Association mapping of local climate-sensitive quantitative trait loci in *Arabidopsis thaliana*

Yan Li[a], Yu Huang[b], Joy Bergelson[a], Magnus Nordborg[b,c], and Justin O. Borevitz[a,1]

[a]Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637; [b]Molecular and Computational Biology, University of Southern California, Los Angeles, CA 90089; and [c]The Gregor Mendel Institute, 1030 Vienna, Austria

Edited* by Joanne Chory, Salk Institute for Biological Studies and Howard Hughes Medical Institute, La Jolla, CA, and approved October 26, 2010 (received for review May 26, 2010)

Flowering time (FT) is the developmental transition coupling an internal genetic program with external local and seasonal climate cues. The genetic loci sensitive to predictable environmental signals underlie local adaptation. We dissected natural variation in FT across a new global diversity set of 473 unique accessions, with >12,000 plants across two seasonal plantings in each of two simulated local climates, Spain and Sweden. Genome-wide association mapping was carried out with 213,497 SNPs. A total of 12 FT candidate quantitative trait loci (QTL) were fine-mapped in two independent studies, including 4 located within ±10 kb of previously cloned FT alleles and 8 novel loci. All QTL show sensitivity to planting season and/or simulated location in a multi-QTL mixed model. Alleles at four QTL were significantly correlated with latitude of origin, implying past selection for faster flowering in southern locations. Finally, maximum seed yield was observed at an optimal FT unique to each season and location, with four FT QTL directly controlling yield. Our results suggest that these major, environmentally sensitive FT QTL play an important role in spatial and temporal adaptation.

genotype by environment interaction | pleiotropy | life history | efficient mixed-model association

Timing of reproduction greatly affects fitness (including survival and fecundity) in many organisms, especially plants (1–3). It also has a major effect on yield in crop species (4, 5). Flowering time (FT) in the model plant *Arabidopsis thaliana* has been shown to be an important adaptive trait (1, 3, 6, 7). *A. thaliana* lives in a wide range of climates and habitats and shows variation in life-history timing across its native species range (6). When behaving as a winter annual, *A. thaliana* germinates in the fall, overwinters as a vegetative rosette, and flowers in spring or summer. The summer annual strategy begins with spring germination and summer flowering followed by extended seed dormancy, whereas a rapid-cycling strategy can have multiple overlapping generations per year. This differentiation in life history is largely controlled by local seasonal cues but also is a genetic requirement for vernalization, which is heavily influenced by variation in major FT loci, *FRIGIDA* (*FRI*) (8) and *FLOWERING LOCUS C* (*FLC*) (9). A recent study has shown that environmental signals create critical windows of sensitivity to local climate and that genetic differences among accessions regulate life-history traits (10). Analysis of the sequences of different accessions suggests that molecular variation in *FRI* has been shaped by selection (11, 12). FT in *A. thaliana* has been shown to exhibit a latitudinal cline, suggesting that natural selection has shaped FT along the continental range to local climatic/geographical conditions (13, 14). Furthermore, an artificial-selection experiment has shown that the natural allelic variation in *FRI* can predict the adaptive evolution in FT under spring conditions (7).

The signaling pathways controlling FT in *A. thaliana*, including photoperiod, gibberellin, vernalization, ambient temperature, and light-quality pathways, have been well studied by forward and reverse genetics, and more than 60 genes have been shown to regulate FT (15–18). However, few specific genes and alleles have been confirmed as controlling natural variation in FT (19–21). The majority of research on natural genetic variation in FT has been done in laboratory conditions with constant photoperiod and temperature, without the cues provided by seasonal changes in day length and temperature (22). Indeed, the genetic basis of FT appears to be very different in laboratory and field conditions (23). Finally, although quantitative trait loci (QTL)–environment interactions (G × E) are often detected, such as day length or vernalization-responsive QTL (24), it has been difficult to directly connect these G × E interactions to plant responses to the climate conditions (cycling day length and temperature throughout the season) in the field.

The goals of this study were to investigate the genetic architecture of FT under simulated local climates. We used genome-wide association (GWA) mapping to detect the major common QTL and G × E in a worldwide mapping sample across seasonal environments that mimic local climate regimes spanning the native range. GWA is powerful in *A. thaliana* because of the availability of naturally occurring inbred lines and high marker density (250,000 SNPs) (25, 26). We used a mapping population of 473 accessions with empirically reduced family structure and a multi-QTL kinship-based mixed-model analysis (27) to reduce the false-positive rate from population structure and linked multiallele haplotypes.

Here, we assayed FT variation apart from germination, which cannot be separated in the field. *Arabidopsis* has many life-history strategies, depending on location, seasonal conditions, and genetics. Multiple strategies can overlap in single site (10). In this study, we synchronize germination to mimic early and late spring cohorts, typical of rapid-cycling and summer annuals that overwinter as seed, to study the genetic effects and environmental sensitivities on FT. We detect 12 QTL, including known candidate alleles, with specific local and seasonal effects. The significant QTL allele latitudinal clines and their pleiotropic effects on seed yield suggest an important role for these seasonal FT genes. The pleiotropic QTL and correlation of FT and seed yield would provide useful information to guide breeding strategies to increase yield in crops (4).

Results and Discussion

Natural Variation of FT and Yield in the GWA Population. A set of accessions (selected to maximize diversity and eliminate unusually close relationships) was grown under four conditions that were designed to mimic a spring (March 1st in Spain and May 1st in Sweden) and summer growing season (April 1st in Spain and June 1st in Sweden) in two locations, Spain and Sweden, that span the native European range (*Materials and Methods*). The study was

Author contributions: Y.L. and J.O.B. designed research; Y.L. performed research; Y.L., Y.H., J.O.B., M.N., and J.B. contributed new reagents/analytic tools; Y.L. and Y.H. analyzed data; and Y.L. and J.O.B. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: borevitz@uchicago.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1007431107/-/DCSupplemental.

replicated in two independent annual experiments: 2008 used 360 accessions (5,418 plants), and 2009 used 473 accessions (7,303 plants; *Materials and Methods*). The broad-sense heritability of FT was high within the same environment (average 98%) while lower for the heritability of yield (73% in spring and 53% in summer). Substantial variation in FT and seed yield was observed (Fig. 1 and Fig. S1). The widest range of FT (from 11 to >171 d) was observed in Sweden in the summer planting season (Fig. 1). The greatest variation in yield was in Sweden in the spring planting season, ranging from 0.001 to 0.616 g (Fig. S1). Environments (including planting season and location), genotype, and G × E significantly ($P < 0.001$) affected FT and yield. Planting season had a great impact on yield in that almost all accessions had higher yield when germinating in the spring relative to the summer season. Latitude showed significant correlation with FT ($r = 0.27$; $P < 0.001$) but was not significant for yield.

Variation in Response to Environments Across Accessions. The FT and yield for each accession depended on the environmental growing conditions (G × E interactions were significant for both traits). In Spain, for example (Fig. S2*A*), some accessions flowered more rapidly when grown in the summer, whereas other accessions were further delayed in the summer growing season, giving rise to a bimodal distribution. This trend is more clear if we plot the plasticity (phenotypic difference of each accession under two contrasting environments) against FT (Fig. S3*A*). The trend was also observed in Sweden, where rapid-flowering accessions flowered relatively more rapidly, whereas late accessions were further delayed flowering in summer (Fig. S2*B* and Fig. S3*B*). Such a delay suggests a vernalization requirement in these accessions absent in the summer growing conditions. When contrasting Spain and Sweden within either growing season, rapid-flowering accessions flowered earlier in Sweden compared with Spain, whereas late accessions were further delayed in Sweden (Fig. S2 *C* and *D* and Fig. S3 *C* and *D*).

GWA Mapping. GWA was carried out by using a mixed model that corrects for population structure and genetic relatedness [as implemented in the efficient mixed-model association (EMMA) software] (27). A list of 281 genes (26) related to FT (a priori genes) were used to evaluate *P* value thresholds from EMMA that would be suitable for selecting candidate SNPs. SNPs with *P* value $\leq 1 \times 10^{-5}$ or *P* value $\leq 1 \times 10^{-4}$, gave relatively high enrichment of a priori candidates (the upper-limit false discovery rate for the priori genes was <25%; Table S1). The GWA maps differed across the four environments but overlapped at several major QTL (Fig. 2). SNPs with <10% minor allele frequency (MAF) were not considered because of possibly elevated false-positive rates (26) (Fig. S4). Ninety-two SNPs with >10% MAF showed *P* values $\leq 1 \times 10^{-5}$ in at least one environment in 2009. All GWA results are available at http://arabidopsis-exp.gmi.oeaw.ac.at:5000/.

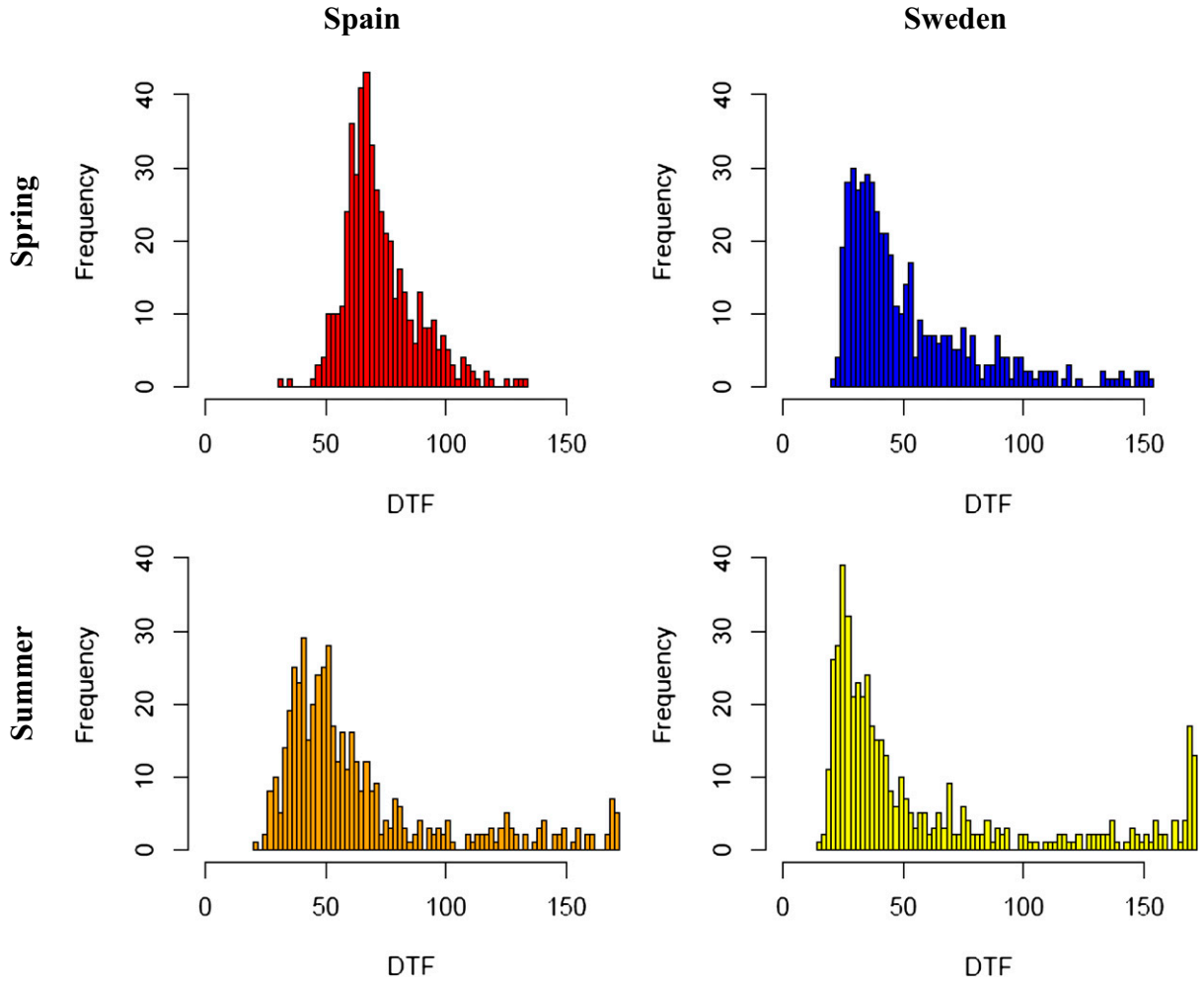

Fig. 1. Histogram of FT in four environments. Shown is the average of four replicates for each accession. DTF, days to flower.

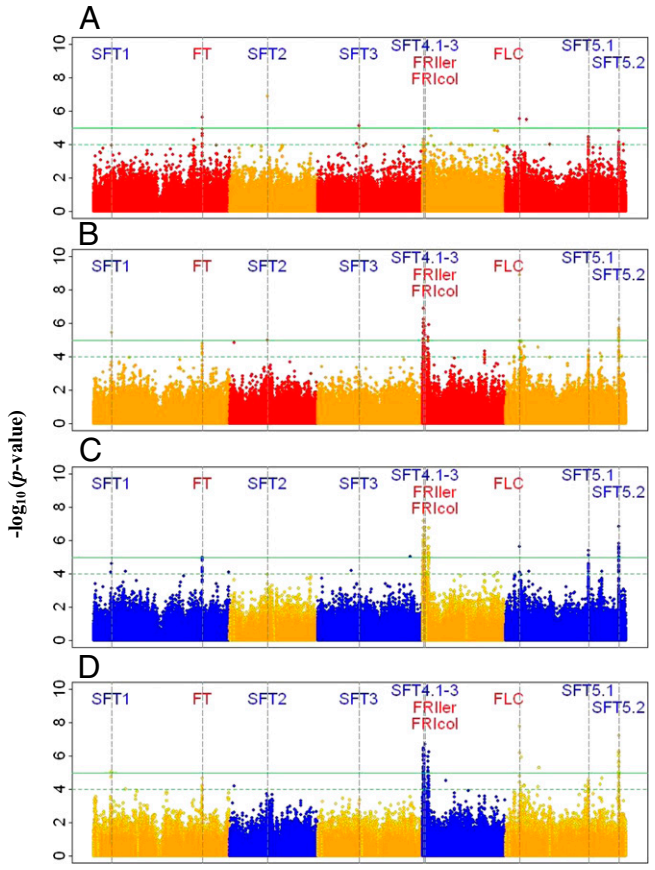

Fig. 2. GWA mapping results by EMMA for FT in four environments (MAF ≥ 0.1). (*A*) Spring in Spain; (*B*) summer in Spain; (*C*) spring in Sweden; and (*D*) summer in Sweden. Four a priori loci (shown in red by the gray dashed lines) for FT showed significant ($P \leq 1 \times 10^{-5}$) in at least one environment. Eight novel loci for seasonal FT (SFT) are shown in blue.

Common and Major QTL of Seasonal FT. To select major candidate loci among all 92 significant (EMMA $P < 1 \times 10^{-5}$) SNPs, these SNPs were clumped by using linkage disequilibrium (LD) as a criterion and the strongest association within each LD set was kept ($r^2 < 0.2$, based on the fact that LD decays within 10 kb; ref. 25). After this clumping of SNPs, 22 loci for FT were significant in at least one environment ($P < 1 \times 10^{-5}$). Fourteen QTL SNP loci for FT were repeated at a significance level of at least $P \leq 1 \times 10^{-4}$ in our 2008 experiment. Seven of these 14 SNPs span 1.2 Mb on chromosome 4, including *FRI*, a region with extended LD likely caused by a selective sweep (26). In our mapping population, these SNPs are unlinked ($r < 0.2$), but multiple extended haplotypes are present (24). We next tested the seven SNPs on chromosome 4 around *FRI* by including the two known causative *FRI* deletion alleles as cofactors. Three SNPs were no longer significant (Table S2) and were dropped. The original *FRI* SNP (4_264496) was in LD with *FRIcol* and therefore substituted by the known *FRIcol* and *FRIler* alleles in the final model. The QTL were named after the known a priori candidates if they were located within ±10 kb of the a priori candidate and then by chromosome and position as seasonal FT (*SFT1*, *SFT2*, *SFT3*, *SFT4.1*, *SFT4.2*, *SFT4.3*, *SFT5.1*, and *SFT5.2*) as novel loci. The tagged SNPs at these QTL are listed in Table S3.

The final model included all 12 QTL as fixed effects and kinship as a random effect, using modified EMMAX (28) for each of the four growing conditions. The effect sizes (the difference in days when replacing homozygous Col allele with homozygous non-Col allele) of each SFT locus differed across four environments with a general trend that the effects were larger in summer vs. spring and Sweden vs. Spain (Fig. 3*A*), where the phenotypic variance was larger (Fig. 1). The allele frequency of the 12 QTL averaged 20% and is shown in Fig. 3. All of these 12 QTL showed significant interactions ($P \leq 0.001$) with season and/or location environmental contrasts (Fig. 3*B*) in a model testing G × E across the four environments. Based on the differential effect sizes of G × season and G × location, these QTL ranged from more seasonally sensitive to more geographically sensitive (Fig. 3*C*). Planting season-sensitive QTL (*FLC*, *SFT1*, and *SFT2*) suggests a spring cold night–sensing function of these alleles; indeed *FLC* functions in the vernalization pathway, whereas location-sensitive QTL (*SFT3*) may reflect the day-length differences between Spain and Sweden because the temperatures are very similar within a season (March 1st in Spain and May 1st in Sweden; Fig. S5).

We next looked to see if QTL alleles showed a pattern on the landscape suggesting selection attributable to geographic location. Four SNPs (*SFT1*, *SFT4.2*, *FLC*, and *SFT5.2*) showed a significant correlation with latitude of origin in the top 1% of the genome-wide distribution (Fig. 4). The non-Col alleles of these four loci delayed FT and were associated with the more northern latitudes.

Correlation Between FT and Yield and Pleiotropic SNPs. Seed yield is a proxy for relative reproductive fitness and depends on FT. In our four growing seasons, two trends were apparent. First, seed yield increased with FT as plants were larger and could set more fruit. Second, seed yield decreased with delayed flowering. This trend was especially evident in spring in Sweden (Fig. 5). To quantify these results, we used segmented linear regression to find optimal breaking points. Seed yield was significantly ($P < 0.001$) positively correlated with FT among early accessions but showed a significant ($P < 0.001$) negative correlation among late accessions (Fig. 5) in all four environments. The pattern suggests that high yield and fitness were obtained at an optimal seasonal FT under our growing conditions. A different relationship is likely under fall germination conditions (experiments ongoing). Here, we show the genetic basis underlying FT and yield in spring and summer growing conditions appropriate to rapid-cycling and summer annuals that overwinter as seed.

Among the 12 FT SNPs identified above, 4 of them (*SFT1*, *FT*, *SFT4.1*, and *FLC*) were also detected as controlling yield (EMMA $P \leq 1 \times 10^{-3}$) in at least one environment. Two SNPs, *FLC* and *SFT1*, showed a correlation with both latitude of origin and yield. Rapid-flowering alleles are more abundant in southern populations and higher seed yield, specifically in spring conditions in Spain.

Candidate Genes and QTL Confirmation. Among the 12 loci, 4 cover a priori alleles (*FRIcol*, *FRIler*, *FLC*, and *FT*), and 8 QTL map to novel genome regions at ±10-kb resolution with an average of eight candidate genes. The effects of *FRIcol* and *FT* were confirmed by the mutants grown in spring in Spain and spring in Sweden in Experiment 1 (Fig. 3*A*, black boxes). Because the Col allele at the *FRIcol* locus is nonfunctional (deletion in *FRI*), the functional non-Col allele at *FRIcol* delayed flowering. The direction of the *FRIler* allele effect is reversed because the non-Col allele (Ler allele) has the deletion in *FRI* and therefore accelerated flowering. The tagged SNP at *FT* was out of phase, but the relative effects were consistent between the mutants and our model (larger effect in spring in Sweden compared with spring in Spain). In addition to the a priori alleles, we could further validate these QTL in independent line crosses by looking at published FT QTL mapping studies (21, 29–31). Ten of 12 seasonal FT QTL regions colocalize with FT QTL identified from other studies as the tagged SNPs also segregate with the two functional alleles of a given cross (Table S3).

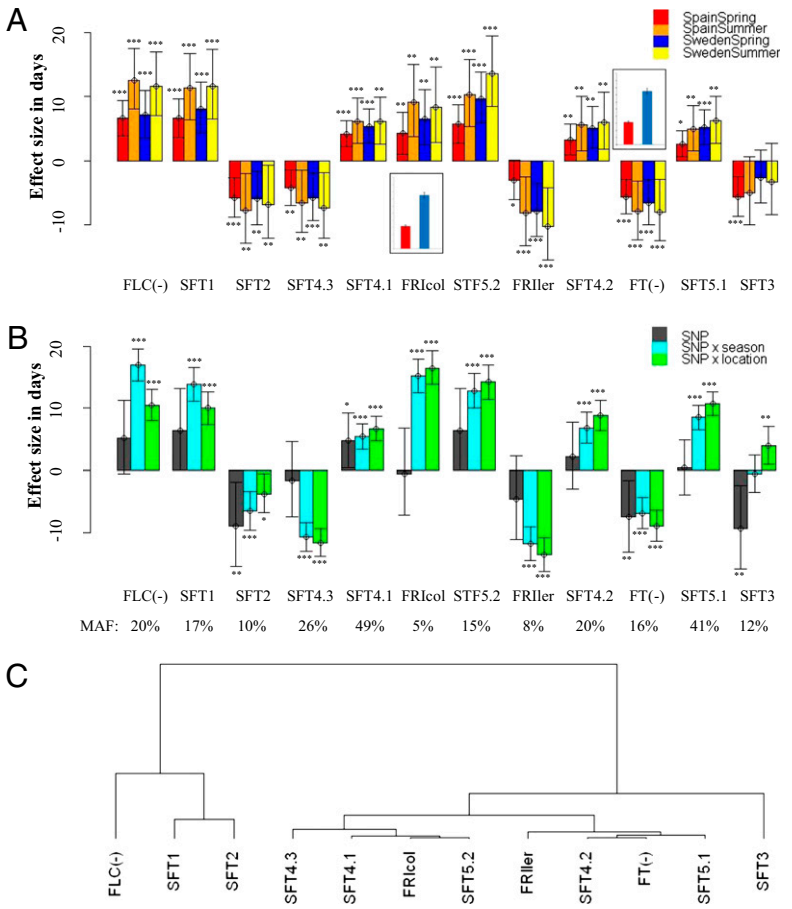

Fig. 3. (*A*) Allele effects of the 12 QTL on FT in each of the four environments (the difference in days when replacing homozygous Col allele with homozygous non-Col allele). The tagged SNPs for these QTL were listed in Table S3. The graphs in the black boxes represent the effects from the mutants at the corresponding loci. (*B*) Effect sizes of SNPs, SNP × season, and SNP × location on FT across four environments. (*C*) Cluster of the 12 QTL by their sensitivity to season vs. location (the difference in effect sizes of SNP × season and SNP × location). Error bars are 95% confidence intervals. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Genetic Architecture of Seasonal FT. The pseudoheritability (the additive component of heritability as estimated with the kinship matrix by using EMMAX) of the FT in the four environments is 90% (spring in Spain), 95% (summer in Spain), 94% (spring in Sweden), and 96% (summer in Sweden), respectively. The proportion of the variance explained by the final 12 QTL is 31%, 34%, 33%, and 32% in spring in Spain, summer in Spain, spring in Sweden, and summer in Sweden, respectively. In the full model with all four environments together, the 12 QTL and environmental dependencies (G + G × E + E) explain 45% of the total phenotypic variation. Genotype or environment alone can explain only 11% or 6%, respectively, whereas G + G × E can explain 32%.

All 66 epistatic pairs among the 12 major QTL were tested, and only 4 pairs were significant ($P < 0.01$); the most significant epistatic term explained only 1.2% of the variation. We therefore have not included epistasis in our final model (Fig. 3). When rare alleles were considered (2% < MAF < 10%), only spring in Spain had 37 additional significant (EMMA $P \leq 1 \times 10^{-5}$) SNPs; however, the false discovery rate also increased (Fig. S4). If these additional SNPs are included (32 SNPs after clumping by LD; $r^2 < 0.2$), an additional 4.8% of the variance can be explained in spring in Spain, whereas only an additional 0.4% was explained across the four environments.

Comparisons of GWA for FT Across Environments and Experiments. The GWA results of FT in our four environments were compared with two common greenhouse growth conditions from our previous study (26), for a total of 15 contrasting pairs among six environments. The results show a greater overlap of significant SNPs among the four environments in this study than between studies (Fig. S6). This finding is because of the large environmental difference between simulated seasonal climates and common greenhouse conditions, but it is also confounded by the different mapping sets used in the two studies. Spring in Spain had less of an overlap with the other three conditions in our study because early flowering was suppressed by noninductive conditions. Among the top 1% of significant SNPs across studies, 10–20% overlapped (depending on the environment), indicating some major effect loci that are present in both studies.

Conclusion and Implications. Using GWA mapping, we identified 12 common and major QTL controlling the natural variation of seasonal FT that show unique environmental sensitivities to growing season and location (Fig. 3), and 10 are supported by previous studies (Table S3). The 12 QTL and environmental dependencies explain 45% of the total phenotypic variation, which is strikingly different from that reported in maize or human studies, where the genetic architecture is dominated by many small additive QTL (32). Our major seasonal FT-QTL sense local seasonal environments, show significant correlation with latitude (Fig. 4), and have pleiotropic effects on yield. Together, they are likely to play important roles in local adaptation. Through various allelic combinations of major loci, with unique environmental sensitivities, *A. thaliana* has spread widely throughout the latitudinal range and into several growing seasons.

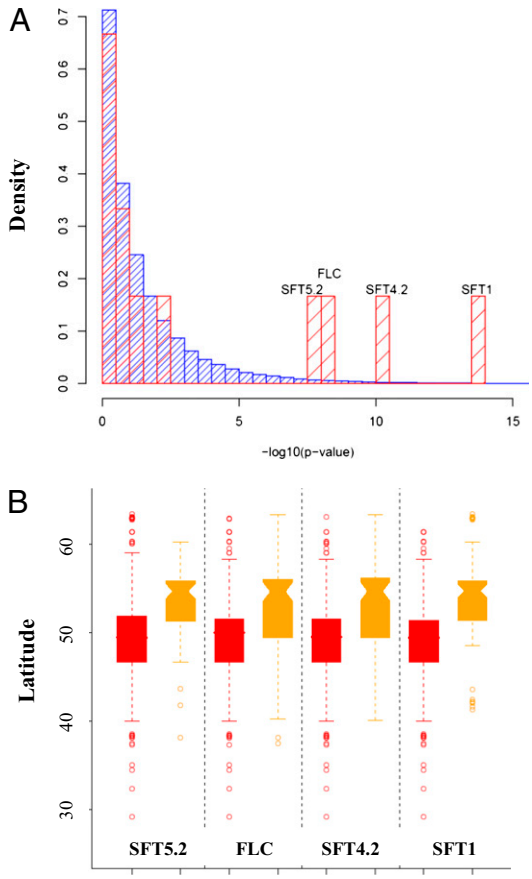

Fig. 4. (*A*) Histogram of the *P* value for correlation between SNP allele and latitude. Histogram in blue represents the genome-wide distribution (172243 SNPs with MAF > 10%), and red represents the 12 candidate QTL SNPs. (*B*) Latitudinal distribution of the alleles at the four QTL (Col allele in red and non-Col allele in orange).

Materials and Methods

Mapping Populations. Two independent experiments were conducted in this study. Experiment 1 was done with a mapping population of 360 accessions (33), and Experiment 2 used a set of 473 accessions. Each accession in each experiment had four replicates under each of the four growth conditions (two planting seasons by two locations). The set of 360 accessions was selected based on the genotypes of 5,810 worldwide accessions at 149 SNPs from a previous study (34), after minimizing the redundancy and close family relatedness (33). To use available genotyped lines to increase mapping power, a mapping set of 473 accessions was chosen that includes unique lines from the diverse cores of 360 and 191 lines (26, 33) to which 67 additional lines were added. The details about the mapping population are available at http://naturalvariation.org/hapmap.

Growth Conditions with Simulated Climates. Both experiments were conducted in two walk-in growth chambers (AR-916, Percival Scientific) that were programmed to cycle the local climates every 5 min from the simulated weather files. The simulated climates were generated by using SolarCalc (35) with sunrise and sunset, light spectrum, temperature, and relative humidity programmed to cycle throughout the day and the season according to 1975–2000 averages (Fig. S5). One chamber was simulating Spain (latitude 41.72091, longitude 2.957075) starting from March 1, and the other chamber was simulating Sweden (latitude 55.71226, longitude 13.207352) starting from May 1st on the first day when plants were put into the chambers. The real growth conditions for 2008 were offset from the SolarCalc input file after about 70 d because of a computer error. The weather files, including input programs and real conditions (recorded using HOBO data loggers), are available at http://naturalvariation.org/hapmap.

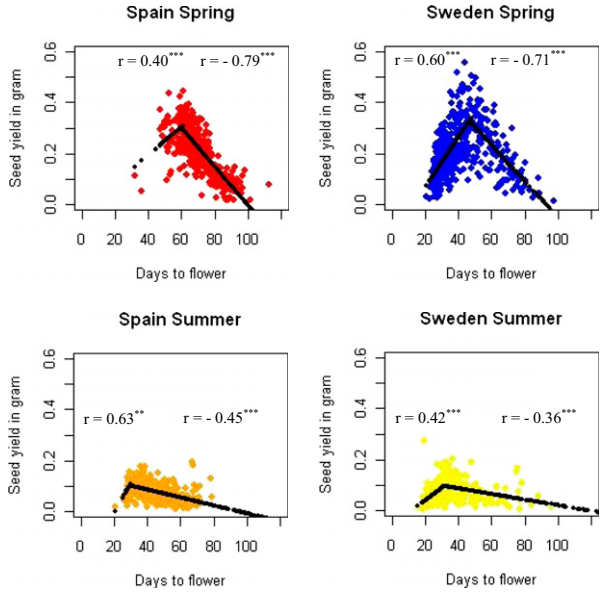

Fig. 5. Correlations between FT and seed yield. Black lines show segmented regression with optimal breaking points. The Pearson's correlation *r* is shown above each regression with the corresponding significance levels: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Phenotyping. FT was recorded daily as days to flower after germination in Experiments 1 and 2 with four replicates under each of the four conditions. Yield was recorded as dry seed weight in grams in Experiment 2 with two replicates under each condition. In detail, before germination, the seeds for core 360 in Experiment 1 were obtained from a previous study (34) where the maternal lines were grown in common greenhouse conditions (long day: 16 h light, 20 °C). The seeds of mutants (CS56 ft-1 and CS6209 FRI-Sf2) were obtained from the *Arabidopsis* Biological Resource Center and grown together with the core 360 accessions in Experiment 1. The seeds for core 473 in Experiment 2 were collected from the maternal lines grown in summer in Spain from Experiment 1. Seeds were stratified in dark at 4 °C in 5mg/L gibberellic acid water (GA_3, Sigma-Aldrich) to synchronize the germination. After 6 d, seeds were put on soil under 24-h white lights at 23 °C for 6 d to synchronize the germination of all accessions. For each accession, three to nine seeds from the same tube were put in each of the eight pots. Most of the seeds germinated within 3 d, and only one seedling was kept for each pot after germination. Germination time was recorded every day after sowing for 2 wk. Plants were transferred into two growth chambers at day 7 after sowing. FT was measured as days from germination to the observation of first flower bud. Germination time was days from sowing seeds on soil to germination. The FT for the accessions that did not flower at the end of experiment was recorded as the last day of the experiment. Seed yield was the total dry seed weight in grams. Plants for yield measurements were kept in sleeves and tied at the bottom to avoid seed dropping and contamination after flowering, and the bottoms were watered until senescence. Seeds were collected after plants and seeds were complete dry. In both experiments, there were four blocks for each environment (two locations × two planting seasons). In 2008, the blocks were located with two levels of shelf-side and two levels of shelf-height. The core 360 accessions were randomized twice a week within each block. The second planting started 35 d later than the first planting. In 2009, the blocks were four levels of shelf-height, and the core 473 accessions were randomized at the beginning of experiments. The second planting started 38 d later than the first planting.

Correlation Between Two Experiments in 2008 and 2009. The correlation of FT between the two experiments (2 y) was high (average 0.91 across the four conditions). The results shown in this article were based on 2009 data with 473 accessions. GWA results for yield and FT, including 2008 data with 360 accessions, are available on http://arabidopsis-exp.gmi.oeaw.ac.at:5000/.

Genotyping. The 473 accessions were genotyped by Atsnptile1 arrays, which contain probe sets for 248,584 SNPs. Details about this SNP-tilling array and methods for array hybridization as well as the SNP-calling algorithm were the

same as described in a previous study (26). In brief, ≈250 ng of genomic DNA was labeled with the BioPrime DNA labeling system (Invitrogen), and 16 μg of the labeled product was hybridized to each array. SNPs were called by using the modified Oligo package. The quality control of the genotypes and imputation of the missing SNPs were according to the same procedure as described before (26) except a 15% mismatch rate was used to filter out bad arrays. After quality control and imputation, 473 accessions had genotypes for at 213,497 SNPs (172,243 SNPs had MAF > 10%).

Statistical Analysis. The broad-sense heritability (h^2) was estimated as $\hat{h}^2 = \hat{\sigma}_g^2 / [(\hat{\sigma}_e^2/r) + \hat{\sigma}_g^2]$, where $\hat{\sigma}_g^2$designates the genotypic variance, $\hat{\sigma}_e^2$is the residual variance, and r is the number of replications. ANOVA for the phenotypic data were done with the R package, lm(stats), and anova.lm (stats). The segmented regression was analyzed by using the R package segmented and Pearson's correlation tests used cor.test (stats). The method of EMMA was used for GWA (27). The candidate SNPs selected based on *P* values were further clumped to remove the SNPs caused by LD using the clumping function in Plink (36).

Model Fitting. Linear multi-SNP mixed-model analysis was performed with modified EMMAX (28). In the model, kinship was used as a random effect, and SNPs, location, planting, shelfTop, shelfMiddle, SNPs:location, SNPs: planting, location:planting, shelfTop: location, shelfMiddle:location, shelfTop: planting, and shelfMiddle:planting were fixed effects.

ACKNOWLEDGMENTS. We thank Dr. Xu Zhang for the array annotation and discussion; John Zdenek, Sandra A. M. Suwanski, and Judy Coswell in the University of Chicago greenhouse for the help preparing soils and watering plants; and all undergraduate students (Lewie Zeng, Tote Hughes, Whitney Panneton, Maria Valdes, Eric Hanss, and Samuel Betcher) for their help in scanning barcodes and weighting seeds. This work was funded by National Institutes of Health Grant R01GM073822 (to J.B.).

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