Plant, Cell & Environment

Plant, Cell and Environment (2012) **35**, 1672–1684 doi: 10.1111/j.1365-3040.2012.02518.x

Novel natural alleles at *FLC* and *LVR* loci account for enhanced vernalization responses in *Arabidopsis thaliana*

EDUARDO SÁNCHEZ-BERMEJO[1]*, BELÉN MÉNDEZ-VIGO[1]*, F. XAVIER PICÓ[2], JOSÉ M. MARTÍNEZ-ZAPATER[1,3] & CARLOS ALONSO-BLANCO[1]

[1]*Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain,* [2]*Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD), Consejo Superior de Investigaciones Científicas (CSIC), 41092 Sevilla, Spain and* [3]*Instituto de Ciencias de la Vid y del Vino (ICVV), Consejo Superior de Investigaciones Científicas (CSIC), Universidad de La Rioja, Gobierno de La Rioja, 26006 Logroño, Spain*

ABSTRACT

Vernalization, the induction of flowering by low winter temperatures, is likely to be involved in plant climatic adaptation. However, the genetic, molecular and ecological bases underlying the quantitative variation that tunes vernalization sensitivity to natural environments are largely unknown. To address these questions, we have studied the enhanced vernalization response shown by the Ll-0 accession of *Arabidopsis thaliana*. Quantitative trait locus (QTL) mapping for several flowering initiation traits in relation to vernalization, in a new L*er* × Ll-0 recombinant inbred line (RIL) population, identified large effect alleles at *FRI*, *FLC* and *HUA2*, together with two small effect loci named as *Llagostera vernalization response* (*LVR*) 1 and 2. Phenotypic analyses of near isogenic lines validated *LVR1* effect on flowering vernalization responses. To further characterize the *FLC* allele from Ll-0, we carried out genetic association analyses using a regional collection of wild genotypes. *FLC*-Ll-0 appeared as a low-frequency allele that is distinguished by polymorphism Del(-57), a 50-bp-deletion in the 5′-UTR. Del(-57) was significantly associated with enhanced vernalization responses and *FLC* RNA expression, as well as with altitude and minimum temperatures. These results are consistent with Del(-57) acting as a novel *cis*-regulatory *FLC* polymorphism that may confer climatic adaptation by increasing vernalization sensitivity.

Key-words: climatic adaptation; flowering; *FLOWERING LOCUS C* (*FLC*); quantitative trait locus (QTL).

INTRODUCTION

The onset of flowering is regulated by several environmental factors that fluctuate seasonally, such as photoperiod or low winter temperature, hence ensuring plant survival and reproduction (Ausin, Alonso-Blanco & Martinez-Zapater 2005; Roux *et al*. 2006; Kobayashi & Weigel 2007). In particular, the induction of flowering by prolonged exposure to low temperature is known as vernalization, and has become a model response to understand the epigenetic regulation of plant development (Kim *et al*. 2009). In the past decade, genetic studies in the model plant *Arabidopsis thaliana* have begun to elucidate the molecular bases of this environmental response (Kim *et al*. 2009; De Lucia & Dean 2011). This has been largely based in the analysis of the natural variation that exists for flowering time, enabling the identification of two major determinants of the vernalization response, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS* (*FLC*) (Michaels & Amasino 1999; Johanson *et al*. 2000). However, the molecular basis of the quantitative variation that fine-tunes vernalization flowering responses to natural environments still remains mostly unknown.

A. thaliana shows substantial amount of genetic variation among wild populations for the timing of flowering initiation (Lempe *et al*. 2005; Shindo *et al*. 2005), which is partly involved in adaptation to the different environments covered by its wide geographic distribution (reviewed in Alonso-Blanco *et al*. 2009; Weigel 2012). Classical studies have described *A. thaliana* as a species with no obligate requirement for flowering induction (Martínez-Zapater *et al*. 1994). However, it has been shown that some accessions from high latitudes or altitudes show an obligate vernalization requirement (Shindo *et al*. 2005; Mendez-Vigo *et al*. 2011). Furthermore, wild accessions have been qualitatively classified in so-called winter- or summer-annuals depending on their flowering behaviour under experimental conditions (Napp-Zinn 1969; Rédei 1970). Genotypes showing a late flowering phenotype that responds to vernalization are presumed to reflect a winter-annual habit, while those showing early flowering and low vernalization response are interpreted as summer-annuals (Rédei 1970). Although this physiological classification does not reflect natural life cycles and growth habits (Montesinos *et al*. 2009; Wilczek *et al*. 2010), its Mendelian genetic analysis allowed the identification of *FRI* and *FLC* genes (Michaels & Amasino 1999; Johanson *et al*. 2000).

Correspondence: C. Alonso-Blanco. Fax: +34 915854506; e-mail: calonso@cnb.csic.es

*These authors contributed equally to this work.

FLC encodes a MADS-box transcription factor that represses flowering initiation by regulating several floral integrator genes (Kim *et al*. 2009), whereas *FRI* encodes a protein that increases *FLC* expression and has no homology to previously characterized genes. It has been shown that FRI is part of a complex that interacts with general transcription factors, chromatin modification factors and the mRNA cap-binding complex, suggesting that FRI participates in several co-transcriptional processes associated with *FLC* expression (Geraldo *et al*. 2009; Choi *et al*. 2011). By contrast, vernalization down-regulates *FLC* expression through histone and chromatin modifications that epigenetically maintain *FLC* silencing. It has been demonstrated that vernalization-mediated silencing of *FLC* also involves multiple non-coding RNAs, including sense (so-called COLDAIR) (Heo & Sung 2011) and antisense transcripts (so-called COOLAIR) (Swiezewski *et al*. 2009; De Lucia & Dean 2011). Sequence analyses of wild genotypes have identified more than 25 FRI truncation alleles generated by indels or non-sense mutations and more than 10 different weak or loss-of-function alleles of *FLC* caused by mutations and transposon insertions (Le Corre, Roux & Reboud 2002; Michaels *et al*. 2003; Lempe *et al*. 2005; Shindo *et al*. 2005; Werner *et al*. 2005; Mendez-Vigo *et al*. 2011; Strange *et al*. 2011).

Current quantitative studies of natural variation for vernalization flowering responses have uncovered a continuous diversity from low to high vernalization sensitivity (Lempe *et al*. 2005; Stinchcombe *et al*. 2005; Shindo *et al*. 2006; Mendez-Vigo *et al*. 2011). This quantitative variation is presumably involved in precise adaptations to present climates, as suggested by its correlations with latitude, altitude and climatic factors (Lempe *et al*. 2005; Stinchcombe *et al*. 2005; Mendez-Vigo *et al*. 2011). Although a large number of quantitative trait locus (QTL) mapping analyses have dissected the overall variation for flowering time (reviewed in Alonso-Blanco *et al*. 2009; Salome *et al*. 2011), only a few QTL studies have specifically addressed the genetic bases of quantitative vernalization responses (Alonso-Blanco *et al*. 1998; Shindo *et al*. 2006; Strange *et al*. 2011). These analyses have detected large effect alleles around *FRI*, *FLC* and *FT* locations, suggesting that additional alleles in these genes underlie the quantitative variation for vernalization response. However, the nucleotide polymorphisms affecting the function of these genes are unknown. In addition, analyses of loci interacting with *FRI* and *FLC* have identified large effect alleles in *HUA2*, *FRL1* and *FRL2* (Poduska *et al*. 2003; Schläppi 2006; Wang *et al*. 2007).

In this work, we address the genetic, molecular and ecological bases of the enhanced vernalization response observed in *A. thaliana* wild genotype Llagostera (Ll-0) from the Iberian Peninsula (Kranz & Kirchheim 1987). To this aim, we combined QTL mapping in a new recombinant inbred line (RIL) population derived from the L*er* × Ll-0 cross, with genetic association analyses in a regional collection of wild accessions (Mendez-Vigo *et al*. 2011). This study illustrates the usefulness of genetic analyses at a regional geographic scale to identify and characterize novel alleles that are ecologically relevant.

MATERIALS AND METHODS

Plant materials

The wild genotype Ll-0 obtained from NASC (N1338) and originally collected around Llagostera (Spain) (Kranz & Kirchheim 1987) and the laboratory strain Landsberg *erecta* (L*er*) derived from a Northern Europe accession (Rédei 1962) were used as parental lines to develop a population of 139 RILs. This population was produced by single seed descent (Alonso-Blanco, Koornneef & van Ooijen 2006) from a single F_1(L*er* × Ll-0) plant obtained using L*er* as the female parent. A single F_8 plant from each L*er* × Ll-0 RIL was genotyped and seeds from 10 F_9 plants per line were bulked to obtain the final F_{10} generation for phenotypic analyses. The population is available through the Nottingham Arabidopsis Stock Centre (http://www.arabidopsis.info).

Four near isogenic lines (NILs) carrying *LVR1*-Ll-0 (NILs 1 and 2) or *LVR1*-L*er* alleles (NILs 3 and 4) in L*er* or Ll-0 background, respectively, were developed from different selected RILs by recurrent backcrossing during three generations and marker-assisted selection. RILs LLL-19 and LLL-132 carrying *LVR1*-Ll-0 alleles were backcrossed to L*er*, whereas RILs LLL-2 and LLL-97 carrying *LVR1*-L*er* alleles were backcrossed to Ll-0. One plant was selected from an F_2 population derived from each cross to obtain the next backcross generation. Genetic selection of *LVR1* genomic region was performed by using the closely linked morphological marker *ERECTA* (*ER*) as Ll-0 carries *ER* functional alleles and L*er* bears recessive and loss-of-function *er*-1 alleles causing compact morphology (Rédei 1962). After three backcrosses, one plant per family was selfed and 10–15 offspring individuals from each family were thoroughly genotyped to select the NILs. All final lines were homozygous for a single introgression fragment around *LVR1* except line NIL-4 that carries an additional introgression of a chromosome 3 region where no flowering locus was detected in the QTL analysis.

Growth conditions and measurements of flowering traits

Seeds were sown in Petri dishes containing a filter paper soaked with demineralized water, and incubated 4 d at 4 °C to break seed dormancy. Thereafter, seeds were transferred to a growth chamber at 21 °C with short-day (SD) photoperiod (8 h light:16 h darkness) for 4 d for germination. Germinated seedlings were planted in 0.9 L pots with a soil : vermiculite mix at 3 : 1 proportion. For the vernalization treatments, pots were placed in a cold chamber at 4 °C and SD photoperiod for 2, 4, 8 or 12 weeks. Subsequently, pots were moved to an air-conditioned greenhouse supplemented with additional light to provide a long-day (LD) photoperiod of 16 h light:8 h darkness (for

the evaluation of RILs and NILs), or to a growth chamber at 21 °C with a similar LD photoperiod (for expression analyses). Non-vernalized plants were moved directly to the same greenhouse or growth chamber as vernalized plants. To reduce developmental differences between vernalization conditions due to environmental factors other than the low temperature treatment, seeds for non-vernalization were sown later than for vernalization and all vernalization treatments were finished right after planting the non-vernalization samples. Thus, plants with different vernalization treatments were developmentally synchronized to share the same environmental conditions during vegetative development.

For evaluation of the RIL population, all RILs and parents were grown simultaneously in a single experiment organized in a two-complete-blocks design. For vernalization treatment, five plants per RIL were grown in one pot in each block, while for non-vernalization 10 plants per line were grown in two pots per block. For evaluation of NILs, all lines were grown simultaneously in an experiment organized in three-complete-blocks. Six plants per line were grown in one pot per block in each vernalization environment.

For analyses of RNA expression, all accessions were grown simultaneously in an experiment organized in two-complete-blocks. Fifty to one hundred seeds per genotype were directly sown on soil in a single pot per block. Pots were placed for germination in a growth chamber at 21 °C and SD photoperiod during 7 d. One month vernalization or non-vernalization treatments were then provided as described previously.

Flowering initiation was measured as leaf number (LN and VLN for non-vernalization and vernalization treatments, respectively) and as days to flowering (FT and VFT for non-vernalization and vernalization treatments, respectively). LN and VLN were calculated as the total number of rosette and cauline leaves in the main inflorescence developed by a plant. FT and VFT were estimated as the number of days from the planting date of the non-vernalization treatment until the opening of the first flower. Vernalization response or sensitivity for LN and FT (SLN and SFT, respectively) was estimated as described by Lempe *et al.* (2005). Briefly, SLN was calculated as [log(LN) – log(VLN)]/[log(mean LN) – log(mean VLN)] with LN and VLN being the mean values of each genotype, and mean LN and mean VLN corresponding to the means of all RILs or parental lines. Similarly, SFT was calculated as [log(FT) – log(VFT)]/[log(mean FT) – log(mean VFT)].

Genotyping and genetic map construction

DNA was isolated as previously described (Bernartzky & Tanksley 1986) without mercaptoethanol. RILs were genotyped with 95 markers selected from different sources and covering 98% of *A. thaliana* physical map (Supporting Information Table S1). In a first step, 58 microsatellites and indels previously reported (Bell & Ecker 1994; Clauss, Cobban & Mitchell-Olds 2002; Loudet *et al.* 2002; Mendez-Vigo *et al.* 2010) and 20 indels developed in this work based on L*er*/Col sequence polymorphisms (Jander *et al.* 2002) were selected at approximate physical intervals of 1.5 Mb. Thereafter, 14 CAPS markers were developed to fill large genetical intervals using described DNA polymorphisms between L*er* and Ll-0 (Nordborg *et al.* 2005). New indel and CAPS markers were named according to BAC clones containing the corresponding sequences. Two additional PCR markers corresponding to known indel polymorphisms in *FRI* (Johanson *et al.* 2000) and *FLC* (Michaels *et al.* 2003) were also genotyped, as well as the *ERECTA* morphological marker segregating in the L*er*/Ll-0 population.

All markers were PCR amplified individually and amplification fragments were separated on standard agarose gels (Konieczny & Ausubel 1993). The 95×139 L*er*/Ll-0 RIL dataset contained an average of 0.14% missing data per marker, and the largest proportion of missing data corresponding to 1.4% (Supporting Information Table S2).

L*er*/Ll-0 linkage map was constructed using JOINMAP 3.0 software package (Van Ooijen & Voorrips 2001) with the RI8 mapping population type. Markers assigned to linkage groups remained on the same group from LOD values of 3 to 7. Markers were arranged within linkage groups using mapping thresholds of REC = 0.45, LOD = 1 and JUMP = 5. Recombination frequencies were converted to genetic distances in cM using Kosambi's mapping function.

NILs were genotyped for a set of 123 polymorphic single nucleotide polymorphisms (SNPs) between L*er* and Ll-0 using Veracode system (Illumina) through CEGEN genotyping service (http://www.cegen.org). SNPs were selected from different sources previously described (Nordborg *et al.* 2005; Schmid *et al.* 2006; Warthmann, Fitz & Weigel 2007) and they were evenly distributed throughout the genome at an average physical interval of 1 Mb.

QTL analyses

QTL mapping was carried out separately for each trait using mean RIL values that were previously log transformed to improve the assumptions of the analyses (Supporting Information Table S3). QTL were located by the multiple-QTL-model method (MQM) implemented in MapQTL v. 4.0 software (Van Ooijen 2000) as described in its reference manual (http://www.mapqtl.nl). QTL were detected using LOD thresholds of 2.5–2.6 corresponding to a genome-wide significance $\alpha = 0.05$ estimated with MapQTL permutation test. The additive allele effect and the percentage of variance explained by each QTL, as well as the total variance explained by the additive effects of all QTL detected for each trait, were obtained from MQM models. QTL additive allele effects correspond to half the differences between the estimated means of the two RIL genotypic groups.

Two-way genetic interactions were tested by two-factor analysis of variance (ANOVA) and the percentage of variance explained by significant interactions was estimated by ANOVA type III variance components analysis. The total variance explained for each trait by additive plus interaction

effects was estimated from a general linear model including all significant effects from detected QTL.

Broad sense heritabilities (h^2_b) were estimated as the variance component among RILs derived from type III ANOVAs. Genotype by environment interaction was tested by two-factor ANOVA using genotypes (RILs) and environments (vernalization and non-vernalization) as classifying factors. Statistical tests were performed with SPSS v. 19 package (SPSS Inc., Chicago, IL, USA).

Gene sequencing

FRI and *FLC* coding regions were sequenced in the parental accessions using the primers and methodology described in Mendez-Vigo *et al*. (2011). Basically, 3.5 and 5.9 kb of *FRI* and *FLC*, respectively, were PCR amplified with 7 and 10 overlapping fragments of 0.5–1 kb. PCR products were then sequenced using an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, CA, USA). DNA sequences were aligned using DNASTAR v.8.0 (DNASTAR Inc., Madison, WI, USA) and alignments were inspected and edited by hand with GENEDOC (Nicholas, Nicholas & Deerfield 1997). Similarly, 2.6 and 0.8 kb segments located upstream and downstream from the *FLC* start and stop codons, respectively, were sequenced in the parents and in 18 Iberian accessions previously sequenced for *FLC* coding region (Mendez-Vigo *et al*. 2011). Primers used to sequence 5′ and 3′ *FLC* regions and to genotype Del(-57) polymorphism are given in Supporting Information Table S4. GenBank accession numbers of DNA sequences generated in this work are JQ663601–JQ663619.

RNA expression analysis

Vegetative tissue of plants growing at 21 °C and LD photoperiod was harvested 5 d after germination for the NV treatment, or 3 d after 1 month of vernalization. Total RNA was extracted using TRIzol reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). For Northern blot analyses, 15 to 20 μg of RNA per sample were fractionated on a 1.4% formaldehyde-agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway, NJ, USA). Hybridizations were carried out at 65 °C overnight. After hybridization and washing, membranes were exposed to X-ray film for 1 d at –80 °C.

The *FLC* probe was a ~700 bp *Eco*RI/*Sph*I fragment from p*FLC* lacking the MADS-box domain (Michaels & Amasino 1999). To normalize RNA loading differences, membranes were stripped in boiling 0.1% SDS and rehybridized with a *β-TUBULIN* probe (Snustad *et al*. 1992). Films were scanned, and intensities of blot signals were quantified with ImageJ version 1.44 (http://www.rsb.info.nih.gov/ij). For analysis of *FLC* expression, the normalized *FLC* intensity of each accession was divided by that of Col without vernalization.

Association analyses

Genetic association between *FLC* polymorphisms and flowering-related traits or *FLC* expression was tested using the mixed-model approach for structured populations (Yu *et al*. 2006) as described in Mendez-Vigo *et al*. (2011). Two levels of genetic relatedness were included in the model: the population structure (Q matrix) and the relative kinship (K matrix) (Zhao *et al*. 2007). Population structure was estimated with STRUCTURE as the Q matrix containing the membership proportions of all genotypes to five ancestral populations. The K matrix was estimated as twice the proportion of shared alleles from 101 segregating SNPs. Mixed models were applied using the software TASSEL version 3.0 (Bradbury *et al*. 2007).

Association between *FLC* polymorphisms and environmental factors or altitude was tested using a similar mixed-model approach (Mendez-Vigo *et al*. 2011). The following 65 climatic variables previously collected from the locations of Iberian populations were tested: 12 monthly mean temperatures and the annual mean temperature; 24 minimum and maximum monthly mean temperatures and the minimum and maximum annual temperatures; 12 monthly total precipitations and the annual total precipitation; and 12 monthly mean solar radiations and the annual mean solar radiation.

RESULTS

Flowering behaviour and vernalization responses of Ll-0, L*er* and Col

Previous flowering studies using collections of wild genotypes identified Ll-0 from the Iberian Peninsula as an accession with enhanced response to vernalization (Shindo *et al*. 2005; Mendez-Vigo *et al*. 2011). To characterize in detail this behaviour, we analysed the flowering phenotypes of Ll-0 and the laboratory strains Col and L*er* exposed to 0 to 12 weeks of vernalization (Fig. 1 and Table 1). Non-vernalized Ll-0 plants flowered on average 20–30 d later and produced ~20 leaves more during vegetative development than L*er* and Col. By contrast, after 4 weeks of vernalization, Ll-0 flowered at the same time as both reference strains but produced fewer leaves than Col ($P < 0.0001$). In addition, a very short vernalization treatment of 2 weeks resulted in Ll-0 exhibiting between 50 and 84% of the maximal reductions of FT and LN, respectively. Longer vernalization treatments barely accelerated flowering initiation in the three accessions (Fig. 1). Vernalization sensitivities SFT and SLN were quantified for FT and LN (see Materials and Methods), showing that Ll-0 is substantially more sensitive than reference strains (Table 1, Fig. 1). Hence, Ll-0 behaves as a late flowering genotype that responds more and faster to vernalization than laboratory strains.

Flowering behaviour and vernalization responses of the L*er* × Ll-0 RIL population

To determine the genetic bases of the enhanced vernalization response of Ll-0, we developed a new population of 139 RILs derived from a L*er* × Ll-0 cross. This population was

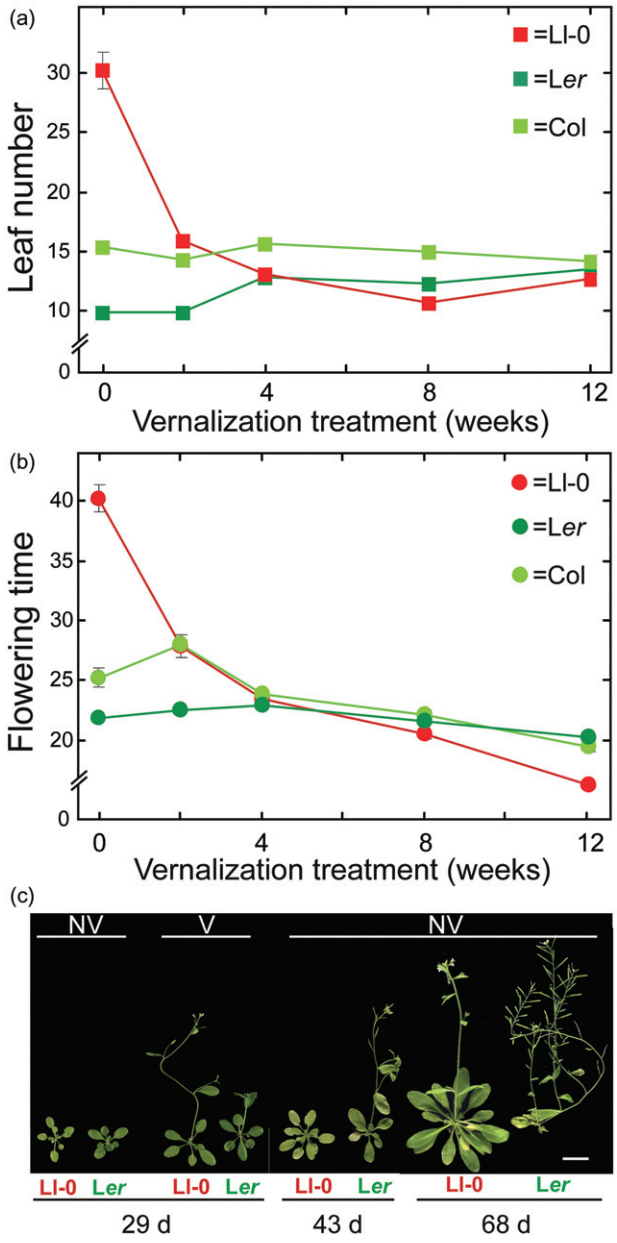

Figure 1. Flowering behaviour of Ll-0, L*er* and Col accessions in relation to vernalization. (a) Leaf number and (b) flowering time, after different vernalization treatments. Data points are means ± SE of 15–18 plants. (c) Representative plants of Ll-0 and L*er* cultivated without vernalization (NV) or with 12 weeks vernalization treatment and photographed in different days after the vernalization treatment. Scale bar in (c) is 2 cm.

grown without vernalization and with 12 weeks of vernalization treatment to estimate LN and FT in both environmental conditions, as well as vernalization sensitivities (Table 1 and Fig. 2). Broad sense heritabilities showed large amounts of genetic variation for most traits, values being considerably higher without than with vernalization (Table 1). As shown in Fig. 2, vernalization strongly reduced FT and LN in most RILs. However, the significant RIL by environment interactions detected for both traits ($P < 10^{-7}$) indicated that RILs respond differently to vernalization (Supporting Information Fig. S1). Genetic variation for vernalization response was supported by the strong positive correlation between LN or FT and the corresponding vernalization sensitivities ($0.91 < r < 0.95$; $P < 10^{-7}$; Supporting Information Fig. S1). Thus, late-flowering lines responded more to vernalization than early-flowering genotypes, in agreement with the phenotypes observed in the parental lines.

Genetic map of L*er* × Ll-0 RIL population

We developed a genetic map including 95 markers evenly distributed at an average distance of 5.1 cM. This map showed a total length of 455 cM, the largest genetic interval between adjacent markers being 15.8 cM (Fig. 3). The genetic order of all markers was similar to that of Col physical map (http://www.arabidopsis.org/), with the exception of three pairs of markers (Supporting Information Fig. S2a), which appeared as inverted segments. Comparison of physical and genetic maps indicated that the recombination rate is homogeneously distributed along most of the length of the five chromosomes with an average value of 375 kb per cM. However, recombination was substantially lower in all five pericentromeric regions. In total, the 139 RILs provided 1204 recombination events on this genetic map, with an average of 8.7 breakpoints per line. As expected for the F_8 generation, heterozygosity of most markers was below 1%, and only two markers presented a higher-than-expected value of 2.9%. Only 10 markers mapping in the lower arms of chromosomes 1, 3 and 5 showed distortion from the expected 1 : 1 segregation of homozygous genotypes ($P < 0.01$; Supporting Information Fig. S2b).

QTL analysis for flowering traits and vernalization responses in the L*er* x Ll-0 RIL population

To identify the loci that determine the variation for flowering traits measured without and with vernalization in the L*er* × Ll-0 RIL population, we used these data for QTL mapping (Fig. 3 and Supporting Information Table S5). Six loci accounted for 83% of the phenotypic variance for LN and FT without vernalization, three of them showing large effect (> 10%) and three other displaying small effect (< 5%). Large-effect QTL located on top of chromosome 4 and the upper arm of chromosome 5 overlapped with the well-known flowering genes *FRI*, *FLC* and *HUA2*, Ll-0 carrying late alleles in all three regions. Sequencing of *FRI*, *FLC* and *HUA2* indicated that Ll-0 carries the most common active *FRI*-H51 allele, as well as presumably functional *FLC* and *HUA2* alleles (Shindo *et al.* 2005; Wang *et al.* 2007). As the L*er* parental line carries loss-of-function alleles that produce early flowering in these three genes (Johanson *et al.* 2000; Michaels *et al.* 2003; Doyle *et al.* 2005), they account, at least partly, for the effect of those QTL. Ll-0 carried early flowering alleles in the other three QTL, two of them

Table 1. Flowering-related traits of L*er*, Ll-0 and the RIL population

	LN	FT	VLN	VFT	SLN	SFT
L*er*	9.8 ± 0.9	21.8 ± 0.8	13.5 ± 1.2	20.3 ± 1.1	–0.76	0.14
Ll-0	30.2 ± 6.0	40.2 ± 4.7	12.7 ± 1.7	16.2 ± 1.0	2.04	1.71
L*er*[a]	8.7 ± 1.0	37.4 ± 3.5	8.9 ± 1.3	34.1 ± 4.5	–0.02	0.12
Ll-0[a]	26.8 ± 4.4	67.1 ± 6.2	8.7 ± 1.2	25.7 ± 3.5	1.48	1.26
RIL mean	21.3 ± 11.9	57.1 ± 20.4	10.0 ± 2.1	26.5 ± 4.0	0.76 ± 0.76	0.92 ± 0.41
Min-max RIL mean	5.0–50.3	27.3–112.1	5.0–16.9	17.8–38.5	–0.71–2.07	–0.15–1.80
h^2_b	0.93	0.91	0.46	0.43	ND	ND

[a]Parental lines grown in the same experiment than the L*er* × Ll-0 RIL population.
Values are mean ± SD.
VLN, VFT, SLN and SFT were estimated after 12 weeks of vernalization treatment.
ND, non-determined; h^2_b, broad sense heritability; RIL, recombinant inbred line; VLN, leaf number for vernalization treatment; VFT, days to flowering for vernalization treatment; SLN, sensitivity for leaf number; SFT, sensitivity for days to flowering.

overlapping with the previously cloned flowering QTL *CRY2* and *MAF2-MAF5* (Fig. 3). Only three genomic regions affected the same traits after vernalization and together explained 35.5–49.1% of the variances. Two of these QTL overlapped with *FLC* and *MAF2-MAF5*, both regions showing a large relative effect after vernalization. The third QTL was detected only for VLN and VFT, its Ll-0 allele accelerating flowering. Thus, a total of seven genomic regions affected FT and LN in both environmental conditions.

To further characterize L*er*/Ll-0 flowering QTL, we analysed two-way genetic interactions (Supporting Information Table S5). Only *FRI*, *FLC* and *HUA2* genomic regions showed significant interactions for some traits (Fig. 4). In particular, *FRI* and *FLC* loci interacted for LN and FT. Lines carrying active alleles in both regions showed lower phenotypic value than expected from the additive effects of both loci, hence *FRI* and *FLC* displayed a less-than-additive interaction in this RIL

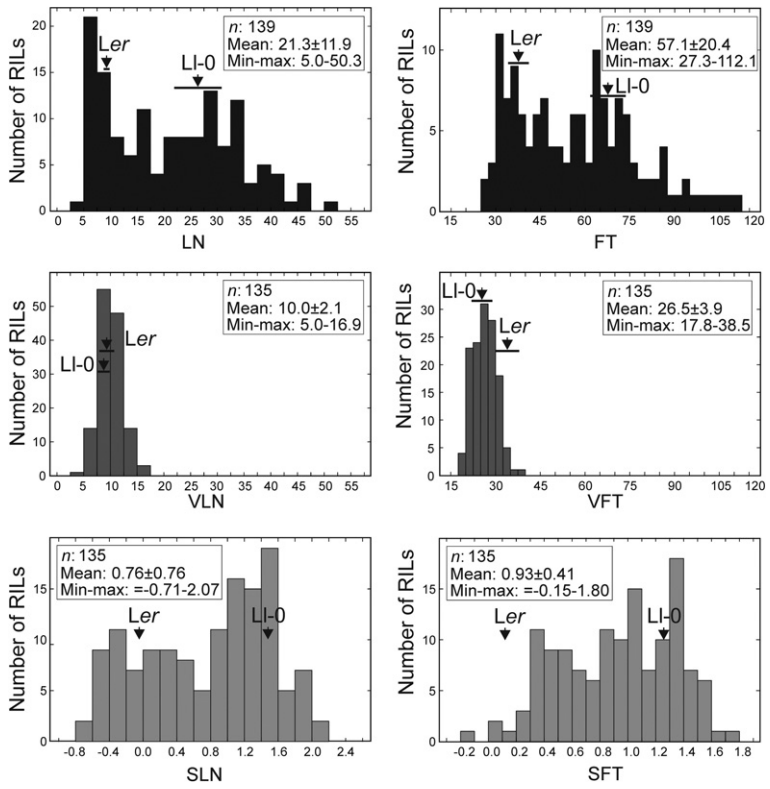

Figure 2. Frequency distributions of flowering related traits in the L*er* × Ll-0 RIL population grown with and without vernalization. Arrows and horizontal bars depict mean ± SD of parental lines. The number of RILs analysed, the population mean, and the minimum and maximum RIL means are indicated inside each panel. VLN, VFT, SLN and SFT were estimated after 12 weeks of vernalization treatment. RIL, recombinant inbred line; LN, leaf number; FT, days to flowering; VLN, leaf number for vernalization treatment; VFT, days to flowering for vernalization treatment; SLN, sensitivity for LN; SFT, sensitivity for FT.

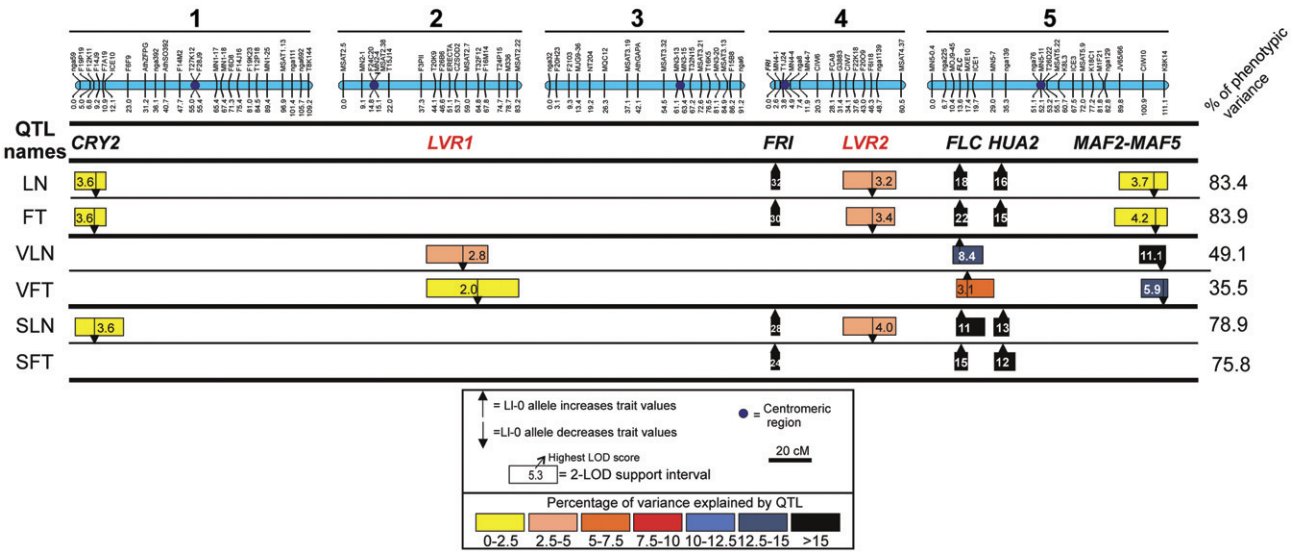

Figure 3. QTL mapping of flowering-related traits in the L*er*/Ll-0 RIL population. Bars on the top depict the genetic map of the five linkage groups. Thick horizontal lines separate groups of traits analysed: flowering initiation without vernalization; flowering initiation after 12 weeks of vernalization treatment; vernalization sensitivity. Column in the right side shows the percentage of phenotypic variance explained by the additive effects of all detected QTL. For each trait, the locations of QTL identified are shown as 2-LOD support intervals. Position of arrows and numbers inside boxes correspond to the highest LOD scores. Colours of QTL boxes depict different ranges of QTL explained variances as described in the legend. Upper and lower arrows indicate that the additive effect of Ll-0 alleles increase or decrease, respectively, the trait values in comparison with L*er* alleles. QTL regions are named according to cloned flowering QTL overlapping with the detected QTL; names appear below the genetic map in black colour text. QTL that do not overlap with previously cloned QTL are named in red colour text. QTL, quantitative trait locus; LN, leaf number; FT, days to flowering; VLN, leaf number for vernalization treatment; VFT, days to flowering for vernalization treatment; SLN, sensitivity for LN; SFT, sensitivity for FT.

population. *FRI* and *HUA2* showed similar patterns of interaction (Fig. 4).

To determine the genetic bases of the variation for the vernalization response, we compared the effect of QTL

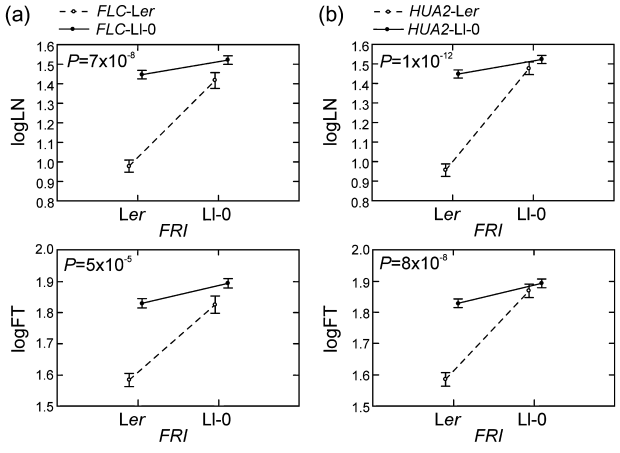

Figure 4. Genetic interactions among *FRI*, *FLC* and *HUA2* genomic regions. (a) Interaction between *FRI* and *FLC* loci. (b) Interaction between *FRI* and *HUA2* loci. Each panel shows the mean ± SE of L*er*/Ll-0 RILs classified into four genotypic classes. To avoid effects derived from the genetic linkage between *FLC* and *HUA2*, only homozygous RILs for active *HUA2*-Ll-0 or *FLC*-Ll-0 alleles are included in a and b, respectively. Statistical significances of interactions are indicated inside the panels. RIL, recombinant inbred line.

between the two environmental conditions. Only *MAF2-MAF5* region showed similar absolute allele effect in both environments, and therefore, it did not contribute to Ll-0 vernalization response (Supporting Information Table S5). However, the remaining six QTL presented three distinct patterns of allelic effects contributing to the variation for vernalization responses. Firstly, Ll-0 late flowering alleles of large effect at *FRI* and *HUA2* loci showed no effect after vernalization, whereas the lateness caused by *FLC*-Ll-0 alleles was strongly reduced by this treatment ($P < 0.001$). Secondly, early flowering Ll-0 alleles at chromosome 2 QTL showed an effect only after vernalization. Thirdly, Ll-0 early flowering alleles at QTL on chromosomes 1 and 4 displayed an effect mainly without vernalization. Given the phenotypic behaviour of Ll-0 and L*er*, the four loci showing the first two patterns of QTL effects appeared as responsible for the larger vernalization response of Ll-0. In contrast, the two QTL displaying the third pattern, which is opposite to the behaviour of parental accessions, likely reduced Ll-0 vernalization responses, hence accounting for the transgression for vernalization sensitivity observed in the RIL population (Fig. 2). As the two regions on chromosomes 2 and 4 did not overlap with previously cloned flowering QTL and they affected vernalization responses, we named them as *Llagostera vernalization response* (*LVR*) 1 and 2, respectively.

The genetic basis of the variation for vernalization response was confirmed by QTL mapping of SLN and SFT (Fig. 3). We detected a total of five genomic regions

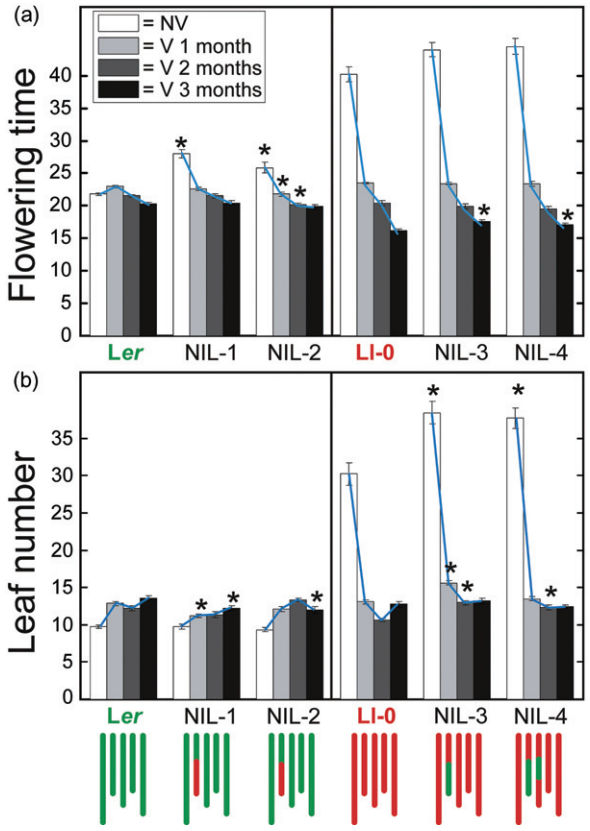

Figure 5. Flowering behaviour of *LVR1* NILs in relation to vernalization. (a) Flowering time and (b) leaf number of NILs carrying *LVR1*-Ll-0 (NILs 1 and 2) or *LVR1*-L*er* alleles (NILs 3 and 4) in L*er* or Ll-0 genetic backgrounds, respectively. Each panel shows the mean ± SE of 10–18 plants grown without vernalization (NV) or after 1, 2 and 3 months of vernalization treatment (V). In each environment, phenotypic differences were tested statistically between L*er* and NILs 1 or 2, and between Ll-0 and NILs 3 or 4, using Student's *t*-test. Significant pair comparisons are indicated with an asterisk on the corresponding NIL ($P < 0.01$). NIL, near isogenic line.

explaining 76–79% of the phenotypic variance for each trait. Ll-0 alleles in *FRI*, *FLC* and *HUA2* genomic regions showed strong effect increasing vernalization sensitivities. On the contrary, regions around *CRY2* and *LVR2* displayed opposite effects in agreement with QTL results for LN and FT.

Genetic validation of the small effect QTL *LVR1*

In order to confirm *LVR1*, we developed four independent NILs carrying *LVR1*-Ll-0 or *LVR1*-L*er* alleles in L*er* or Ll-0 genetic backgrounds, respectively. NIL phenotypic analyses in relation to vernalization (Fig. 5) detected three small effects of *LVR1* that were in agreement with allelic effects estimated in the QTL analysis. Firstly, the two NILs carrying *LVR1*-Ll-0 alleles displayed significantly lower LN than L*er* plants only after 3 months of vernalization. Secondly, although these two lines flowered later than L*er* when they were not vernalized, both NILs showed increased FT response to vernalization than L*er*, as estimated from the slopes of regression lines between FT and vernalization time (Fig. 5a). Thirdly, the two NILs with *LVR1*-L*er* alleles showed higher LN than Ll-0 when grown without vernalization and after several vernalization treatments. Together, these results validated *LVR-1* as a small effect QTL at which Ll-0 alleles accelerate flowering mainly after vernalization and, consequently, increase vernalization responses.

Molecular, physiological and climatic characterization of *FLC*-Ll-0 allele

To determine the type of *FLC* allele present in Ll-0, we sequenced *FLC* coding region and compared it with available sequences. *FLC*-Ll-0 was identical to a moderate frequency haplotype previously found in a collection of 182 populations from the Iberian Peninsula, and referred to as Indel-1075 (Mendez-Vigo *et al.* 2011). This is an Iberian-specific haplotype that belongs to the major *FLC*-A haplogroup (Caicedo *et al.* 2004) but differs from all other Iberian *FLC* alleles in two intronic polymorphisms showing complete linkage disequilibrium (Fig. 6a).

To further characterize *FLC*-Ll-0 allele, we sequenced 2.6 kb of its promoter and 5′-UTR region, as well as 0.8 kb of the 3′-UTR, in Ll-0 and other 18 Iberian accessions. *FLC*-Ll-0 was characterized by a specific 50-bp-deletion, named as Del(-57), which is located 57 bp upstream from the start codon and that spans the first half of *FLC* 5′-UTR (Fig. 6a). Genotyping of Del(-57) in the 182 accessions of the Iberian collection detected Ll-0 allele only in 7 out of 14 accessions with haplotype Indel-1075. Analysis of the geographic distribution of *FLC* alleles shows that haplotype Indel-1075 occurs in a wide area of 350 × 120 km in north-eastern (NE) Spain (Fig. 6b). However, *FLC*-Ll-0 allele displayed stronger geographic structure as it was just found in a smaller area of 40 × 35 km in the eastern part of Indel-1075 domain. The average proportion of allelic differences (genetic distance) among pairs of accessions carrying *FLC*-Ll-0 allele was 0.23 ± 0.05, as estimated from a set of 101 genome-wide SNP markers previously genotyped in this collection. This value was only slightly lower than the average distance observed among all 182 Iberian genotypes (0.26 ± 0.05), indicating that accessions carrying *FLC*-Ll-0 are genetically differentiated throughout their genome. Therefore, *FLC*-Ll-0 allele probably originated from haplotype Indel-1075 in NE Spain and, after outcrossing and recombination, it was expanded over a limited geographic area where it is currently present at high relative frequency (>25%).

Genetic association analyses using the Iberian regional collection of local populations had previously suggested that *FLC* Indel-1075 contributes to the variation for several flowering traits, including vernalization sensitivity (Mendez-Vigo *et al.* 2011). Similar analyses with Del(-57) (see Table 2 and Materials and Methods) showed significant associations, *FLC*-Ll-0 allele accelerating flowering only after vernalization and, consequently, increasing vernalization sensitivity. As Del(-57) explained larger or comparable

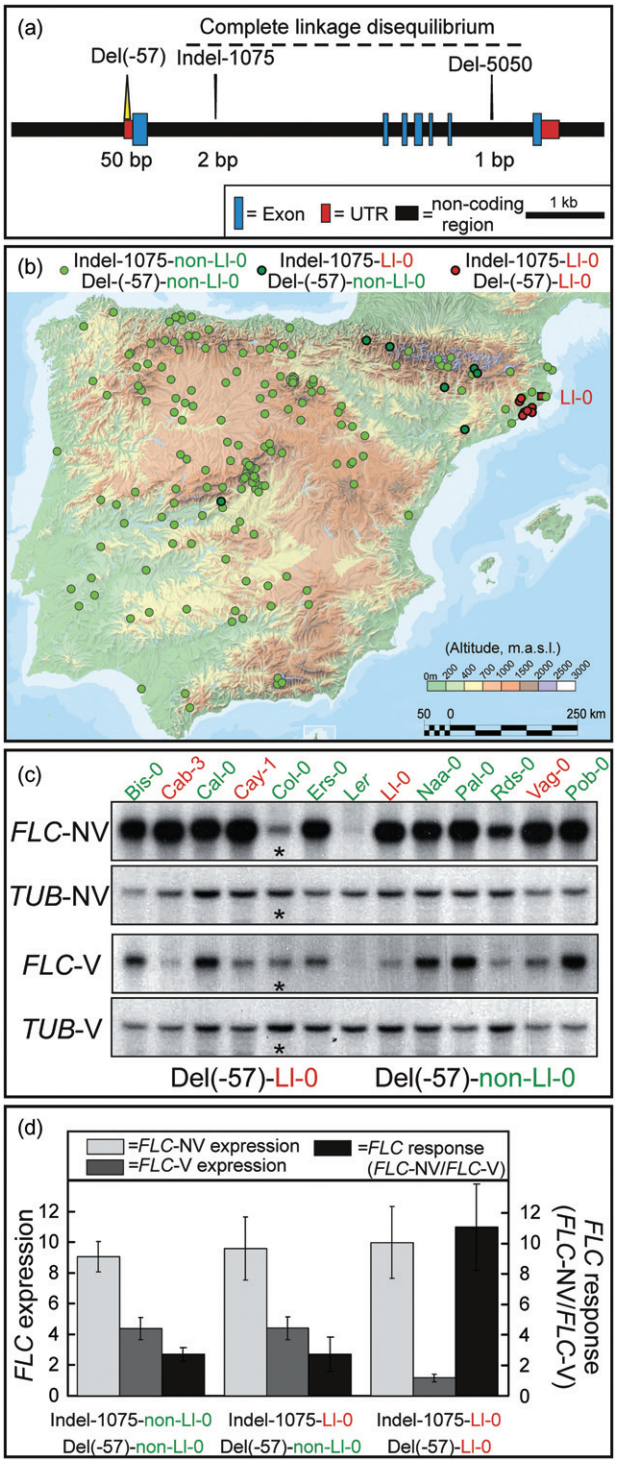

Figure 6. Geographic distribution and gene expression of *FLC* Iberian alleles. (a) *FLC* genomic structure showing Ll-0 specific polymorphisms. Names and sizes of polymorphisms are shown above and below the gene, respectively. (b) Geographic distribution of the three *FLC* haplotypes detected according to Ll-0 specific polymorphisms. (c) Northern analysis of *FLC* expression without vernalization (*FLC*-NV) and after 1 month of vernalization (*FLC*-V) treatment, in 11 representative Iberian accessions differing in Del(-57) and in the control laboratory strains Col and L*er*. The latter is characterized by a strong reduction of *FLC* expression caused by a transposable element insertion undetected in the Iberian Peninsula (Michaels *et al.* 2003; Mendez-Vigo *et al.* 2011). (d) Quantification of *FLC*-NV and *FLC*-V expressions, and *FLC* vernalization response (measured as the *FLC*-NV/*FLC*-V ratio) in the three *FLC* haplotypes. Bars correspond to mean ± SE of 6 to 13 accessions per haplotype. *FLC* expression in each accession was normalized with *β-TUBULIN* (*TUB*) abundance and relativized to Col *FLC*-NV expression. Asterisks in panel (c) indicate Col NV control samples included in each blot.

amounts of phenotypic variances than Indel-1075, this appears as the most likely mutation affecting *FLC* function, whereas flowering associations of Indel-1075 are probably consequence of the partial linkage disequilibrium between both polymorphisms.

Because Del(-57) does not affect FLC protein structure but it involves a non-coding genomic sequence, we hypothesized that this polymorphism might affect the *cis*-regulation of *FLC* expression. To test this, we analysed *FLC* expression and its response to vernalization in a set of 27 Iberian accessions and the laboratory strains L*er* and Col (Fig. 6 and Supporting Information Fig. S3). These accessions included all 14 genotypes with Ll-0 alelles in Del(-57) and/or Indel 1075, as well as 13 accessions with the common non-Ll-0 allele in both polymorphisms and distributed in the same NE Iberian subregion. Accessions carrying the three haplotypes defined by Del(-57) and Indel-1075 polymorphisms showed similar high *FLC* expression in non-vernalized plants (Fig. 6). In contrast, after 1 month of vernalization, accessions carrying the *FLC*-Ll-0 haplotype showed fourfold lower *FLC* expression than accessions with the non-Ll-0 allele in Del(-57) ($P < 0.004$), which indicates that Ll-0 carries an *FLC* allele that responds substantially more to vernalization. To test if this higher Ll-0 response is determined by *FLC* polymorphisms, we also carried out genetic association analyses with expression data but taking into account the genetic relatedness among genotypes (Table 2). Despite the small sample size, Del(-57) appeared marginally associated with *FLC* expression after vernalization and with *FLC* response; this polymorphism accounted for ~13% of the variation for each trait. On the contrary, Indel-1075 did not show any significant effect on *FLC* expression. Therefore, Ll-0 carries a hyper-responsive *FLC* allele whose functional alteration is probably caused by Del(-57).

To evaluate if *FLC*-Ll-0 allele is involved in adaptation to particular environmental conditions, we carried out association analyses between *FLC* polymorphisms and altitude or the climatic conditions of the local populations where the accessions were collected (Table 2). Indel-1075 was not associated with any of these variables ($P > 0.01$). However, Del(-57) was significantly associated with altitude, with *FLC*-Ll-0 accessions occurring at an average altitude 425 m lower than the rest of Iberian populations. Furthermore, Del(-57) was significantly associated with annual and autumn minimum temperatures (Table 2), *FLC*-Ll-0

Table 2. Association analyses between *FLC* and flowering-related traits or environmental factors

Polymorphism	Gene location	N	MAF (%)	Associated variables	*P*	R^2 (%)
Indel-1075	First intron	182	7.7	VLN, SLN, VFT	7.0×10^{-4}–0.002	5.7–4.9
		182	7.7	Altitude	ns	–
		182	7.7	Annual and autumn minimum temp	ns	–
		26	50.0	*FLC*-V, *FLC*-NV/*FLC*-V	ns	–
Del(–57)	5′-UTR	182	3.8	VLN, SLN, VFT	1.0×10^{-4}–0.007	7.5–3.6
		182	3.8	Altitude	4.6×10^{-3}	4.1
		182	3.8	Annual and autumn minimum temp	2.7×10^{-3}–6.3×10^{-3}	4.2–3.4
		26	26.9	*FLC*-V, *FLC*-NV/*FLC*-V	0.06–0.08	12–14

Table includes the statistical significance and R^2 of significant associations ($P < 0.001$) detected for *FLC* polymorphisms and phenotypic traits, *FLC* expression (*FLC*-V and *FLC*-NV/*FLC*-V), altitude or climatic factors. Polymorphisms are named according to type of mutation and nucleotide position; the first coding nucleotide corresponds to position 1.
MAF, minor allele frequency; VLN, leaf number for vernalization treatment; VFT, days to flowering for vernalization treatment; SLN, sensitivity for leaf number; ns, not significant.

appearing in locations with higher minimum temperatures than the remaining *FLC* alleles. As the two ecological variables associated with *FLC* are highly correlated, we concluded that minimum temperature is the climatic factor determining the altitudinal association of *FLC*.

DISCUSSION

The enhanced vernalization response of Ll-0 is determined by large and small effect loci

Current studies of the quantitative variation for vernalization response in *A. thaliana* have been based in accessions showing extremely late or early flowering when plants are not vernalized and weak to strong vernalization responses (Alonso-Blanco *et al*. 1998; Shindo *et al*. 2006; Strange *et al*. 2011). Ll-0 displayed a different flowering phenotype characterized by a moderate late flowering of non-vernalized plants and a very rapid response to short periods of vernalization, thus extending the responses that have been genetically dissected. QTL mapping in the L*er* × Ll-0 RIL population indicates that the enhanced Ll-0 vernalization response is largely determined by major effect alleles at *FRI*, *FLC* and *HUA2*, in agreement with previous studies (Shindo *et al*. 2006; Salome *et al*. 2011; Strange *et al*. 2011). In addition, we detected two small effect QTL, *LVR1* and *LVR2*, that have not been previously involved in vernalization sensitivity, the effect of *LVR1* to increase Ll-0 vernalization response being validated by NIL analyses. The low number of small effect loci found in this work is in accordance with recent studies emphasizing that few loci with large effect alleles account for most variation for flowering time and vernalization sensitivity in *A. thaliana* (Brachi *et al*. 2010; Salome *et al*. 2011; Strange *et al*. 2011). However, the statistical power to detect small effect QTL in current mapping populations is rather limited, due not only to their minor effect, but also because large effect loci might mask their detection (Keurentjes *et al*. 2007). Additional studies using different mapping strategies, such as genome-wide association mapping (Atwell *et al*. 2010; Li *et al*. 2010) or whole genome NIL populations (Keurentjes *et al*. 2007), are required to estimate the overall contribution of small *versus* large effect loci to *A. thaliana* adaptations by genetic modifications of vernalization sensitivity (Orr 2005).

The vernalization hyper-responsive *FLC*-Ll-0 allele is characterized by a regulatory 5′-UTR deletion likely involved in local climatic adaptation

Sequencing and expression analyses carried out in this work showed that Ll-0 bears an Iberian-specific *FLC* allele that is hyper-responsive to vernalization, its phenotypic effects suggesting that this is not a loss-of-function but a change-of-function allele. Genetic association results support that a 50-bp-deletion in the 5′-UTR of this allele is the nucleotide polymorphism causing more rapid vernalization-mediated down-regulation of *FLC* expression and, subsequently, the stronger Ll-0 vernalization sensitivity. Nevertheless, the causal polymorphism might only be in linkage disequilibrium with this *FLC* deletion. Previous comparative QTL mapping and association analyses indicate that a series of *FLC* alleles with different effects on gene function contribute to flowering variation (Shindo *et al*. 2006; Mendez-Vigo *et al*. 2011; Salome *et al*. 2011). This study provides the first natural *FLC* polymorphism that might affect the vernalization-mediated *cis*-regulation of *FLC*, although further studies are needed to conclusively demonstrate the effects and mechanisms of Del(-57) polymorphism.

Analyses of artificially induced deletions have previously identified *FLC* segments in the promoter and first intron that are required for its silencing by vernalization (Sheldon *et al*. 2002; He, Doyle & Amasino 2004; Sung *et al*. 2006). However, no DNA motif has been found that negatively regulates *FLC* repression and silencing, and Del(-57) suggesting the presence of such *cis*-element within the deleted sequence. We hypothesize that *FLC*-Ll-0 deletion might limit *FRI*-mediated activation of *FLC* during vernalization, as supported by several results. Firstly, *FRI* has been recently shown to interact with the 5′-cap-binding complex

that is bound to the first nucleotides of mRNAs (Geraldo *et al*. 2009). Secondly, the absence of *FRI* QTL in the L*er* × Ll-0 RIL population after vernalization, but not in non-vernalized plants, indicates a vernalization-dependent-suppression of *FRI* effects. This mechanism might also explain the less-than-additive genetic interaction detected between *FRI* and *FLC* in the L*er* × Ll-0 RIL population grown without vernalization, which contrasts with the synergistic interaction described in previous studies comparing L*er* loss-of-function alleles and active alleles from other accessions (Koornneef *et al*. 1994; Lee *et al*. 1994; Poduska *et al*. 2003). As Ll-0 carries the most common active *FRI*-H51 allele (Shindo *et al*. 2005; this work), this particular interaction is likely to be determined by *FLC*-Ll-0, probably through certain Del(-57) effect on *FLC* activation also without vernalization.

Environmental analyses indicate that *FLC*-Ll-0 is associated with high minimum temperatures characteristic of low altitude locations, suggesting that the increased vernalization response associated with this allele is involved in adaptation to the warm climate of the Mediterranean coast. However, as the strongest association was found with autumn minimum temperature, it is possible that this climatic factor determines the spatial pattern of *FLC*-Ll-0 via pleiotropic effects on temperature-dependent germination (Chiang *et al*. 2009). The role of this allele in climatic adaptation is further supported by its temporal permanence in this region, because we collected *FLC*-Ll-0 ~50 years after Laibach's original Mediterranean samplings (Kranz & Kirchheim 1987). This climatic association is also in agreement with previous correlations found between vernalization sensitivity (SLN) and latitude, altitude or minimum temperature (Lempe *et al*. 2005; Stinchcombe *et al*. 2005; Mendez-Vigo *et al*. 2011), which suggest that *FLC*-Ll-0 allele contributes to described geographic and climatic clines. In addition, *FLC*-Ll-0 appeared distributed only in a small geographic area, further suggesting that it might be involved in adaptation to a particular local environment. Alternatively, we cannot discard that the restricted distribution of *FLC*-Ll-0 may be a consequence of its limited demographic expansion.

Most alleles identified as involved in *A. thaliana* natural variation for flowering traits appear as rare or unique in global collections of wild genotypes (reviewed in Alonso-Blanco *et al*. 2009; Salome *et al*. 2011). This is in agreement with the low frequency of most *A. thaliana* nucleotide polymorphisms and the strong geographic structure of such low frequency alleles (Nordborg *et al*. 2005; Mendez-Vigo *et al*. 2011). It can then be expected that alleles affecting adaptive traits will often show moderate frequency only at a subregional or local geographic level. Therefore, these alleles will be amenable for genetic and environmental associations mainly in dense regional collections but not in current global studies. The analysis of *FLC*-Ll-0 in the Iberian Peninsula illustrates the usefulness of such regional collections to reveal ecologically relevant alleles. However, demonstrating the ecological significance of these genetic variants awaits further studies under natural conditions, such as field-based QTL mapping analyses (Brachi *et al*. 2010) or long-term genetic studies of wild populations (Gomaa *et al*. 2011). It can be expected that such analyses will eventually reveal the genetic and molecular bases underlying plant adaptation by fine-tuning vernalization responses to current and future climates.

ACKNOWLEDGMENTS

The authors thank Mercedes Ramiro and Jenifer Pozas for their excellent technical assistance. This work was funded by grants BIO2010-15022 and TRANSPLANTA CSD-2007-00057 from the Ministerio de Ciencia and Innovación of Spain to C.A-B.

REFERENCES

Alonso-Blanco C., El-Assal S.E., Coupland G. & Koornneef M. (1998) Analysis of natural allelic variation at flowering time loci in the Landsberg erecta and Cape Verde Islands ecotypes of *Arabidopsis thaliana*. *Genetics* **149,** 749–764.

Alonso-Blanco C., Koornneef M. & van Ooijen J.W. (2006) QTL analysis. *Methods in Molecular Biology* **323,** 79–99.

Alonso-Blanco C., Aarts M.G., Bentsink L., Keurentjes J.J., Reymond M., Vreugdenhil D. & Koornneef M. (2009) What has natural variation taught us about plant development, physiology, and adaptation? *The Plant Cell* **21,** 1877–1896.

Atwell S., Huang Y.S., Vilhjalmsson B.J., *et al*. (2010) Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature* **465,** 627–631.

Ausin I., Alonso-Blanco C. & Martinez-Zapater J.M. (2005) Environmental regulation of flowering. *International Journal of Developmental Biology* **49,** 689–705.

Bell C.J. & Ecker J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. *Genomics* **19,** 137–144.

Bernartzky R. & Tanksley S. (1986) Genetics of acting-related sequences in tomato. *Theoretical and Applied Genetics* **72,** 314–324.

Brachi B., Faure N., Horton M., Flahauw E., Vazquez A., Nordborg M., Bergelson J., Cuguen J. & Roux F. (2010) Linkage and association mapping of *Arabidopsis thaliana* flowering time in nature. *PLoS Genetics* **6,** e1000940.

Bradbury P.J., Zhang Z., Kroon D.E., Casstevens T.M., Ramdoss Y. & Buckler E.S. (2007) TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23,** 2633–2635.

Caicedo A.L., Stinchcombe J.R., Olsen K.M., Schmitt J. & Purugganan M.D. (2004) Epistatic interaction between *Arabidopsis FRI* and *FLC* flowering time genes generates a latitudinal cline in a life history trait. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 15670–15675.

Chiang G.C., Barua D., Kramer E.M., Amasino R.M. & Donohue K. (2009) Major flowering time gene, *flowering locus C*, regulates seed germination in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 11661–11666.

Choi K., Kim J., Hwang H.J., Kim S., Park C., Kim S.Y. & Lee I. (2011) The FRIGIDA complex activates transcription of *FLC*, a strong flowering repressor in *Arabidopsis*, by recruiting chromatin modification factors. *The Plant Cell* **23,** 289–303.

Clauss M.J., Cobban H. & Mitchell-Olds T. (2002) Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabis* (*Brassicaeae*). *Molecular Ecology* **11,** 591–601.

De Lucia F. & Dean C. (2011) Long non-coding RNAs and chromatin regulation. *Current Opinion in Plant Biology* **14,** 168–173.

Doyle M.R., Bizzell C.M., Keller M.R., Michaels S.D., Song J., Noh Y.S. & Amasino R.M. (2005) *HUA2* is required for the expression of floral repressors in *Arabidopsis thaliana*. *The Plant Journal* **41,** 376–385.

Geraldo N., Baurle I., Kidou S., Hu X. & Dean C. (2009) *FRIGIDA* delays flowering in *Arabidopsis* via a cotranscriptional mechanism involving direct interaction with the nuclear cap-binding complex. *Plant Physiology* **150,** 1611–1618.

Gomaa N.H., Montesinos-Navarro A., Alonso-Blanco C. & Pico F.X. (2011) Temporal variation in genetic diversity and effective population size of Mediterranean and subalpine *Arabidopsis thaliana* populations. *Molecular Ecology* **20,** 3540–3554.

He Y., Doyle M.R. & Amasino R.M. (2004) PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in *Arabidopsis*. *Genes and Development* **18,** 2774–2784.

Heo J.B. & Sung S. (2011) Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. *Science* **331,** 76–79.

Jander G., Norris S.R., Rounsley S.D., Bush D.F., Levin I.M. & Last R.L. (2002) *Arabidopsis* map-based cloning in the post-genome era. *Plant Physiology* **129,** 440–450.

Johanson U., West J., Lister C., Michaels S., Amasino R. & Dean C. (2000) Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* **290,** 344–347.

Keurentjes J.J., Bentsink L., Alonso-Blanco C., Hanhart C.J., Blankestijn-De Vries H., Effgen S., Vreugdenhil D. & Koornneef M. (2007) Development of a near-isogenic line population of *Arabidopsis thaliana* and comparison of mapping power with a recombinant inbred line population. *Genetics* **175,** 891–905.

Kim D.-H., Doyle M.R., Sung S. & Amasino R.M. (2009) Vernalization in plants. *Annual Review of Cell and Developmental Biology* **25,** 277–299.

Kobayashi Y. & Weigel D. (2007) Move on up, it's time for change–mobile signals controlling photoperiod-dependent flowering. *Genes and Development* **21,** 2371–2384.

Konieczny A. & Ausubel F.M. (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *The Plant Journal* **4,** 403–410.

Koornneef M., Blankestijn-de Vries H., Hanhart C.J., Soppe W. & Peeters A.J.M. (1994) The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the *Landsberg erecta* wild-type. *Plant Journal* **6,** 911–919.

Kranz A.R. & Kirchheim B. (1987) *Genetic resources in Arabidopsis* (vol. 24), Frakfurt am Main.

Le Corre V., Roux F. & Reboud X. (2002) DNA polymorphism at the *FRIGIDA* gene in *Arabidopsis thaliana*: extensive nonsynonymous variation is consistent with local selection for flowering time. *Molecular Biology and Evolution* **19,** 1261–1271.

Lee I., Michaels S.D., Masshardt A.S. & Amasino R.M. (1994) The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDENS* is suppressed in the *Landsberg erecta* strain of *Arabidopsis*. *Plant Journal* **6,** 903–909.

Lempe J., Balasubramanian S., Sureshkumar S., Singh A., Schmid M. & Weigel D. (2005) Diversity of flowering responses in wild *Arabidopsis thaliana* strains. *PLoS Genetics* **1,** 109–118.

Li Y., Huang Y., Bergelson J., Nordborg M. & Borevitz J.O. (2010) Association mapping of local climate-sensitive quantitative trait loci in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 21199–21204.

Loudet O., Chaillou S., Camilleri C., Bouchez D. & Daniel-Vedele F. (2002) Bay-0 x Shahdara recombinant inbred line population: a powerful tool for the genetic dissection of complex traits in *Arabidopsis*. *Theoretical and Applied Genetics* **104,** 1173–1184.

Martínez-Zapater J.M., Coupland G., Dean C. & Koornneef M. (1994) The transition to flowering in *Arabidopsis*. In *Arabidopsis* (eds E.M. Meyerowitz & C.R. Somerville), pp. 403–434. Cold Spring Harbor Lab Press, Cold Spring Harbor, NY, USA.

Mendez-Vigo B., de Andres M.T., Ramiro M., Martinez-Zapater J.M. & Alonso-Blanco C. (2010) Temporal analysis of natural variation for the rate of leaf production and its relationship with flowering initiation in *Arabidopsis thaliana*. *Journal of Experimental Botany* **61,** 1611–1623.

Mendez-Vigo B., Pico F.X., Ramiro M., Martinez-Zapater J.M. & Alonso-Blanco C. (2011) Altitudinal and climatic adaptation is mediated by flowering traits and *FRI, FLC*, and *PHYC* genes in *Arabidopsis*. *Plant Physiology* **157,** 1942–1955.

Michaels S.D. & Amasino R.M. (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *The Plant Cell* **11,** 949–956.

Michaels S.D., He Y., Scortecci K.C. & Amasino R.M. (2003) Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 10102–10107.

Montesinos A., Tonsor S.J., Alonso-Blanco C. & Picó F.X. (2009) Demographic and genetic patterns of variation among populations of *Arabidopsis thaliana* from contrasting native environments. *PLoS ONE* **4,** e7213.

Napp-Zinn K. (1969) Arabidopsis thaliana (L.) Heynh. In *The Induction of Flowering: Some Case Histories* (ed. L.T. Evans), pp. 291–304. Macmillan, Melbourne, Vic., Australia.

Nicholas K.B., Nicholas H.B.J. & Deerfield D.W. (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNEW.NEWS*, 4.

Nordborg M., Hu T.T., Ishino Y., *et al*. (2005) The pattern of polymorphism in *Arabidopsis thaliana*. *PLoS Biology* **3,** e196.

Orr H.A. (2005) The genetic theory of adaptation: a brief history. *Nature Reviews. Genetics* **6,** 119–127.

Poduska B., Humphrey T., Redweik A. & Grbić V. (2003) The synergistic activation of *FLOWERING LOCUS C* by *FRIGIDA* and a new flowering gene *AERIAL ROSETTE 1* underlies a novel morphology in *Arabidopsis*. *Genetics* **163,** 1457–1465.

Rédei G. (1970) *Arabidopsis thaliana* (L.) Heynh. A review of the genetics and biology. *Bibliographia Genetica* **2,** 1–151.

Rédei G.P. (1962) Single locus heterosis. *Zeitschrift fur Vererbungslehre* **93,** 164–170.

Roux F., Touzet P., Cuguen J. & Le Corre V. (2006) How to be early flowering: an evolutionary perspective. *Trends in Plant Science* **11,** 375–381.

Salome P.A., Bomblies K., Laitinen R.A., Yant L., Mott R. & Weigel D. (2011) Genetic architecture of flowering-time variation in *Arabidopsis thaliana*. *Genetics* **188,** 421–433.

Schläppi M.R. (2006) *FRIGIDA LIKE 2* is a functional allele in *Landsberg erecta* and compensates for a nonsense allele of *FRIGIDA LIKE 1*. *Plant Physiology* **142,** 1728–1738.

Schmid K.J., Torjek O., Meyer R., Schmuths H., Hoffmann M.H. & Altmann T. (2006) Evidence for a large-scale population structure of *Arabidopsis thaliana* from genome-wide single nucleotide polymorphism markers. *Theoretical and Applied Genetics* **112,** 1104–1114.

Sheldon C.C., Conn A.B., Dennis E.S. & Peacock W.J. (2002) Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *The Plant Cell* **14,** 2527–2537.

Shindo C., Aranzana M.J., Lister C., Baxter C., Nicholls C., Nordborg M. & Dean C. (2005) Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of *Arabidopsis*. *Plant Physiology* **138,** 1163–1173.

Shindo C., Lister C., Crevillen P., Nordborg M. & Dean C. (2006) Variation in the epigenetic silencing of *FLC* contributes to natural variation in *Arabidopsis* vernalization response. *Genes and Development* **20,** 3079–3083.

Snustad D.P., Haas N.A., Kopczak S.D. & Silflow C.D. (1992) The small genome of *Arabidopsis* contains at least nine expressed *β*-tubulin genes. *The Plant Cell* **4,** 549–556.

Stinchcombe J.R., Caicedo A.L., Hopkins R., Mays C., Boyd E.W., Purugganan M.D. & Schmitt J. (2005) Vernalization sensitivity in *Arabidopsis thaliana* (*Brassicaceae*): the effects of latitude and *FLC* variation. *American Journal of Botany* **92,** 1701–1707.

Strange A., Li P., Lister C., Anderson J., Warthmann N., Shindo C., Irwin J., Nordborg M. & Dean C. (2011) Major-effect alleles at relatively few loci underlie distinct vernalization and flowering variation in *Arabidopsis* accessions. *PLoS ONE* **6,** e19949.

Sung S., He Y., Eshoo T.W., Tamada Y., Johnson L., Nakahigashi K., Goto K., Jacobsen S.E. & Amasino R.M. (2006) Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1. *Nature Genetics* **38,** 706–710.

Swiezewski S., Liu F., Magusin A. & Dean C. (2009) Cold-induced silencing by long antisense transcripts of an *Arabidopsis* Polycomb target. *Nature* **462,** 799–802.

Van Ooijen J.W. (2000) *MapQTL Version 4.0: User Friendly Power in QTL Mapping: Addendum to the Manual of Version 3.0*. Plant Research International, Wageningen, the Netherlands.

Van Ooijen J.W. & Voorrips R.E. (2001) *JoinMap® 3.0, Software for the Calculation of Genetic Linkage Maps*. Plant Research International, Wageningen, the Netherlands.

Wang Q., Sajja U., Rosloski S., Humphrey T., Kim M.C., Bomblies K., Weigel D. & Grbic V. (2007) *HUA2* caused natural variation in shoot morphology of *A. thaliana*. *Current Biology* **17,** 1513–1519.

Warthmann N., Fitz J. & Weigel D. (2007) MSQT for choosing SNP assays from multiple DNA alignments. *Bioinformatics* **23,** 2784–2787.

Weigel D. (2012) Natural variation in *Arabidopsis*: from molecular genetics to ecological genomics. *Plant Physiology* **158,** 2–22.

Werner J.D., Borevitz J.O., Uhlenhaut N.H., Ecker J.R., Chory J. & Weigel D. (2005) *FRIGIDA*-independent variation in flowering time of natural *Arabidopsis thaliana* accessions. *Genetics* **170,** 1197–1207.

Wilczek A.M., Burghardt L.T., Cobb A.R., Cooper M.D., Welch S.M. & Schmitt J. (2010) Genetic and physiological bases for phenological responses to current and predicted climates. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **365,** 3129–3147.

Yu J., Pressoir G., Briggs W.H., *et al*. (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nature Genetics* **38,** 203–208.

Zhao K., Aranzana M.J., Kim S., *et al*. (2007) An *Arabidopsis* example of association mapping in structured samples. *PLoS Genetics* **3,** e4.

Received 28 February 2012; received in revised form 2 April 2012; accepted for publication 4 April 2012

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Relationships between flowering related traits in the L*er* × Ll-0 RIL population.
Figure S2. L*er*/Ll-0 linkage map.
Figure S3. Gene expression of *FLC* Iberian alleles in relation to vernalization.
Table S1. Markers used to construct L*er* × Ll-0 genetic map.
Table S2. Genotypes of the L*er* × Ll-0 RILs for 95 markers used to develop the genetic map.
Table S3. Quantitative data used for QTL mapping in the L*er* × Ll-0 RIL population.
Table S4. Oligonucleotides used for sequencing the promoter, 5′-UTR and 3′-UTR regions of *FLC* gene.
Table S5. QTL affecting flowering related traits in relation to vernalization in the L*er* × Ll-0 RIL population.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.