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Cis-regulatory Changes at *FLOWERING LOCUS T* Mediate Natural Variation in Flowering Responses of *Arabidopsis thaliana*

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ABSTRACT

Flowering time, a critical adaptive trait, is modulated by several environmental cues. These external signals converge on a small set of genes that in turn mediate the flowering response. Mutant analysis and subsequent molecular studies have revealed that one of these integrator genes, *FLOWERING LOCUS T* (*FT*), responds to photoperiod and temperature cues, two environmental parameters that greatly influence flowering time. As the central player in the transition to flowering, the protein coding sequence of *FT* and its function are highly conserved across species. Using QTL mapping with a new advanced intercross-recombinant inbred line (AI-RIL) population, we show that a QTL tightly linked to *FT* contributes to natural variation in the flowering response to the combined effects of photoperiod and ambient temperature. Using heterogeneous inbred families (HIF) and introgression lines, we fine map the QTL to a 6.7 kb fragment in the *FT* promoter. We confirm by quantitative complementation that *FT* has differential activity in the two parental strains. Further support for *FT* underlying the QTL comes from a new approach, quantitative knockdown with artificial microRNAs (amiRNAs). Consistent with the causal sequence polymorphism being in the promoter, we find that the QTL affects *FT* expression. Taken together, these results indicate that allelic variation at pathway integrator genes such as *FT* can underlie phenotypic variability and that this may be achieved through *cis*-regulatory changes.

MOLECULAR analysis of the phenotypic variation in life history traits is key to understanding how plants evolve in diverse natural environments. Among such traits, flowering time is critical for the reproductive success of the plant and is highly variable among natural *Arabidopsis thaliana* strains, providing an attractive paradigm for studying adaptive evolution (Johanson *et al.* 2000; Hagenblad and Nordborg 2002; Stinchcombe *et al.* 2004; Lempe *et al.* 2005; Shindo *et al.* 2005; Werner *et al.* 2005a). Two major environmental parameters that modulate flowering time are light and temperature (Koornneef *et al.* 1998). Temperature and light conditions vary substantially within the geographical range of *A. thaliana*, and natural populations presumably need to adapt to the local environment to ensure reproductive success. Flowering in *A. thaliana* is generally accelerated by long photoperiods, vernalization (exposure to winter-like conditions), and elevated ambient temperatures (Bäurle and Dean 2006). All these cues favor flowering of *A. thaliana* during spring or early summer, although the contribution from each individual cue and the interactions among them vary depending on the local environmental conditions (Wilczek *et al.* 2009).

Flowering time is controlled through several genetic cascades that converge on a set of integrator genes including *FLOWERING LOCUS T* (*FT*), which encodes a protein that is highly conserved in flowering plants (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999; Ahn *et al.* 2006). FT and its homologs are very likely an integral part of the mobile signal (florigen) that is produced in leaves and travels to the shoot apex to induce flowering (Abe *et al.* 2005; Wigge *et al.* 2005; Lifschitz *et al.* 2006; Corbesier *et al.* 2007; Jaeger and Wigge 2007; Lin *et al.* 2007; Mathieu *et al.* 2007; Tamaki *et al.* 2007; Notaguchi *et al.* 2008). In

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A. thaliana, *FT* expression is controlled by photoperiod, vernalization, and ambient growth temperature. Photoperiod in conjunction with the circadian clock promotes daily oscillations in *FT* RNA levels, which are greatly elevated at the end of long days. The central role of *FT* in determining the timing of flowering appears to be conserved in many species, making *FT* an attractive target for altering flowering time in cereals and other plants of economic importance (recently reviewed by Kobayashi and Weigel 2007; Turck *et al.* 2008).

Wild strains of *A. thaliana* show extensive variation in flowering time and much of this is due to variation in the activity of the floral repressor *FLOWERING LOCUS C* (*FLC*). While some of this variation maps to *FLC* itself, much of it is due to differential activity at the epistatically acting *FRIGIDA* (*FRI*) locus (Michaels and Amasino 1999; Sheldon *et al.* 1999; Johanson *et al.* 2000; Michaels *et al.* 2003; Lempe *et al.* 2005; Shindo *et al.* 2005, 2006). Flowering is typically substantially delayed when the *FRI/FLC* system is active, unless these plants are first vernalized. However, *FRI* and *FLC* do not explain all of the flowering time variation seen in wild strains, and functionally divergent alleles of several additional flowering regulators, including *CRYPTOCHROME 2* (*CRY2*), *HUA2*, *FLOWERING LOCUS M (FLM)*, *PHYTOCHROME C* (*PHYC*), and *PHYTOCHROME D (PHYD)*, have been identified in different strains of *A. thaliana* (Aukerman *et al.* 1997; Alonso-Blanco *et al.* 1998; El-Assal *et al.* 2001; Werner *et al.* 2005b; Balasubramanian *et al.* 2006a; Wang *et al.* 2007). Finally, there are many genotype-by-environment interactions that dramatically affect the contribution of a specific locus to the overall phenotype.

The study of natural variation in *A. thaliana* has been greatly facilitated through the use of recombinant inbred line (RIL) populations (Koornneef *et al.* 2004). We have recently established two advanced intercross (AI)-RIL sets, in which the genetic map is greatly expanded, allowing for high-resolution QTL mapping (Balasubramanian *et al.* 2009). Here we use one of the new AI-RIL populations along with an independent F_2 population to identify the molecular basis of a light and temperature-sensitive flowering time QTL that mapped to the promoter of the *FT* gene. We show that *FT* is likely the causal gene for variation in light and temperature-sensitive flowering. Our results, in combination with those from other species, suggest that *cis*-regulatory variation rather than structural variation at *FT* contributes to phenotypic variation in natural populations.

MATERIALS AND METHODS

Plant material and growth conditions: Two early flowering accessions, Est-1 [Estland (Estonia); European Arabidopsis Stock Center N6701] and Col-0 (Columbia; WT-2, Lehle Seeds, Tucson, AZ), were used to create the AI-RIL population for QTL analysis. The population consists of 279 individual lines, genotyped at 221 markers (Balasubramanian *et al.* 2009). A second F_2 population, derived from Dra-1 (Drahonin, Czechoslovakia; N1119), a strain that behaves similarly to photoperiodic mutants (Lempe *et al.* 2005), and L*er* (Landsberg *erecta*; N8581), consisted of 190 F_2 plants that were genotyped at 77 markers. The markers and the *ft* mutant alleles have been previously described (Koornneef *et al.* 1991; Yoo *et al.* 2005; Balasubramanian *et al.* 2009).

Flowering time was determined under 23° in long days (LD, 16 hr light/8 hr dark) in growth rooms and under LD conditions in a greenhouse in La Jolla, CA, and under 16° LD in growth rooms in Tübingen, Germany. Short day (SD, 8 hr light/16 hr dark) experiments and flowering time QTL confirmation studies with HIF plants were conducted in a Percival Series 942 growth chamber at 16° or 22° in Madison, WI. For quantitative complementation experiments, F_1 populations of Est-1, Col-0, Dra-1, L*er* introgression lines, and other arbitrarily chosen strains crossed with various *ft* mutants were grown in growth rooms, and flowering time was measured as total leaf number at 16° LD for 4–10 plants. Quantitative complementation and knockdown studies with amiR-ft-1 (Schwab *et al.* 2006) were carried out in Tübingen and Madison in 16° LD or 23° LD.

QTL analyses: Ten plants per RIL were grown in a completely randomized design and flowering time was measured as days to flowering and as total number of leaves, which were partitioned into juvenile, adult, and cauline leaves. QTL analyses (*scanone* for simple interval mapping and *scantwo* for two-dimensional scans) were carried out using the r-qtl package in R (http://www.r-project.org). QTL significance was determined by permutation testing (1000 runs). The *scantwo* plots are presented as heat maps of additive and epistatic interactions between markers. A color scale for the LOD scores allows comparison to genomewide averages. Thermosensitivity, a measure of the response of a particular RIL line to a change from 16° to 23° compared to the average response of all RILs, was calculated using the slope of the reaction norms as previously described (Lempe *et al.* 2005). Thermosensitivity, which is a quantifiable measure of temperature response, was then used as a trait in QTL mapping.

HIF experiments: To confirm the F5I14*/FT* QTL, two heterogeneous inbred families (HIFs) (Tuinstra *et al.* 1997; Loudet *et al.* 2005) segregating only for the QTL region and derived from RIL 110 and RIL 133 were characterized. Initially, 12 seeds from the S8 generation were genotyped to isolate plants homozygous for each parental allele as well as a plant heterozygous at marker F5I14. Seeds were collected from these plants for subsequent experiments, and 200 segregating progeny were analyzed for an association between flowering time and allele status. SD flowering time was measured using only progeny from the two homozygous lines.

Fine mapping with NILs: To fine map the F5I14*/FT* QTL, we generated near isogenic lines (NILs). Est-1 was crossed to Col-0 and seeds were collected from the F_1 and F_2 generations. F_2 plants were genotyped at F5I14 for the QTL region and at one marker from each of the other four chromosomes. A single plant was selected that was heterozygous at F5I14, and Col-0 homozygous at the other markers, and backcrossed to Col-0. This was repeated for the BC_1 to BC_3 generations, selecting for heterozygosity at F5I14 and Col homozygosity for all other regions. From the BC_3F_2 generation, plants that belonged to the earliest and latest quartile (192 plants) were genotyped at marker F5I14. From this experiment, two lines for each F5I14 allele combination (Est-1/Est-1, Est-1/Col-0, and Col-0/Col-0) were chosen as NILs for subsequent experiments. Progeny testing showed that the flowering behavior of the NILs was stable and that the direction and effect of the alleles agreed with the QTL

mapping results. One NIL-Est was genotyped at 94 genome-wide loci, and all but 17 of 182 alleles were Col-0.

Second round of fine mapping: To reduce the QTL region further, 700 NIL plants heterozygous at F5I14 were genotyped at two markers (24.1 Mb and 24.6 Mb), identifying 28 plants with a recombination event between the two markers. It was necessary to phenotypically classify each plant by progeny testing because of the relatively small effect of the QTL (15% difference between NIL-Col and NIL-Est). Therefore, 12 progeny from each of 28 recombinants were used to classify each recombinant as early, intermediate, or late flowering. Heterozygous parents were readily apparent due to a relatively large standard deviation of the flowering time of their progeny. Further SNP genotyping combined with DNA sequencing reduced the QTL region to the final interval of 6.7 kb.

Quantitative complementation and quantitative knockdown: *FT* activity was assessed by combining specific natural *FT* alleles with laboratory-induced *ft* mutant alleles. *ft-1*, *ft-2*, and *ft-3* are EMS-induced alleles in the L*er* background (Koornneef *et al.* 1991) and were crossed to Est-1 and Col-0. *ft-10* is a T-DNA insertion line in the Col-0 background (Yoo *et al.* 2005) and was crossed to L*er* and Dra-1. Similar experiments were conducted using the two homozygous NILs. Line × cross interaction was determined by the following analysis of variance (ANOVA) model: Total leaf number ~ Line + Cross + Line × Cross. An artificial miRNA, amiR-ft-1, which specifically reduces *FT* expression (Schwab *et al.* 2006), was introduced into Est-1, Col-0, and the homozygous NILs, and flowering time assayed in 20 or more T1 plants for each genotype. The interaction between the genotype and the presence of the amiR-ft-1 transgene was assessed by the following ANOVA model: Total leaf number ~ Line + Transgene + Line × Transgene.

***FT* expression studies:** Plants were analyzed over a time course as previously described (Michael *et al.* 2008). Briefly, seeds from each genotype were vapor sterilized, plated on half strength MS 0.8% agar plates, and stratified for 4 days at 4° in the dark. Plates were then transferred to continuous white light at 70 μmol m^{-2} s^{-1} with a daily 12 hr 22°/12 hr 12° temperature regime and grown for 14 days. On the 15th day, plants were harvested into liquid nitrogen every 4 hours starting at the transition from 12° to 22°, for a total of six time points (0, 4, 8, 12, 16, and 20 hr). Frozen tissue was disrupted in 2-ml Eppendorf tubes containing three ball bearings using a Retsch (Hann, Germany) shaker. RNA was extracted with RNeasy (QIAGEN, Valencia, CA) and 5 μg of RNA was used to prepare cDNA (Invitrogen, Carlsbad, CA). cDNA was diluted 1:20, and *FT* RNA expression was quantified by quantitative real time PCR (qRT-PCR) with SYBRgreen on a MyIQ system (Biorad, Hercules, CA). qRT-PCR protocol and primers have been described (Mockler *et al.* 2004). The *FT* primers used were: FT_Q254F, 5′-ATCTCCATTGGTTGGTGACTGATA and FT_Q306R, 5′-GCCAAAGGTTGTTCCAGTTGTAG.

Statistical analysis: Statistical analysis was carried out using JMP (SAS Institute, Raleigh, NC) or R (http://www.r-project.org). Student's *t*-tests as implemented in Microsoft Excel were used to determine the significance of the *FT* expression difference between NILs.

Sequencing: Genomic DNA of two recombinants and the parental accessions, Est-1 (GenBank accession nos. GQ377110 and GQ395499) and Col-0, were sequenced from 24,327,174 to 24,337,983 bp on chromosome 1. Multiple overlapping fragments of 0.6- to 1.0-kb size were amplified by PCR with Advantage cDNA Polymerase Mix (Clontech, Palo Alto, CA), DNA fragments were purified with a spin column (QIAGEN). Sequencing reactions were performed in house with Big Dye terminator (Applied Biosystems, Foster City, CA). Alignments were generated using MegAlign (DNAStar, Madison, WI). To sequence *FT* from 24 wild strains (GenBank accession nos. GQ395468 to GQ395500), four overlapping fragments covering the coding and upstream region were amplified, purified by gel electrophoresis, and sequenced using nested primers on both strands. Sequences were aligned with the ABI Prism 2.1 Autoassembler (Applied Biosystems), and the alignment was manually verified and edited in SeAl (http://tree.bio.ed.ac.uk/).

RESULTS

QTL analysis of flowering behavior for the Est-1 × Col-0 AI-RIL population: Flowering time for the Est-1/Col-0 AI-RIL population was measured under inductive LD at two different temperatures (16° and 23°) and in two different light environments (growth room and greenhouse). The distribution of flowering times, measured as days to flowering or total leaf number, was continuous and showed in all conditions transgression well beyond the parental values (Figure 1A, supporting information, Table S1). QTL analysis identified a large-effect locus on chromosome 1 that influenced flowering time under all conditions, with the Est-1 allele causing a delay in flowering (Figure 1B). Single marker association with closely linked markers as factors and flowering time as the response revealed that the QTL effect was stronger at 16°, with the QTL explaining as much as 45% of the total variation in days to flowering, while it accounted for about 25% at 23°.

The 1.5 LOD confidence interval of this QTL at 16° in LD spanned a 600-kb region that included the well-known floral regulator, *FT* (At1g65480) (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999). Twenty-seven RILs carried recombinant chromosomes in this interval. An ANOVA using these 27 lines with flowering time as the response and allelic state at the markers as factors revealed that the F5I14 marker, which is tightly linked to *FT* (36 kb away), had the strongest association among the seven surrounding markers (Table S2). To confirm the QTL, we compared the flowering times of individuals from two HIFs (Tuinstra *et al.* 1997; Loudet *et al.* 2005) that were segregating for the parental alleles only at this marker. Among 200 segregating progeny for each HIF, plants homozygous for the Est-1 allele flowered 3–5 days later at 23° LD and 16° LD than plants with the Col-0 allele (Figure 1, C and D). The differences in flowering time for the homozygous plants were highly significant ($P < 0.0001$), while the heterozygous plants had an intermediate phenotype (Figure 1, C and D). In addition, the QTL was specific to LD conditions (Figure 1, C and D).

Flowering time data from 23° LD suggested a second QTL on chromosome 1 at 20.8 Mb (linked to marker nga280), north of F5I14, which is located at 24.3 Mb (Figure 1B). This QTL accounted for ~25% of variation in flowering time, with the Est-1 allele delaying flowering compared to the Col-0 allele (Table S3). The effect

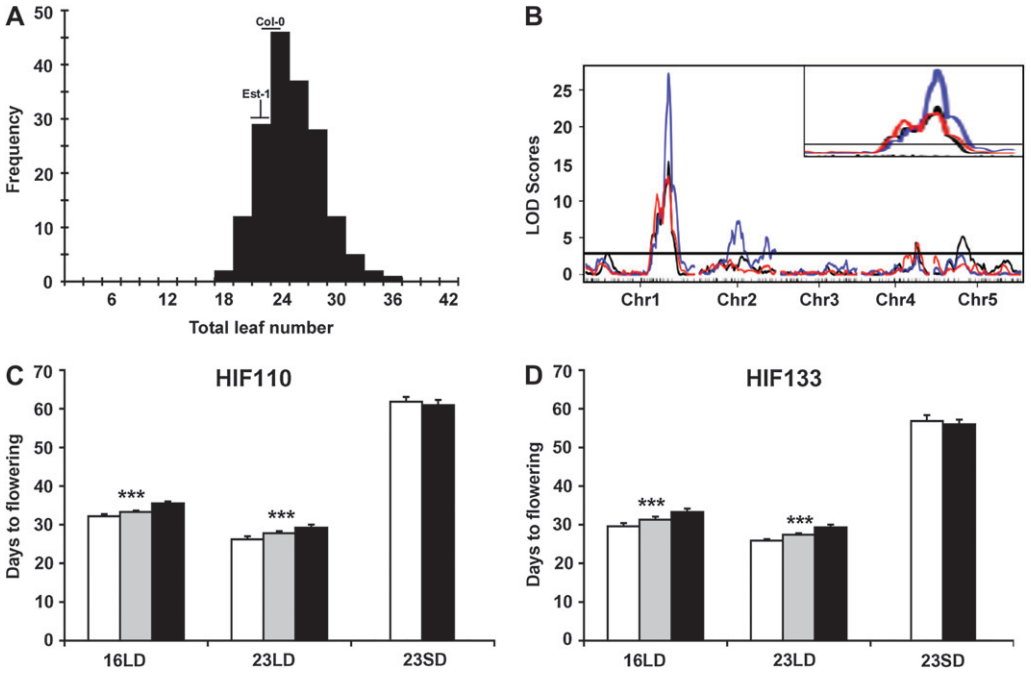

FIGURE 1.—QTL analysis of flowering time in Est-1/Col-0 AI-RIL population. (A) Distribution of flowering time (expressed as leaf number) in the AI-RIL population grown in the greenhouse under 23° LD, including the means for the Est-1 and Col-0 parents. (B) QTL maps of flowering time (measured as days to flower) under three different growth conditions: blue, 16° LD in growth room; red, 23° LD in growth chambers; black, 23° LD in green house. The horizontal line represents the significance threshold for the LOD score. (C and D) Average flowering times of the HIFs based on the genotype of the F5I14 marker. White bars, homozygous for Col-0; gray bars, heterozygous; black bars, homozygous for Est-1. Significant differences ($P < 0.0001$) between the Est-1 and Col-0 genotypes are indicated by asterisks. Error bars represent standard error of mean (SEM).

of the nga280 QTL largely disappeared at 16° LD, while the F5I14/*FT* QTL effect became more pronounced. A two-dimensional genome scan revealed distinct QTL interactions at 16° and 23°. At 23°, a significant additive interaction was detected between the two QTL on chromosome 1 (Figure 2A), while at 16° the F5I14/*FT* QTL interacted additively with a QTL linked to the marker PLS2 on chromosome 2, for which the Est-1 allele promoted early flowering (Figure 2B, Table S3). This QTL was near the *PHYB* locus, which is known to affect flowering (REED *et al.* 1993). Since there were two interacting QTL that appeared to modulate thermal responses, we calculated the temperature sensitivity in flowering time for each of the RILs (Table S1) and mapped QTL for the same (Figure 2C). This analysis revealed that the F5I14/*FT* QTL and the PLS2/*PHYB* QTL on chromosome 2, but not the nga280 QTL on chromosome 1, affected temperature sensitivity.

Colocalizing QTL for flowering time in a Dra-1/L*er* F_2 population: We have previously identified Dra-1 as a strain that behaves similarly to photoperiodic mutants in the Col-0 and L*er* genetic backgrounds (LEMPE *et al.* 2005). Dra-1 flowers in short days at a similar time as Col-0 and L*er*, but flowers later than these strains in long days (although still earlier than in short days). Analysis of an F_2 population derived from a cross between Dra-1 and L*er* revealed a continuous distribution of flowering times at 23° LD. However, at 16° LD, there was a group of late-flowering plants comprising almost 25% of the population (Figure 2D). Linkage analysis indicated a strong association between flowering time and marker F5I14, with the Dra-1 allele conferring later flowering at 16° LD. QTL analysis at 16° LD with 192 F_2 plants identified several significant QTL across the genome (Figure 2E). The most robust QTL was centered on F5I14/*FT*, with the confidence interval overlapping that of the Est-1/Col-0 QTL. In addition, a QTL that colocalized with the PLS2/*PHYB* QTL detected in the Est-1/Col-0 RIL population was observed, with the Dra-1 allele conferring late flowering. Two QTL were found on chromosome 5, one linked to marker CA72 near the floral repressor *FLC*, and one linked to MBK5 near the *MAF2-4* cluster of *FLC* homologs (Figure 2E). The Dra-1 allele conferred late flowering at the CA72/*FLC* QTL, and early flowering at the MBK5/*MAF* QTL (Table S3). A two-dimensional genome scan of the Dra-1/L*er* population confirmed the epistasis between the F5I14/*FT* QTL and the PLS2/*PHYB* QTL on chromosome 2 and revealed additional additive interactions with other QTL (Figure 2F).

Fine mapping of the F5I14 QTL in Est-1 × Col-0: To fine map the F5I14/*FT* QTL, we introgressed the QTL interval from Est-1 into Col-0. We classified the progeny of 392 descendants of a single BC_3 plant, which had been heterozygous at marker F5I14, for extreme flowering behavior. We found that the QTL in this backcross population roughly segregated in a Mendelian manner, with all but 4 of the 96 latest plants being either homozygous for Est-1 at F5I14 (65 plants) or heterozygous (27 plants). To generate NILs, we propagated a single plant for each of the three possible F5I14 genotypes (Col-0/Col-0, Est-1/Est-1, and Col-0/Est-1). The NIL-Est (homozygous for Est-1 allele at *F5I14*, with the rest of the genome being mostly Col-0; see MATERIALS

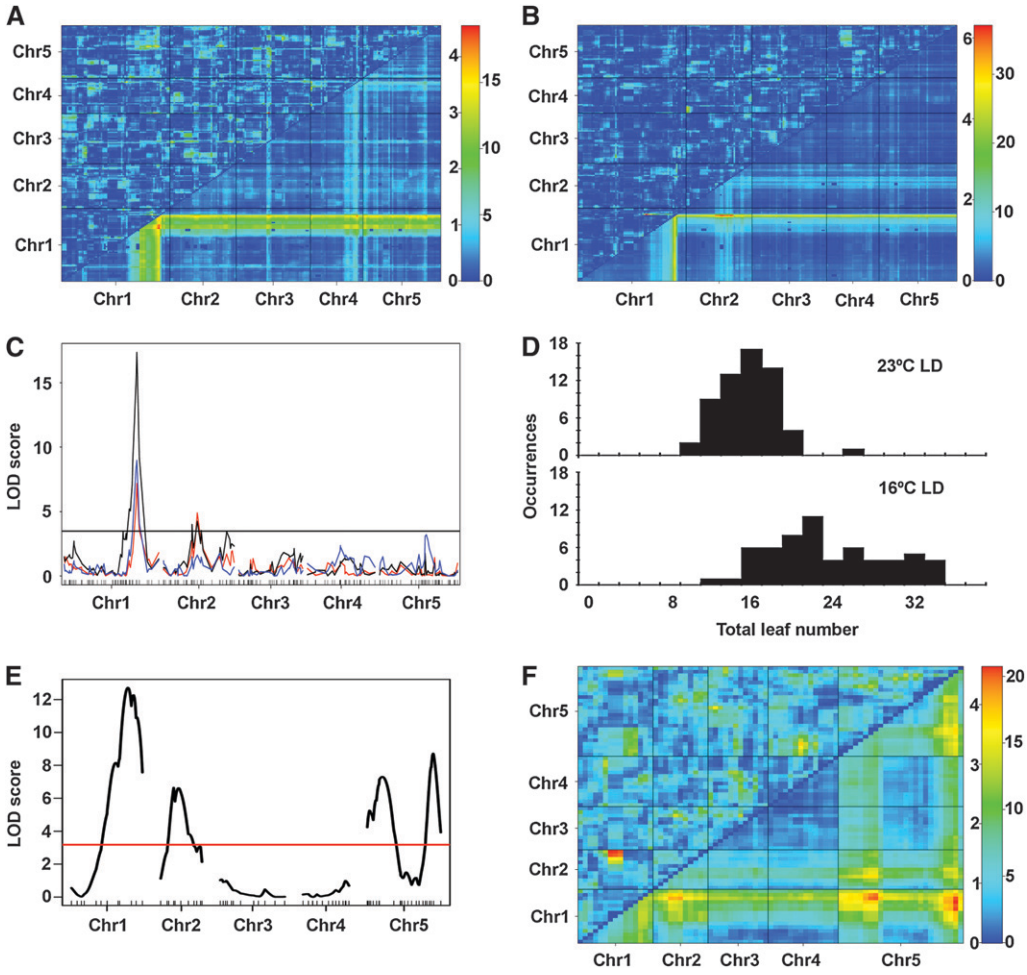

FIGURE 2.—Genetic interactions between flowering time QTL. (A and B) Two-dimensional genome scan in Est-1/Col-0 AI-RIL population in 23° LD (A) and 16° LD (B), with epistatic interactions on top, and additive interactions on the bottom. Interactions between markers on each chromosome are shown. Color scale indicates LOD scores for epistatic (left) and additive interactions (right). (C) QTL analyses of thermosensitivity in Est-1/Col-0 AI-RIL population, contrasting flowering at 16° and 23°, using three different 23° data sets: black, growth room 1; red, growth room 2; blue, green house. The thermosensitivity QTL colocalizes with the QTL in the F5I14/*FT* region (Figure 1B). (D) Distribution of flowering time in Dra-1 × L*er* F_2 population at 23° and 16° LD. (E) QTL analysis of flowering time in Dra-1 × L*er* F_2 population. (F) Two-dimensional genome scan in Dra-1 × L*er* F_2 population (see A and B for legend).

AND METHODS) was consistently later than Col-0 or the NIL-Col (Figure 3A). In addition, the NIL-Est flowered later than either parent, suggesting that Est-1 contains alleles at other loci that accelerate flowering compared to the Col-0 allele(s) (Figure 3, B and C).

For fine mapping, we identified 28 plants with a recombination event within the 600-kb interval surrounding *FT* and phenotyped 12 progeny each from these recombinants. Combining the flowering time information of these 28 families with additional genotyping with SNP markers around *FT* reduced the QTL to an interval of 9 kb (Figure 3D). The entire 9-kb interval was sequenced in the two last recombinants, which further reduced the QTL interval to a 6.7-kb region upstream of the *FT* coding region (Figure 3D).

***FT* as the causal gene for the F5I14/*FT* QTL:** Since fine mapping identified a noncoding fragment upstream of *FT* as the QTL, we tested whether *FT* was the causal gene underlying the QTL in both the Est-1 × Col-0 and Dra-1 × L*er* populations. First, we performed quantitative complementation experiments (LONG *et al.* 1996) using crosses between strains containing *ft* mutant alleles and Est-1 or Dra-1. We compared the effect of *FT* alleles in Est-1 with Col-0, and of Dra-1 with L*er*, at 16° LD, the environment where the QTL effect was strongest in both populations. There was a significant line × cross interaction ($P < 0.0001$), indicating quantitative noncomplementation of the *ft* mutant alleles by the Est-1 and Dra-1 alleles compared to Col-0 and L*er* alleles, respectively (Figure 4, A and B).

Second, we performed a similar set of experiments with the NILs. These experiments again confirmed a significant line × cross interaction ($P < 0.01$), with the NIL-Est being unable to fully complement the *ft* mutant at 16° LD. We included a number of additional strains as controls in this analysis and observed significant interactions only with Est-1 and Dra-1, and the respective NILs (Figure 4, A and B; Figure S1, A and B).

Third, we adopted a novel approach, quantitative knockdown, where we tested directly whether inactivating the Est-1 allele of *FT* was less effective in delaying flowering than inactivation of the Col-0 allele, using an artificial microRNA against *FT* (amiRNA-ft-1) (SCHWAB *et al.* 2006). We transformed the amiR-ft-1 construct into Est-1, Col-0, and the NILs and analyzed the flowering time of at least 20 independent T1 lines in each background. An ANOVA revealed a significant interaction between genetic background and presence of the transgene,

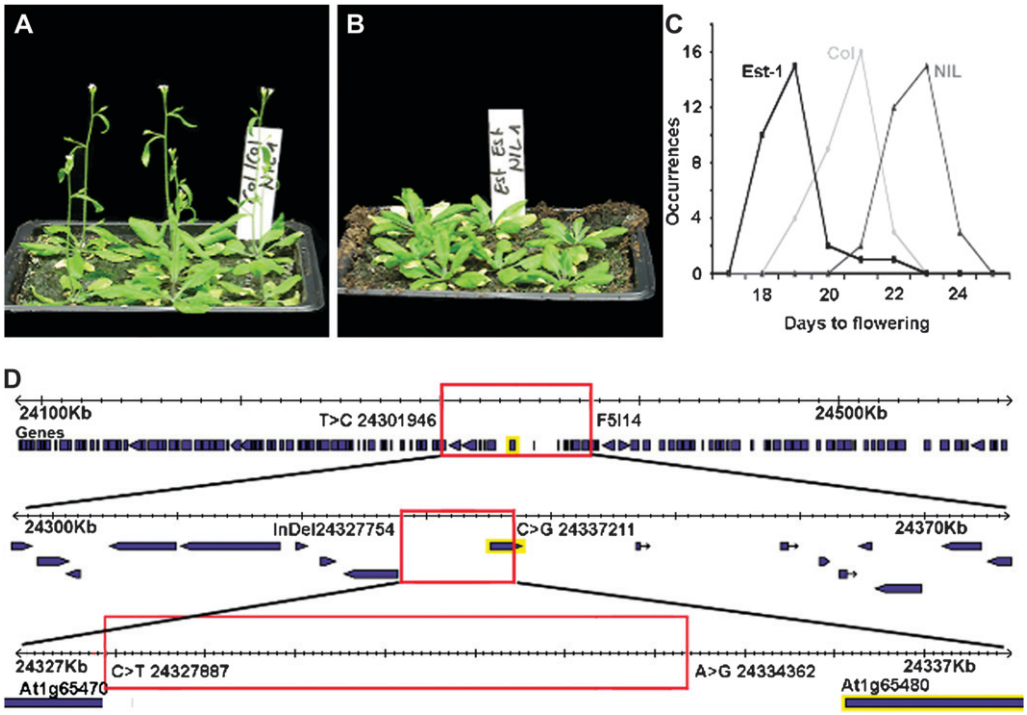

FIGURE 3.—Fine mapping of the chromosome 1 Est-1/Col QTL. (A) Four-week-old NIL-Col. (B) NIL-Est of same age and grown in parallel. (C) Distributions of flowering time for Est-1, Col-0, and the Est-NIL. (D) Fine mapping of the QTL. Transcription units are in purple. The *FT* gene (At1g65480) is highlighted in yellow. The three levels reflect the progressive rounds of fine mapping, with the final 6.7-kb mapping interval in the *FT* promoter shown on the bottom. The flanking markers used for mapping are shown. *FAS1* (At1g65470) is the gene to the left.

with the flowering time of the NIL-Est being the least affected by knocking down *FT* activity (Figure 4C).

Finally, consistent with the flowering behavior of *ft* loss-of-function mutants, the effects of allelic variation largely disappeared in SD, with the NILs flowering at the same time as Col-0 (Figure 4D). On the basis of these results, we conclude that *FT* underlies the detected F5I14/*FT* QTL.

Allelic variation leads to *FT* expression differences: Since the QTL interval did not include any coding sequences, we tested whether the phenotype resulted from a difference in *FT* RNA levels. *FT* expression, which is critically correlated with flowering time, is highest at the end of long days (YANOVSKY and KAY 2002; IMAIZUMI *et al.* 2003). Since the parental strains, Est-1 and Col-0, flowered at similar times, and the effect of the QTL was modest, we reasoned that the differences in *FT* expression conferred by the two alleles might be small. Therefore, we used conditions where such differences are likely to be most obvious. Temperature is known to affect the circadian clock, which in turn is an important factor in the regulation of *FT* expression (BLÁZQUEZ *et al.* 2003; BALASUBRAMANIAN *et al.* 2006b; MICHAEL *et al.* 2008). The clock can be entrained by both light and temperature (MICHAEL *et al.* 2003), and thermocycles (which are normally composed of cool nights and warm days) affect >50% of the *A. thaliana* transcriptome (MICHAEL *et al.* 2008). Thermocycles have a strong

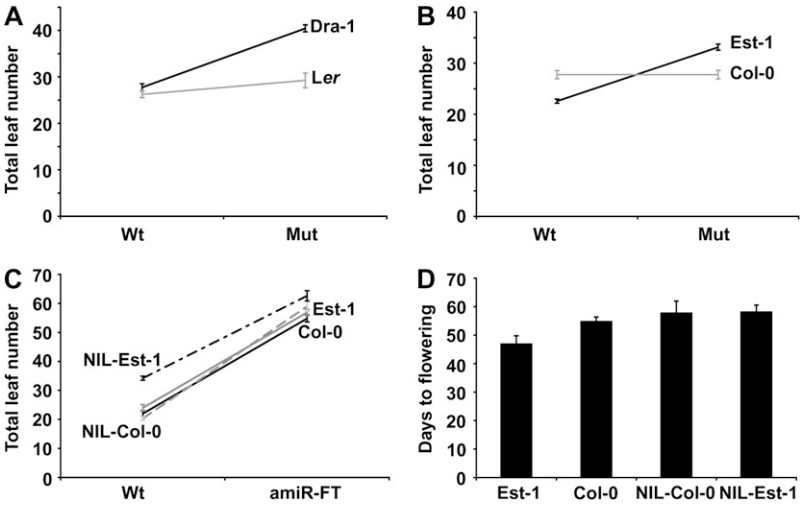

FIGURE 4.—Genetic evidence for *FT* being causal for the chromosome 1 QTL. (A and B) Quantitative complementation assays. (A) Flowering time of F_1 plants from crosses of Dra-1 and L*er* to Col-0 ("wild type") and the isogenic *ft*-10 mutant. (B) Flowering time of F_1 plants from crosses of Est-1 and Col-0 to L*er* ("wild type") and the isogenic *ft*-1 mutant. (C) Quantitative knockdown experiment with artificial miRNA against *FT* (amiR-ft-1) introduced into the two NILs and the two parents. (D) Flowering time of the different genotypes under short days.

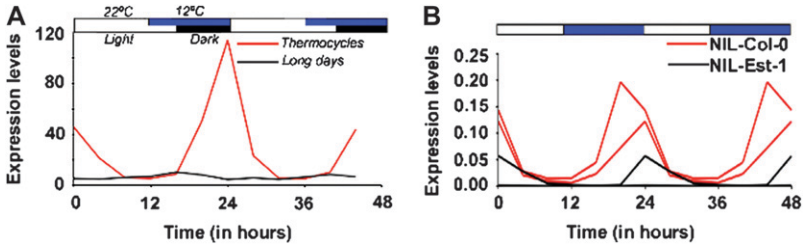

FIGURE 5.—Allelic variation affects *FT* expression. (A) Comparison of *FT* expression in Col-0 under thermocycles (12 hr 22°/12 hr 12°, continuous white light) and light cycles (16 hr light/8 hr dark, constant 23°). (B) Comparison of *FT* expression levels in NIL-Col and NIL-Est under thermocycles. Two different lines for each NIL are shown. The second NIL-Est line had very low *FT* expression, and its values are barely visible.

inducing effect on *FT* expression coupled with causing early flowering (Figure 5A; T. P. MICHAEL, unpublished results). Consistent with the QTL affecting *FT* expression, the NIL-Est had lower *FT* RNA levels compared to the NIL-Col (Figure 5B). However, there was little difference in the parents, suggesting that other loci compensate for this allelic variation, which is consistent with the flowering behavior of Est-1 being almost identical to Col-0 at both 16° LD and 23° LD.

Sequence variation at the *FT* locus in *A. thaliana*: To investigate the basis of the *FT* QTL, we analyzed in more detail sequence diversity at *FT*, by sequencing the coding region in 24 strains with variable flowering times in long days, and by sequencing a 4-kb promoter fragment from 12 strains. We found two large overlapping deletions and several other polymorphisms in the upstream region, but few variants in the coding region. We compared the level of polymorphism in our sequence data with published estimates of genomewide sequence polymorphisms. The genomewide average is about five nonsynonymous variants per kilobase coding sequence among 20 divergent strains of *A. thaliana* (NORDBORG *et al.* 2005; CLARK *et al.* 2007). In the 531-bp *FT* coding region, we found four synonymous changes, but no nonsynonymous changes. In contrast, the noncoding sequence was more variable. Within the final 6.7-kb QTL interval, there were many polymorphisms that differentiated the Est-1 and Col-0 alleles, including a 29-bp and 17-bp deletion in Est-1 relative to Col-0, and one insertion of 10 bp (Table S4). Partial sequencing of the Dra-1 promoter region revealed a small number of shared polymorphisms that differentiated Est-1 and Dra-1, strains with less active *FT* alleles, from Col-0 and L*er*, strains with more active *FT* alleles.

DISCUSSION

Complex genetic interactions modulate flowering time variation in *A. thaliana*: In the Est-1/Col-0 AI-RIL population, we have detected at least three distinct QTL that interact in an environment-dependent manner. The *FT* QTL has the largest effect, explaining ~20–40% of the phenotypic variance depending on the environment, but its impact is modulated by additional loci, as might be expected for a gene that integrates multiple environmental signals. For example, the PLS2/*PHYB* QTL detected at 16° on chromosome 2 (Figure 1B and Figure 2, B and E) displays significant epistatic interaction with the *FT* QTL. The effect of the Est-1 allele at the PLS2/*PHYB* QTL is in the opposite direction of the *FT* allele of Est-1, providing an explanation for why the two parental strains have very similar flowering times. In addition, our analysis at different temperatures revealed interactions between the two closely linked QTL on chromosome 1. While the *FT* QTL appeared to be the major factor determining variation at lower temperature, the effect of the linked nga280 QTL increased with higher temperature (M. TODESCO, S. BALASUBRAMANIAN and D. WEIGEL, unpublished results). A similar picture could also be seen in the Dra-1 × L*er* population, in which multiple QTL were mapped and significant interactions between all QTL were detected. While it is conceivable that many of the QTL effects may eventually be mediated through changes in *FT* expression levels, these results underscore the complexity of the genetic architecture of flowering time variation in *A. thaliana*.

Some of the complexity might reside within *FT* itself. The genomic interval surrounding *FT* is unusual (Figure 3D), with long noncoding regions both upstream and downstream of the *FT* coding sequence. The *FASCIATA1* (*FAS1*) gene is 7.3 kb upstream of *FT* and transcribed in the opposite direction of *FT*, while there are no *bona fide* open reading frames downstream of *FT* for >20 kb. Chromatin structure likely plays a prominent role in the regulation of *FT*, since mutations in *LIKE HETEROCHROMATIN PROTEIN1/TERMINAL FLOWER2* (*LHP1/TFL2*), which is required for epigenetic silencing, cause ectopic *FT* expression, as do mutations in *EARLY BOLTING IN SHORT DAYS* (*EBS*), a gene encoding a putative chromatin remodeling factor (KOTAKE *et al.* 2003; PIÑEIRO *et al.* 2003; SUNG *et al.* 2006; TURCK *et al.* 2007; ZHANG *et al.* 2007). An analysis of chromatin identified extensive histone modifications in the 3′ region of *FT* (TURCK *et al.* 2007). These are indicative of epigenetic gene regulation, consistent with a large and complex set of 5′ and 3′ sequences required for proper *FT* expression. They likely reflect the integrator function of *FT*, which is the target of many different pathways affecting flowering time (TURCK *et al.* 2008).

Flowering-time QTL in the *FT* region: The analysis of the Dra-1 × L*er* and the Est-1 × Col-0 populations, and reports of colocalizing QTL in other populations

(Werner *et al.* 2005a; El-Lithy *et al.* 2006; Shindo *et al.* 2006; Simon *et al.* 2008), suggest that a QTL near *FT* contributes to natural variation in flowering behavior of several *A. thaliana* strains. In total, seven independent QTL mapping experiments (including this work), with nine different strains, identified the *FT* region as a flowering time QTL when Col-0 and L*er* are the common parental strains, although the directionality of the QTL varied. Thus, it needs to be determined whether these QTL reflect linked genes affecting flowering in natural strains, or whether they are indeed due to functionally divergent alleles at *FT*.

Natural variation at a highly connected gene: By integrating multiple environmental signals, *FT* has a central position in the genetic network that controls flowering time. *FT* is expressed predominantly in leaves and it is thought that the small FT protein moves to the shoot apex, where it induces flowering by interacting with the FD transcription factor (Abe *et al.* 2005; Wigge *et al.* 2005; Lifschitz *et al.* 2006; Corbesier *et al.* 2007; Jaeger and Wigge 2007; Lin *et al.* 2007; Mathieu *et al.* 2007; Tamaki *et al.* 2007; Li and Dubcovsky 2008; Notaguchi *et al.* 2008). A major role for *FT* in flowering time regulation has been shown in many species, including rice, wheat, and poplar (Kojima *et al.* 2002; Böhlenius *et al.* 2006; Yan *et al.* 2006). FT is related in sequence to TERMINAL FLOWER1 (TFL1), which has the opposite effect on flowering (Bradley *et al.* 1997; Ohshima *et al.* 1997; Kardailsky *et al.* 1999; Kobayashi *et al.* 1999). The 537-bp coding sequence of *TFL1* has been analyzed previously in a sample of 15 different strains. In this collection, one synonymous change and three nonsynonymous changes were reported, with one of the nonsynonymous changes surprisingly affecting a residue that appears to be invariant in the entire *TFL1/FT* gene family across all flowering plants (Olsen *et al.* 2002). In contrast, among a similar size sample of 22 strains, we did not detect a single nonsynonymous change in the 531-bp *FT* coding region. These data suggest that *FT* is highly conserved at the protein level and more constrained than *TFL1*, consistent with what has been observed at larger phylogenetic distances (Ahn *et al.* 2006).

The role of *cis*-regulatory *vs.* coding sequence variation: Among genes responsible for natural variation in *A. thaliana* flowering, variant alleles at six loci, *CRY2*, *HUA2*, *FLM*, *FRI*, *PHYC*, and *PHYD*, are affected in protein activity, which in some cases is absent altogether (Aukerman *et al.* 1997; Johanson *et al.* 2000; El-Assal *et al.* 2001; Gazzani *et al.* 2003; Werner *et al.* 2005b; Balasubramanian *et al.* 2006a; Wang *et al.* 2007). The exception is the *FRI* and *HUA2* target *FLC*, where most alleles are affected in expression levels, and only a minority in protein function (Gazzani *et al.* 2003; Liu *et al.* 2004; Shindo *et al.* 2005, 2006; Lempe *et al.* 2005). *FT*, which encodes a highly conserved protein (Ahn *et al.* 2006), is the most downstream component of flowering time control for which natural variants have been identified in *A. thaliana*. Functional variation at *FT* is associated with expression differences, and in this study the final QTL interval included only regulatory sequences. The identification of the *FT* QTL increases the slowly growing number of examples where regulatory sequences contribute to natural phenotypic variation in *A. thaliana* (Kliebenstein *et al.* 2001; Lambrix *et al.* 2001; Koornneef *et al.* 2004).

Outside of *A. thaliana*, differences in expression of the *FT* orthologs *Heading date 3a* (*Hd3a*) in rice and *VERNALIZATION3* (*VRN3*) in wheat are responsible for strain-specific flowering differences in these two grasses (Kojima *et al.* 2002; Yan *et al.* 2006; Takahashi *et al.* 2009). In contrast, natural variants at the upstream acting loci *Hd1*, which encodes the ortholog of *CONSTANS* (*CO*) in *A. thaliana*, and *Early heading1* (*Ehd1*), which has no clear *A. thaliana* equivalent, are associated with simple loss-of-function mutations in rice (Yano *et al.* 2000; Doi *et al.* 2004; Takahashi *et al.* 2009), as is the case for the upstream acting wheat *VRN2* gene (Yan *et al.* 2004). Finally, further downstream, regulatory variation has also been identified in natural alleles of *VRN1*, the wheat ortholog of the *FT* target *APETALA1* (*AP1*) in *A. thaliana* (Yan *et al.* 2003). The theme that emerges from these observations is that downstream factors might be more likely to exhibit regulatory sequence variation, while functional diversification at upstream factors might more often involve changes in protein function. These observations might influence the ongoing debate on the importance of regulatory *vs.* coding sequence variation in adaptation and the evolution of development (Hoekstra and Coyne 2007; Wray 2007; Stern and Orgogozo 2008).

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GENETICS

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Cis-regulatory Changes at *FLOWERING LOCUS T* Mediate Natural Variation in Flowering Responses of *Arabidopsis thaliana*

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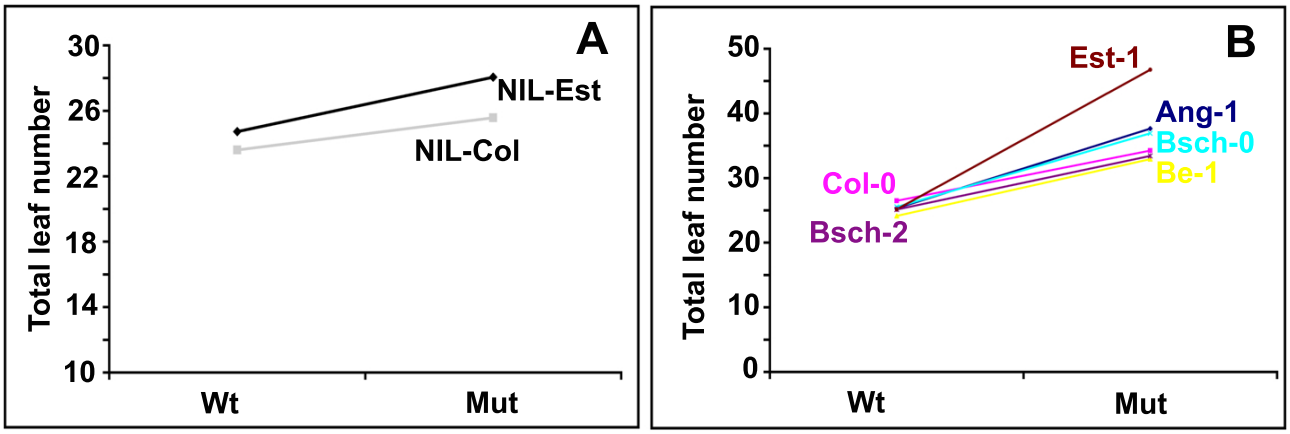

FIGURE S1.—Quantitative complementation analysis. (A) Quantitative complementation analysis comparing NIL-Est and NIL-Col. L*er* and *ft-1* in the same background are used as wild type and mutant for the reaction norms representing the mean flowering times of the F_1 progeny. (B) Quantitative complementation analysis with wild strains. Col and *ft-10* are used as wild type and mutant.

TABLE S1

Quantitative complementation analysis

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.109.104984/DC1.

TABLE S2

ANOVA results using the 27 lines that contain recombination events in the F5I14/*FT* QTL region

S.No	Marker	Position	Variation explained (TLN)	p-value	Variation explained (DTF)	p-value
1	44607889	23.16c	5%	0.5356	21%	0.0569
2	44607380	23.27b	4%	0.2663	15%	0.0447
3	44606313	24.19b	9%	0.1275	19%	0.0206
4	F5I14	24.374	48%	0.0004	51%	0.0002
5	21607700	24.4	11%	0.0874	25%	0.0069
6	21607386	26.6	2%	0.7711	2%	0.6971
7	21607030	27.3	2%	0.7589	1%	0.8161

DTF- Days to flowering, TLN- Total leaf number. The model flowering time ~ marker genotype was used for the analysis.

TABLE S3

A summary of the detected QTL and their allelic effects and confidence intervals

S.No	Population	Chr	Name of the QTL	LODint Markers	Physical position	Closest marker	Variance explained	Allele conferring later flowering
1	EstC RIL	1	nga280 QTL	44607364 to genea	19672910 to 22400757	nga280	9%	Est-1
2	EstC RIL	1	F5I14/FT QTL	44606313 to 21607386	24190997 to 26641963	F5I14	45%	Est-1
3	EstC RIL	2	PLS2/PHYB QTL	21607013 to GPA	8439620 to 11204074	44606590	13%	Col-0
4	Dra/Ler F2	1	F5I14/FT QTL	AtMSQT_NW_44 to AtMSQT_NW_66	21908668 to 30116495	AtMSQT_NW_56	25%	Dra-1
5	Dra/Ler F2	2	PLS2/PHYB QTL	AtMSQT_NW_81 to AtMSQT_NW_95	7551659 to 13265124	AtMSQT_NW_84	14%	Dra-1
6	Dra/Ler F2	5	MBK5/MAF QTL	AtMSQT_NW_249 to AtMSQT_NW_258	23270994 to 26708459	AtMSQT_NW_253	19%	Ler
7	Dra/Ler F2	5	CA72/FLC QTL	AtMSQT_NW_208 to AtMSQT_NW_219	3603967 to 9448109	AtMSQT_NW_212	18%	Dra-1

The variance explained in the total leaf number at 16ºC long days is given for all the detected QTL. The QTL for thermosensitivity co-localizes within the same confidence interval as the *FT-QTL*.

TABLE S4

A list of sequence changes in Est-1 compared to Col-0 in the region around *FT*

S.No	Genomic Position	Polymorhphism in Est	Length Change	Sequence Context
	24327172		START	
1	24327556	insert	2bp	agaaacttcgtccatcgcaaaaaaaaaaa(--/aa)ctagaaaaattcgggaatctataaac
2	**24327754**	**deletion**	**1bp**	**gagtgggaagatggataggtttg(a/-)aaaaaaaaaaggataaaaa**
3	24329015	g->A		gtaaattatgacactaaat(g/a)gtgcgtttaaacgattaaa
4	24329775	a->G		tttcttgttgttaaagataatg(a/g)gatctttcacattattgagag
5	24329902	t->G		gtgcaaagtatcgagacgtc(t/g)ctacttggttgcgttt
6	24329918	insert	1bp	ctacttggttgcgtttc(-/t)ttctatttttatattc
7	24329967	insert	29bp	ctacttcctatttttatatt(-/InDel)cgtttttacatac
8	24329992	a->C		tttacatacttcctatttccaa(a/c)cgtttttaaaatttattt
9	24330265	deletion	1bp	tgatattatattatt(t/-)atttaaatttatatat
10	24330301	c->T		acgaaaaacgtaacttt(c/t)ttacggttttctcagggc
11	24330344	ta->CG		tttccctagttttttaaaata(ta/cg)atgttattttaaa
12	24330359	insert	2bp	atgttattttaaa(--/at)gtaatccttttagaacaag
13	24330465	t->A		ggaactgttgatatttt(t/a)tagccaccaacttcaca
				atataaacacacac(----------
14	24330504	insert	10bp	acacatgg/gcacgagagccaaaaagc)gaacaagacgactgaa
15	24331136	insert	17bp	gatcgtggttttttagattttt(-/aaaaaaaaaaaaaaaaa)tttttaaaactcatcataa
16	24331200	insert	2bp	aatcaagcttttgtt(--/ca)ggacattcagttccgga
17	24331452	c->G		aagctaaactatctaaaatct(c/g)tt--atattatttgagaagtcgcaat
18	24331455	deletion	2bp	aagctaaactatctaaaatctgtt(ac/--)atattatttgagaagtcgcaat
19	24331793	deletion	1bp	gaagaagaagaaaaaa(a/-)gagaagaaaatctatcaagatc
20	24331966	g->C		ctatgtttaattatcttcta(g/c)attttaaggcacaacggattga
21	24333515	c->T***		aagtgcatatagcagattt(c/t)gagttttttttaattgcga
22	24333553	c->T***		ttcgattatcaatggt(c/t)gatcgtagacgtcttt
23	24333609	t->A		ttctatgaaggttaccaa(a/t)tattttggatttcaacct
24	24333698	g->A		gataaagttcaatat(g/a)aatacatcactgagatgtcacaaaaaaaaaaacta
25	24333706	t->C		gataaagttcaatataaatacat(t/c)actgagatgtcacaaaaaaaaaaacta
26	24333717	t->C		gataaagttcaatataaatacatcactgagatgt(t/c)acaaaaaaaaaaacta
27	24333725	insert	4bp	gataaagttcaatataaatacatcactgagatgtcacaaaaaa(-/aaaa)cta
28	24333749	a->C***		catcactgaataattccttt(a/c)ttttccagtttggacag
29	24333779	c->T		acagtagaacctatcg(c/t)taaatatttaattaccaat
30	24333783	t->A		acagtagaacctatcgttaa(t/a)tatttaattaccaat
31	24333800	insert	1bp	atttaattaccaatt(-/a)aaaagaaaaagaaa
32	**24334362**	**a->G**		**tttgtttgtcg(a/g)ccatataacacaaacggctagaaaaa**
33	24334376	g->A		tttgtttgtcggccatataacacaa(g/a)cggctagaaaaa
34	24334523	a->T		tgatttctatctcaacttttttttt(a/t)gtttttt

35	24334524	g->A		tgatttctatctcaacttttttttta(g/a)tttttt
36	24334525	t->G		tgatttctatctcaacttttttttttag(t/g)ttttt
37	24334532	c->T		tttagttttttt(c/t)ttttagaacgttttcgctttcg
38	24334568	deletion	11bp	ttttcgctttcgaat(tttttaagaat/-)ttttttttttctttata
39	24334731	insert	4bp	ataaattatgttaatatatggcaaaactaa(-/atat)atatatatgaaactac
40	24334840	g->A***		aggaaataaatcaaaaatta(g/a)tggctaccaagtgggaga
41	24335472	t->G***		tagaaattatttttccttttt(t/g)ggggtatactgaaaatatt
42	24335550	a->T		ttttctctgttttttt(a/t)tgatttatttggttttacttt
43	24335551	Insert	1bp	ttttctctgttttttta(-/a)tgatttatttggttttacttt
44	24335612	t->G***		aatttatatttcatttc(t/g)tttttcatgaagatggacc
45	24335801	insert	1bp	ttgcatttgtttttttttg(-/t)ttttttttttaagtataa
46	24336161	a->T		ataatcttatcttcttat(a/t)attcttcgtcttatttgt
47	24336605	deletion	1bp	ctgatcgcacatagattctatagaa(t/-)gtataggacgtgacttgaact
48	24337211	c->G		tgtttatattgtttcgacagcttgg(c/g)aggcaaacagtgtatgcaccag
49	24337967	insert	2bp	gaaaaacgtttttttttt(--/tt)aatttgggataaatacg
50	24338090	c->T***		tagatcactgattcaa(c/t)gcctaaaatagtataa
51	24338506	g->A***		ctgtgcattcaacc(g/a)atatatttttgcgtac
	24338523		END	

A list of sequence changes in Est-1 compared to Col-0 in the region around *FT*. Common polymorphisms that differentiate Est-1/Dra-1 from Col-0/L*er* are shown by ***.