observed consistently and was even more pronounced in two other experiments not shown). Reciprocal differences have been observed previously in crosses between other Arabidopsis ecotypes suggesting a certain influence of maternal factors on flowering (Waterman 1970; Clarke and Dean 1994), but they have not been further analyzed.

Although the flowering differences between Ler and Cvi are small, transgressive variation in both directions was observed in the RIL population under the three environments, indicating the presence in the two parental lines of alleles increasing and reducing flowering time (Figure 1; Table 1). A large amplification of the flowering range was observed in the RIL population when grown under SDs, and three major classes of flowering time appeared. In contrast, a reduction in the flowering range occurs when vernalizing the RILs (Figure 1; Table 1). The G × E interactions were highly significant (P < 0.001) when the flowering responses to vernalization or to photoperiod length were compared in the RIL population. This indicates the presence of allelic variation, whose effect is expressed differentially with the environments to control the different responses of the RILs to photoperiod length changes and to vernalization treatment.

The flowering phenotype was measured as FT and as TLN. As shown in Figure 2, both traits are tightly correlated in the RIL population and therefore both are expected to be mostly under the same genetic control as that observed previously with mutant genotypes (Koornneef et al. 1991).

Mapping loci that control the flowering behavior differences between Ler, Cvi, and the RILs: To identify and locate the loci controlling the flowering behavior differences between Ler and Cvi, the flowering marker values of the 162 RILs, collected under the three environments, were used for QTL analysis. Four flowering-related traits (FT, TLN, RLN and CLN) were analyzed separately for each environment (LD with and without vernalization treatment, and SD) using the MQM method of MapQTL (see materials and methods). The use of cofactors strongly improved the mapping accuracy of linked QTLs, which could not be separated with interval mapping. Figure 3 shows the QTL likelihood maps obtained for TLN under the three environmental conditions, indicating the genetic intervals where the putative QTLs
TABLE 1
 Phenotypic values for flowering traits of the parental lines, reciprocal F1 hybrids, and the RIL population grown in three different environments (10 plants were used per RIL; 20 plants for the rest of lines)

<table>
<thead>
<tr>
<th></th>
<th>Long day</th>
<th>Long day + vernalization</th>
<th>Short day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flowering time (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ler</td>
<td>25.0 ± 1.0</td>
<td>21.6 ± 1.0</td>
<td>49.4 ± 2.9</td>
</tr>
<tr>
<td>Cvï</td>
<td>28.0 ± 1.8</td>
<td>21.6 ± 0.9</td>
<td>45.4 ± 6.6</td>
</tr>
<tr>
<td>F1 Ler × Cvï</td>
<td>25.1 ± 0.9</td>
<td>21.1 ± 0.9</td>
<td>21.0 ± 0.9</td>
</tr>
<tr>
<td>F1 Cvï × Ler</td>
<td>22.2 ± 1.9</td>
<td>19.8 ± 0.7</td>
<td>21.0 ± 0.9</td>
</tr>
<tr>
<td>RIL mean</td>
<td>24.8 ± 5.1</td>
<td>21.4 ± 2.9</td>
<td>38.4 ± 14.7</td>
</tr>
<tr>
<td>Min.-max. RIL mean</td>
<td>18.1-44.8</td>
<td>16.3-32.0</td>
<td>21.1-78.0</td>
</tr>
<tr>
<td>RIL LSD</td>
<td>1.7</td>
<td>1.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Total leaf number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ler</td>
<td>10.4 ± 1.0</td>
<td>9.1 ± 1.0</td>
<td>32.5 ± 2.4</td>
</tr>
<tr>
<td>Cvï</td>
<td>11.6 ± 1.3</td>
<td>8.1 ± 1.0</td>
<td>27.0 ± 8.9</td>
</tr>
<tr>
<td>F1 Ler × Cvï</td>
<td>10.9 ± 0.8</td>
<td>8.7 ± 0.6</td>
<td>27.0 ± 8.9</td>
</tr>
<tr>
<td>F1 Cvï × Ler</td>
<td>10.2 ± 1.7</td>
<td>9.5 ± 0.8</td>
<td>27.0 ± 8.9</td>
</tr>
<tr>
<td>RIL mean</td>
<td>10.5 ± 5.2</td>
<td>8.6 ± 2.3</td>
<td>20.8 ± 14.0</td>
</tr>
<tr>
<td>Min.-max. RIL mean</td>
<td>5.7-32.1</td>
<td>5.4 ± 18.1</td>
<td>5.4-55.6</td>
</tr>
<tr>
<td>RIL LSD</td>
<td>1.9</td>
<td>0.6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. RIL mean, minimum and maximum, and least significant differences at P ≤ 0.01 (LSD) for mean RIL comparisons are also shown.

were mapped. A total of 11 QTLs were detected along the five linkage groups. However, a clear distinction can be made between large effect (major) and small effect (minor) loci (Table 2). Allelic variation at four loci mapping, respectively, on top of chromosome 1, and on top, middle and bottom of chromosome 5, had a large effect on both TLN and FT (15% of the phenotypic variance could be attributed in at least one environment). We have named them EDI, and FLF, FLG and FLH, respectively. Cvï alleles produce earliness at EDI and FLF and lateness at FLG, this allelic variation accounting for nearly all the RIL phenotypic variance in the three environments and for the parental phenotypes (see Figure 4 in which FLH has not been included but its effect is in agreement with the phenotypes of Ler and Cvï). The remaining seven QTLs had small additive effects (in general less than 5% of the variance could be attributed to each one) and were detected under only the LD with vernalization environment.

The QTLs detected for FT and TLN were in most cases mapped in the same intervals, indicating pleiotropy at these loci. The four main QTLs showed comparable contributions to the phenotypic variance of both traits (Table 2). However, two small effect QTLs on chromosome 2 appeared as significantly affecting FT but not TLN (markers FD.81 and DF.140C) and two others as significant for TLN but not for FT (BF.325L and HH.171C-Col). These putative QTLs were considered either significant or not on the basis of the 2.4 LOD threshold, but the likelihood values for both traits always increased around the corresponding positions (see, for instance, chromosome 2 in Figure 3). In agreement with this, one of the small QTLs affecting FT but not TLN (DF.140C) was significant for RLN. Only the QTL located at the bottom of chromosome 4 (around DHS1) appeared to affect CLN but not RLN and FT in the LD conditions. Therefore, most of the QTLs identified affected FT and TLN, although small differ-

Figure 2.—Relationship between flowering time means and total leaf number means in the RIL population. r, Pearson correlation coefficient.
Figure 3.—QTL likelihood maps for total leaf number in the three different environments. The abscissas correspond to the genetic maps in cM, the linkage group number being indicated in the right upper corner of each map. Horizontal dashed line corresponds to the LOD score threshold of 2.4. Two LOD support intervals for the significant QTLs are shown as solid bars along abscissas. The largest effect QTLs have been named EDI, FLF, FLG, and FLH.

ences might exist in their relative effect on both traits, or in their relative contribution to RLN or CLN.

Epistasis between QTLs was analyzed by performing a genome-wide search for two-way interactions. The two major QTLs located on the top and middle of chromosome 5 (FLF and FLG) show very significant synergistic interaction for all traits and all environments (P < 0.0001; see Table 2 and Figure 4). These loci have relatively small additive effects individually (FLF shows practically no effect while FLG has small effect), and lateness in the three environmental conditions is mainly observed when both Cvi alleles are present. Interactions were also detected between these regions and markers at the bottom of chromosome 1. However, because pseudo-linkage is observed in the RIL population between markers at the bottom of chromosome 1 and the top of chromosome 5 (22% recombinant frequency due to the lack of RILs of one of the recombinant genotypes) these interactions were rejected as not true epistasis. Another significant epistatic interaction was detected between the QTL linked to BF.325L on chromosome 2, and the marker HH.440L on chromosome 3, which had not been associated previous to flowering.

The significant interaction of the three environments with EDI, FLF, and FLG (Table 2) indicates that these are the loci responsible for the different flowering responses in the RILs. The QTL on chromosome 1 around AD.121C also showed significant QTL × E interaction but it was due to its genetic linkage with EDI, since it was not significant when analyzing the interaction of both QTLs simultaneously. The remaining QTLs did not show significant interactions with the environments and therefore were not considered as environment specific. The overall effect of the three major loci on the flowering responses was examined. The responses of each RIL were quantified as the difference in TLN between the LD and SD conditions (photoperiod length response) and between the LD and the LD with vernalization treatment (vernalization response). Figure 4 shows the TLN frequency distributions of the RILs classified according to these three loci under the three environments. Several conclusions can be summarized as follows:

1. EDI, FLF, and FLG are the loci controlling the differences in photoperiod length response. RILs carrying late alleles at EDI, or at FLF and FLG, not only flower later but responded more to photoperiod length than the RILs carrying early alleles at these loci. An extremely low response was shown by the genotypes EDI-Cvi, FLF-Ler, FLG-Ler, which led to the naming of this locus as early, day-length insensitive (EDI). Therefore, to “abolish” the photoperiod response in the Ler/Cvi RILs required early alleles at the three loci.

2. FLF and FLG are the main loci controlling the differences in vernalization response. The FLF and FLG effects are much smaller under vernalization conditions than in normal LDs. In other words, the lateness observed under LDs in RILs carrying FLF-Cvi, FLG-Cvi alleles, is very much diminished by a 3-wk vernalization treatment. It is expected that a longer vernalization
Natural Variation at Flowering Loci

Figure 4.—Frequency distributions of TLN means of the RILs grown in three environments with different photoperiod length and/or vernalization treatment. The RILs have been classified according to their genotype at the closest markers to the loci EDI, FLF and FLG. The four distributions within each environment (vertical) correspond to the distributions of the four RIL classes obtained according to their genotypes at FLF and FLG (legend in the right part of the figure). Within each graph, the RILs are classified in relation to the genotype at EDI and the two distributions are overlaid. Arrows indicate the parental line means (20 plants per parent) and the horizontal bars represent their ranges of variation.

Characterization of Cvi early alleles: the loci EDI and FLH: Near isogenic lines containing Cvi alleles at EDI, and/or FLH in a Ler genetic background were constructed by phenotypic and genotypic selection (see Figure 5 and material and methods). The introgression line containing Cvi alleles only in the EDI region was used for further genetic mapping, analyzing an F$_2$ population under SD conditions where the flowering segregation could be classified qualitatively and behaved as monogenic. The location of EDI was narrowed to a segment smaller than 10 cM comparable to the 2 LOD support interval established in the QTL analysis (data not shown). The genetic length of the introgression segment in the monogenic FLH-Cvi NIL (10 cM approximately) confirmed the FLH position obtained in the MQM analysis of the RILs.

The NILs and the line S10, from which they were derived, were analyzed under LD and SD photoperiod conditions, with and without vernalization treatment (Figure 5). The Cvi allele of EDI was largely dominant, which was particularly manifest under SD conditions where Ler plants flowered on average with 18.9 more leaves than the EDI-Cvi plants. EDI-Cvi plants flowered with almost the same TLN under both photoperiod length conditions, thus behaving as an almost day-length neutral genotype. These plants responded little to vernalization, showing a comparable response to Ler. At the FLH locus, the slight earliness produced by the Cvi allele behaved on average codominantly. However, its effect was almost absent under LD conditions without vernalization, differing from the effect estimate obtained in the RIL population. This suggests FLH might be involved in some undetected epistatic interaction, or that some introgressed fragment not detected in the extensively genotyped lines affected flowering time. In contrast, under SDs, FLH-Cvi plants flower on average with 3.4 fewer leaves than Ler plants, an effect not detected in the QTL analysis. These plants responded to photoperiod length in a comparable way to Ler. However, it is remarkable that they responded more than Ler to vernalization, an effect that was mainly observed under SD conditions. The allelic effects at EDI and FLH were basically additive because plants of the EDI-Cvi,FLH-Cvi line flowered earlier than the monogenic introgression lines in all environments.

Characterization of Cvi late alleles: the loci FLF and FLG: Three RILs were selected on the basis of their genotype as being Ler at EDI and FLH (and as much as
TABLE 2
QTLs detected for four flowering related traits in three environments differing in photoperiod length and/or vernalization treatment

<table>
<thead>
<tr>
<th>Trait</th>
<th>QTL</th>
<th>Long-day + vernalization</th>
<th>Long-day</th>
<th>Short-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Map position</td>
<td>% of variance</td>
<td>Additive allele effect</td>
<td>% of variance</td>
</tr>
<tr>
<td>Flowering time</td>
<td>AXR-1 (ED1)</td>
<td>1-7.5</td>
<td>43.9</td>
<td>-3.5</td>
</tr>
<tr>
<td></td>
<td>AD.121C</td>
<td>1-40.5</td>
<td>3.8</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>BF.325L</td>
<td>2-7.2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>FD.81L</td>
<td>2-18.7</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>DF.140C</td>
<td>2-62.3</td>
<td>3.0</td>
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<td>HH.171C-Col</td>
<td>3-78.4</td>
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<td>NS</td>
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<tr>
<td></td>
<td>BH.92L-Col</td>
<td>4-30.2</td>
<td>0.8</td>
<td>-0.7</td>
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<td></td>
<td>DHS1</td>
<td>4-80.2</td>
<td>NS</td>
<td>NS</td>
</tr>
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<td></td>
<td>BH.325L (FLF)</td>
<td>5-15.7</td>
<td>2.9</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>GH.121L-Col (FLG)</td>
<td>5-41.5</td>
<td>14.8</td>
<td>14.8</td>
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<tr>
<td></td>
<td>FLF × FLG</td>
<td></td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>DF.119L (FLH)</td>
<td>5-110</td>
<td>14.5</td>
<td>-2.0</td>
</tr>
<tr>
<td>Total leaf number</td>
<td>AXR-1 (ED1)</td>
<td></td>
<td>33.6</td>
<td>-2.4</td>
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<tr>
<td></td>
<td>AD.121C</td>
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<td>1.1</td>
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<td>BF.325L</td>
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<td>DF.140C</td>
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The closest marker to each QTL is shown and its location is indicated by the linkage group number followed with its map position. Only QTLs with LOD score > 2.4 are reported. The QTLs with the largest effects have been designated as EDI, FLF, FLG, and FLH and are indicated between parentheses. For each trait and environment, the relative contribution of each QTL was estimated by analysis of variance components. Because of the epistatic interaction between FLF and FLG (see text) the relative contribution of their interaction was included in the model and it is shown (FLF × FLG). The additive allele effects of FLF and FLG have been added together. The additive allele effects are estimated as the mean differences between the two RIL genotypic groups carrying the Cvi and Ler alleles (a positive value implies Cvi allele increasing the corresponding phenotypic value; a negative value, Cvi allele decreasing). Allele effects are shown in the original scale of measurement (days for flowering time and number of leaves for leaf numbers). For each trait, the QTLs interacting with the environments are indicated by * (P < 0.005). NS, not significant.
Figure 5.—(A) Phenotype of the parental lines Ler and Cvi, and the introgression lines in Ler genetic background containing Cvi alleles at EDI and/or FLH. Plants were grown in four different environmental conditions: LD photoperiod (left side); SD photoperiod (right side); V−, without vernalization treatment; V+, with vernalization treatment. Plants were photographed 25 days after planting. (B) Total leaf number of the parental lines Ler and Cvi, the introgression lines in Ler genetic background containing Cvi alleles at EDI and/or FLH, and the F1 hybrids with Ler. The line S10 from which the introgression lines were derived is also included (see materials and methods). Plants were grown in four different environmental conditions: LD photoperiod (left side); SD photoperiod (right side); without vernalization treatment (dark columns); with vernalization treatment (light columns). Graphical genotypes of the lines are shown in the left lower side, each of the five bars corresponding to one linkage group. Total leaf numbers are the mean of 20–24 plants and the standard errors are represented by error bars.

possible in the rest of the genome), but carrying Cvi alleles at FLF and/or FLG. RIL 130 was selected as genotype FLF-Cvi,FLG-Cvi, RIL 104 as FLF-Cvi,FLG-Ler, and RIL 40 as FLF-Ler,FLG-Cvi (the chromosome 5 regions of RILs 40 and 104 are not overlapping). To confirm the presence of two linked flowering loci we performed a reconstruction experiment, under LD conditions, to obtain the expected late flowering genotype when the homozygotes FLF-Cvi and FLG-Cvi are combined. For that, an F1 hybrid between the genotypes FLF-Cvi (RIL
ecotypes, and late
FRI
er
no QTL was identi®ed in the L
FRI
is very closely linked to
LD
i.e.
their allelic effects are dosage in their ¯owering phenotype (measured as both TLN
and
FLF
and
FLF
other crosses between early ecotypes such as L
LD light conditions. The genotypes at markers closely linked to
FLF
(nga158) and
FLG
(nga139) were determined for 64 out of the 142 plants of the population (not filled columns). The symbols for genotypes at these markers in the segregating gametes are indicated in the upper part. The TLN mean ± standard deviation of the four genotypic classes are also shown. Arrows indicate the line means of the parents and some hybrids; the horizontal bars represent their ranges of variation.

Another locus, FLC, at which natural allelic variation has been reported previously, maps in the region of FLF (Koornneef et al. 1994; Lee et al. 1994a). FLC-Ler alleles are known to be early in relation to most other tested ecotypes, and late FLC alleles interact synergistically with late FRI alleles and with mutant alleles at the LD locus. The FRI locus maps on top of chromosome 4, where no QTL was identi®ed in the Ler/Cvi material, and it is very closely linked to LD (Clarke and Dean 1994; Lee et al. 1994b). To determine whether FLC might be FRI, we studied the genetic interactions between FLF and FLG and the loci FRI and Id. We analyzed the flow-
ering phenotype of F1 hybrids and derived F2 populations between the three selected RILs and the late flowering introgression lines in Ler genetic background, FRI-M73 and Id (Figures 7 and 8). F1 and F2 populations were grown under LD conditions in different experiments and therefore they are comparable only indirectly, through the corresponding common controls. Transgression over the latest parent was observed in all F2 populations indicating the effect of Cvi late alleles. The latest flowering plants of each F2 population were genotyped for molecular markers closely linked to FLF, FLG, FRI, and Id (Figure 8; see material and methods). Thus, it was con®rmed that the late flowering phenotype was due to the effects of FLG-Cvi and/or FLG-Cvi and not to interactions of FRI-M73 or Id with Cvi alleles in other genomic regions (either detected in the QTL analysis or not) that might be segregating. Taking together the ®owering phenotype of the F1 hybrids and of the latest F2 plants, and the proportion of toward-lateness transgression in these populations, several conclusions can be summarized as follows:

(1) FLG-Cvi behaves additively with Id to produce lateness and shows a weak synergistic interaction with FRI-M73. The phenotypes of the corresponding F1 hybrids and F2 populations were in agreement, con®rming that both FLG-Cvi and FRI-M73 are partly dominant and Id is recessive.

(2) FLF-Cvi behaves as a late allele of FLC in its synergistic interaction with FRI-M73, and with Id, although it must be a weaker allele than FLC-Sf2 or FLC- Col when compared with TLNs reported previously (Koornneef et al. 1994; Lee et al. 1994a). The phenotypes of the corresponding F1 hybrids and F2 populations were again in agreement with FLG-Cvi and FRI-M73 being partly dominant and Id recessive. Therefore, it is likely that FLF and FLC are the same locus.

DISCUSSION
In this article we have analyzed the ®owering behavior of two early Arabidopsis ecotypes: the laboratory strain Ler originating from Northern Europe (Rédei 1992) and the ecotype Cvi (Lobin 1983). They hardly differ in their ®owering phenotype (measured as both TLN and FT) and in their responses to photoperiod length and vernalization treatment. However, segregating populations derived from crosses between these ecotypes show a much larger variation than that observed in other crosses between early ecotypes such as Ler and Col (Jansen et al. 1995). The ®owering behavior differences between the Ler/Cvi lines can be mainly attributed to four loci referred to as EDI, FLF, FLG, and FLH. Cvi alleles at EDI and FLH produce earliness while at FLF and FLG Cvi alleles produce lateness, thus explaining the similar behavior of the parental lines and the transgression in the RILs. Another seven putative minor QTLs might contribute secondarily to these differences,
but they were found only in the environment with the lowest phenotypic variation and further confirmation is necessary. This is, at least partly, due to the limitations for detecting minor QTLs in small populations where several QTLs with large effects are segregating, as seen, for instance, with the effect of FLH, which was not detected under SD conditions in the RIL population but was present in the Ler genetic background NILs. Alleles with major effect at the loci FRI and FLC have appeared responsible previously for most flowering differences between several very late, vernalization-responsive ecotypes and early ones (classified according to their flowering behavior under long-day light conditions; Napp-Zinn 1969; Burn et al. 1993; Koornneef et al. 1994; Lee et al. 1994a; Clarke et al. 1995; Kuittinen et al. 1997; Sanda et al. 1997). It was shown before that large allelic effects can also be present in crosses between some early ecotypes (Van Der Veen 1965; Kowalski et al. 1994; this study). Furthermore, it can be predicted that strong effect alleles will probably segregate in crosses between late ecotypes, since some of them carry large effect late alleles with genetic behavior different than the allelic variation at FRI and FLC (Burn et al. 1993; C. Alonso-Blanco and M. Koornneef, unpublished results). Therefore, major effect mutations seem to contribute frequently to the natural flowering variation observed among Arabidopsis ecotypes, although how many loci are involved is still unknown.

Late alleles at two of the major loci identified in the Ler/Cvi population, FLF and FLG, interact synergistically. A similar type of interaction has been previously shown to occur between natural late alleles at FRI and FLC (Koornneef et al. 1994; Lee et al. 1994a) and in addition, FLF-Cvi and FLG-Cvi interact synergistically with late alleles at FRI. Epistasis has also been detected in two previous crosses where it has been analyzed (Clarke et al. 1995; Kuittinen et al. 1997) and therefore, epistasis among natural alleles might account for an important proportion of the phenotypic variation, as shown among alleles of mutant loci (Koornneef et al. 1998a), and among induced and natural alleles (Sanda and Amasino 1996a,b).

The Cvi ecotype shows a slightly reduced response to photoperiod length changes and a more pronounced vernalization response than the Ler ecotype. The three major loci, EDI, FLF, and FLG, control most of the response differences to photoperiod and vernalization, as shown by their strong QTL × E interactions. Early alleles at these loci not only reduced flowering time but also diminished the response to photoperiod length. In fact, as shown with the near isogenic line EDI-Cvi in Ler genetic background, the combination of EDI-Cvi alleles with FLF-Ler, FLG-Ler is able to render Arabidopsis practically day-length neutral in its flowering behavior. On the other hand, FLF, FLG accounted for much of the vernalization response, the late-flowering effect of Cvi alleles being eliminated by a 3-wk vernalization treatment. In agreement with these results, the Cvi ecotype flowered at almost similar times under LD and SD conditions when vernalized; i.e., Cvi eventually behaved as almost day-length neutral when the effect of FLF-Cvi, FLG-Cvi was physiologically removed by the vernalization treatment. In other Arabidopsis populations where QTL × E interactions have been analyzed, the largest effect QTLs also showed significant interaction (Clarke et al. 1995; Jansen et al. 1995). In addition, allelic variation at FLG and FRI is differentially expressed depending on the vernalization treatment (Koornneef et al. 1994; Lee et al. 1994a; Lee and Amasino 1995). Therefore, major effect loci controlling the flowering
Figure 8.—Frequency distributions of TLN in F2 populations derived from crosses between the three RILs selected as genotypes FLG-Cvi (upper part), FLF-Cvi (middle part) and FLF-Cvi, FLG-Cvi (lower part) and the two late flowering introgression lines FRI-M73 (left side) and ld (right side). Plants were grown under LD light conditions. The genotypes at molecular markers closely linked to FLF, FLG, FRI, and ld were determined for the latest flowering plants of each F2 (not filled columns). In each graph, the cross involved and the symbols for the deduced genotypes at the corresponding flowering loci are indicated in the right upper corner. Arrows indicate the parental line means and the horizontal bars represent their ranges of variation. Hom, homozygote; het, heterozygote.

differences among Arabidopsis populations seem to interact with the environment, which might be an important factor for maintaining natural genetic variation (Mitchell-Olds 1995).

Many of the Arabidopsis flowering loci have been characterized genetically and physiologically in relation to the vernalization and photoperiod responses and a model for the control of the transition from the vegetative to the reproductive phase is being developed (reviewed in Martinez-Zapater et al. 1994; Coupland 1995; Amasino 1996; Koornneef et al. 1998b). Three major flowering promotion pathways with partly additive and partly redundant functions have been defined, namely, the autonomous (also called constitutive or endogenous), the photoperiod (or long-day), and the vernalization pathways. The vernalization flowering promotion is thought to act on certain targets common to the autonomous pathway, and it has been suggested they might involve gibberellin metabolism or sensitivity. Mutants of loci involved in the autonomous flowering promotion pathway (fca, fdl, fpa, fve, fy, and ld) are more responsive to day-length and vernalization than the Ler wild type, whereas mutations in the photoperiod pathway (co, fd, fe, fha, ft, fwa, and gi) are less responsive to day-length changes. The analysis of EDI suggests it might be involved in the photoperiod flowering promotion pathway given the lack of photoperiod response observed in the EDI-Cvi NIL in Ler genetic background.
The dominance associated with the flowering behavior of the EDI-Cvi allele indicates that its product might promote flowering (or repress the vegetative phase) and this function would be reduced in the EDI-Ler allele. The EDI-Cvi line flowers earlier than Ler under both LD and SD conditions and somehow resembles the phenotype of transgenic lines carrying the CO gene under control of a 3SS-promoter (Simon et al. 1996), suggesting that the photoperiodic promotion pathway is over-functioning under both day-length conditions, leading to the earliness and day-length insensitivity observed. In other words, EDI function could be controlled by photoperiod length when encoded by the Ler allele but might be expressed independently of day-length when encoded by the Cvi allele.

Late alleles at the FLF and FLG loci are very responsive to vernalization and confer a more pronounced response to photoperiod length, as seen from the behavior of the EDI-Ler, FLF-Cvi, FLG-Cvi RILs, features also shared with the late alleles at FRI and FLC (Lee and Amasino 1995). The similar physiological behavior of the FLF-Cvi and FLG-Cvi alleles and the late mutant alleles of the autonomous flowering promotion pathway suggest that they act in the same pathway. Given the codominance of these Cvi late alleles it is not possible to speculate whether they might promote or repress the flowering process. However, recessive early alleles at the FLC locus have been obtained by mutagenesis (Sanda and Amasino 1995) and candidate mutant alleles at the positions of FRI and FLG are not known, which might indicate that their gene products play a role in inhibiting the flowering process. The similar physiological and genetic behavior of late alleles at the FLF and FLC loci, together with their matching map positions, suggests they are probably the same locus. In addition, the similar genetic and physiological characteristics of FLF-Cvi and FLG-Cvi and the late alleles at FRI, and the fact that they are partly interchangeable in their genetic interactions, suggest they have certain redundant functions repressing flowering within the autonomous promotion pathway. As proposed by Lee et al. (1994a) and Sanda and Amasino (1996a), the effect of FLC/FLF would be counteracted by the autonomous pathway mutant genes, such as LD, given their epistatic interaction. Since FLG-Cvi does not interact with Id, LD might act directly on FRI, FLC/FLF but probably not on FLG.

Considering together the behavior of the three loci EDI, FLF, and FLG, it is worth noting that RILs EDI-Cvi, FLF-Cvi, FLG-Cvi respond to photoperiod length, in contrast to the EDI-Cvi NILs. Under the discussed model, in such genotypes the photoperiod pathway would be promoting flowering at the same level in both day-lengths. This photoperiod response would therefore imply that under SDs there is also an inhibition (or lack of promotion) of the autonomous flowering pathway, which would operate through FLF,FLG. In agreement with this, similar genetic behavior has been observed in double mutants between nonresponsive and responsive loci, which show mostly an intermediate, additive, day-length response (Koornneef et al. 1998a).

The allelic variation at the FLH locus has a rather mild effect on flowering. Cvi alleles responding like Ler to day-length changes. The additive behavior of EDI and FLH together with the more pronounced response of FLH-Cvi alleles to a vernalization treatment, suggest that FLH might be involved in the autonomous flowering promotion pathway. However, opposite to FLF, FLG and to other vernalization responsive loci, at FLH it is the early allele which increases the response; i.e., FLH-Cvi early alleles make Ler more vernalization responsive. This might suggest its role in the control of the vernalization response.

Figure 9 shows a scheme of the current general model for the control of flowering initiation (Koornneef et al. 1998b), where the possible role of EDI, FLF, FLG, and FLH is indicated.

We have shown that the Ler/Cvi allelic variation probably concerns loci involved in different flowering pathways. Comparison of map positions between identified QTLs and mutant loci might suggest putative candidate genes at which this natural variation occurs. Nevertheless, caution must be taken given the inaccuracy of the QTL mapping and the large number of known mutant loci affecting flowering behavior, which appeared scattered over the five linkage groups (Koornneef et al. 1998b). Similar considerations must be taken when comparing QTL positions in different populations, and fine-scale mapping and allelism tests are required to determine the locus (or tightly linked loci) involved in the corresponding allelic variation. Two mutant loci, LHY and FHA (Simon and Coupland 1996; Koornneef et al. 1991), assigned to the photoperiod flowering promotion pathway, have been mapped on chromosome 1 in the EDI region, although preliminary fine mapping has left LHY out. Furthermore, a flowering QTL has been mapped on this genomic region in the cross between Ler and Col ecotypes (Jansen et al. 1995). The FLF locus maps in the same region as FLC, and the

![Figure 9](image)
similar physiological and genetic behavior of late alleles at both loci suggests they are probably the same locus. Several other loci have been identified by mutagenesis close to FLC, such as FY and CO (Koornneef et al. 1994). Allelic natural variation has been also assigned to this region in all crosses analyzed previously (Kowalski et al. 1994; Clarke and Dean 1994; Jansen et al. 1995; Kuittinen et al. 1997). It is unknown whether this eco-
type variation belongs only to the FLC locus, which would indicate the existence of multiple alleles with different flowering effects, or to several closely linked loci. One natural variant, ART-Sy0, has been mapped in the region of FLG (Grbic and Eecker 1996; Grbic and Gray 1997). ART-Sy0 gives rise to aerial rosettes when combined with late alleles at another locus on chromosome 4, probably FRI. In addition, it seems to produce lateness in the absence of late alleles at the chromosome 4 locus, but taking into account the gen-
etic linkage to FLC it is unclear whether this lateness involved FLC and whether late FLC alleles are also necessary to produce the aerial rosette phenotype. Nevertheless, aerial rosette phenotypes were not observed in late plants of the crosses FLG-Cvi with FLF-Cvi, FLG-Cvi with FRI-M73. No known mutant locus maps at the FLH position, although QTLs have been identified in this region in most crosses analyzed previously (Kowalski et al. 1994; Clarke et al. 1995; Mitchell-Olts 1996; Jansen et al. 1995).

It is expected that part of the natural variation will correspond to alleles of mutant flowering genes. Never-
theless, it is evident that the spectrum of natural genetic variation will be different from the spectrum obtained by artificial mutational analyses, partly due to the limita-
tions of the small number of ecotypes used to generate mutants. Some alleles might not be functional in some ecotypes, as is likely to be the case for FRI alleles in many early ecotypes (Koornneef et al. 1994), and the epistatic interactions hamper the detection by mutagen-
esis of flowering loci, such as was shown previously with LD or FLD, which interact with FLC (Koornneef et al. 1994; Lee et al. 1994a; Sanda and Amasino 1996b), and therefore their mutations were detected in Col but not in Lr. Further analysis of the loci identified in this study and in other populations is to come and the final identification of individual natural alleles at the molecular level is still needed. This will provide tools not only for the developmental and physiological dissection of the flowering process, but also for understanding the molecular mechanisms and the ecological and evolu-
tionary significance of this quantitative natural varia-
tion.

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ment of Egypt.

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