Environmental and genetic interactions reveal *FLC* as a modulator of the natural variation for the plasticity of flowering in Arabidopsis

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ABSTRACT

The timing of flowering initiation depends strongly on the environment, a property termed as the plasticity of flowering. Such plasticity determines the adaptive potential of plants since it provides phenotypic buffer against environmental changes, and its natural variation contributes to evolutionary adaptation. We addressed the genetic mechanisms of the natural variation for this plasticity in *Arabidopsis thaliana* by analysing a population of recombinant inbred lines derived from Don-0 and L*er* accessions collected from distinct climates. QTL mapping in four environmental conditions differing in photoperiod, vernalization treatment and ambient temperature, detected: i) *FLC* as a large effect QTL affecting flowering time differentially in all environments; ii) numerous QTL displaying smaller effects specifically in some conditions; iii) significant genetic interactions between *FLC* and other loci. Hence, the variation for the plasticity of flowering is determined by a combination of environmentally sensitive and specific QTL, and epistasis. Analysis of *FLC* from Don identified a new and more active allele likely caused by a *cis*-regulatory deletion covering the non-coding RNA *COLDAIR*. Further characterization of four *FLC* natural alleles showed different environmental and genetic interactions. Thus, *FLC* appears as a major modulator of the natural variation for the plasticity of flowering to multiple environmental factors.

Keywords:

Arabidopsis, natural variation, *Flowering locus* C (*FLC*), phenotypic plasticity, genotype by environment interaction, epistasis, temperature, vernalization, photoperiod

Introduction

The timing of flowering initiation is a crucial developmental trait of annual plants because it determines the individual reproductive outcome and, ultimately, the survival of most plant species. Accordingly, the transition from vegetative to reproductive phase is regulated by multiple seasonal cues including light components such as photoperiod (Ausin *et al*., 2005; Andrés and Coupland, 2012). In addition, temperature plays an essential regulatory role because prolonged exposures to either low non-freezing temperatures, called vernalization, or to higher ambient temperatures during the growth season, affect flowering time (Kim *et al*., 2009; Verhage *et al*., 2014). Hence, annual plants display substantial phenotypic plasticity for this trait, defined as the differential phenotypic expression of a genotype depending on the environmental conditions (Casal *et al.*, 2004). More than 150 genes affecting flowering initiation and its environmental plasticity have been identified in the model plant *Arabidopsis thaliana*. These genes have revealed a complex regulatory network that includes, among others, the photoperiod, the vernalization and the temperature pathways that promote flowering in response to such environmental signals (Posé *et al*., 2012). Together, these pathways regulate the expression of the so-called flowering integrator genes *FT* and *SOC1* depending on the environment, which will subsequently act on the flower identity genes (Andrés and Coupland, 2012).

Classical garden studies of the natural quantitative variation for flowering time within species distributed across a wide range of climates have shown that this variation is involved in the genetically-based (evolutionary) adaptation of plants to different climates (Stebbins, 1950; Briggs and Walters, 1997; Aspinwall *et al*., 2013; Shrestha *et al*., 2014). This is best illustrated with the wild species *A. thaliana*, whose native range spans Eurasia (Weigel, 2012). The large amount of intraspecific flowering time variation of this plant exhibits significant geographical clines associated with latitude and altitude, as well as climatic clines related with temperature and precipitation

(Stinchcombe *et al*., 2004; Samis *et al*., 2008; Hancock *et al*. 2011; Mendez-Vigo *et al*., 2011; Samis *et al*., 2012; Manzano-Piedras *et al*., 2014). In addition, phenotypic analyses in different environmental conditions have shown that wild genotypes (accessions) differ also in their flowering plasticity, which is detected by significant genotype by environment (GxE) interactions. In particular, the genetic variation for the plasticity in relation to vernalization treatments, measured as vernalization sensitivity or response to vernalization, has been associated with geographical and climatic factors (Lempe *et al*., 2005; Mendez-Vigo *et al*., 2011). This variation for the phenotypic plasticity determines the adaptive potential of plants to climate change through a double contribution: as a buffer against it, and as evolutionary adaptation (Nicotra *et al*., 2010; Anderson *et al*., 2012). Thus, understanding the genetic architecture of the plasticity of flowering in a changing environment has become a relevant question in plant biology (Nicotra *et al*. 2010; Lee *et al*., 2014; El-Soda *et al*., 2014).

Quantitative trait locus (QTL) analyses of flowering time have identified dozens of loci in numerous *A. thaliana* mapping populations derived from crosses among accessions from distant locations (Simon *et al*., 2008; O'Neill *et al*., 2008; Schwartz *et al*., 2009; Mendez-Vigo *et al* 2010; Salomé *et al.*, 2011; Sanchez-Bermejo *et al*., 2012; Li *et al*., 2014). The molecular dissection of these QTL has already found 14 genes contributing to the natural variation for flowering time (reviewed in Mendez-Vigo *et al*., 2013). An even larger number of nucleotide polymorphisms affecting the function of those genes has been also identified because multiple independent mutations, both structural and/or *cis*-regulatory, occur in *FRI*, *FLC*, *HUA2*, *MAF2-MAF5* and *ELF3* (Shindo *et al*. 2005; Wang *et al*. 2007; Caicedo *et al*. 2009; Rosloski *et al*., 2010; Mendez-Vigo *et al*., 2011; Undurraga *et al*., 2012; Li *et al*., 2014, 2015, Schmalenbach *et al*., 2014). The role of some of these functional polymorphisms in climatic adaptation has been supported by their geographic patterns associated with climatic variables (Samis *et al*., 2008; Mendez-Vigo *et al*., 2011; Samis *et al*. 2012; Sanchez-Bermejo *et*

al., 2012; Li *et al*., 2014, 2015). In addition, several of these QTL affect the plasticity of flowering since they show significant QTL by environment (QTLxE) interactions in relation to specific environmental factors (Balasubramanian *et al*., 2006; Schwartz *et al*., 2009; Sanchez-Bermejo *et al*., 2012). Furthermore, field experiments in different climatic regions have shown that flowering QTL often display pleiotropic indirect effects on fitness traits (Dittmar *et al*., 2014). Environmental conditions affect not only the direct QTL effects on flowering time but also QTL indirect effects and direction on fitness traits (Fournier-Level *et al*., 2013). Several genes have been demonstrated to contribute to the natural variation for the plasticity of flowering in relation to photoperiod and temperature. However, these QTL and genes have been mostly analysed in independent studies, using different experimental populations, and addressing plasticity to single environmental factors. Consequently, the general genetic mechanisms underlying flowering plasticity remain largely unknown (Nicotra *et al*., 2010; El-Soda *et al*., 2014).

In this study we have addressed systematically the genetic bases of the natural variation for the plasticity of flowering time in *A. thaliana* with respect to the relevant climatic factors, light and temperature. To this end, we selected the accessions Don-0 and L*er* collected from the Southern and Northern limits of the species range in Europe, respectively, and representing contrasted Mediterranean and Atlantic climates. QTL mapping for flowering time was carried out in a Don-0/L*er* RIL population grown under four environmental conditions differing in photoperiod, vernalization treatment and growth ambient temperature, to estimate: i) the relative contribution of environment-specific loci (showing effect only in one particular environmental condition) and environment-sensitive loci (showing different quantitative effect depending on environment); ii) the contribution of genetic interactions (QTL by QTL) to the plasticity of flowering. In addition, we characterized the effect of several natural alleles of the

main gene detected in these analyses, *FLC*, on the plasticity of flowering to multiple environmental signals.

Materials and methods

Plant materials

The wild accession Don-0, collected in Southern Spain (Picó *et al*., 2008) and the laboratory strain Landsberg *erecta* (L*er*) from Poland (Alcazar *et al*., 2014) were used as parental lines to develop a population of 377 RILs. This population was produced by single seed descent from a F_1(L*er* x Don-0) plant obtained using Don-0 as the male parent (198 lines) and a F_1(Don-0 x L*er*) obtained using Don-0 as mother (179 lines). A single F_6 plant from each RIL was genotyped and seeds from 10 F_9 plants per line were bulked to obtain the final F_{10} generation used for phenotypic analyses. The population is available through the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). The collection of Iberian accessions used for *FLC*-Don screen has been previously described (Mendez-Vigo *et al*. 2011). Don and Bon local populations analysed in this study were originally sampled in 2002 and 2008 respectively (Picó *et al*., 2008). Both locations were resampled by collecting nine and six individuals in 2008 and 2009, respectively.

The *flc*-Don mutant in Don-0 genetic background was obtained by γ-radiation (400 Gy) and selection for early flowering in a population of 5000 M_2 plants. *flc*-Don was genotyped with 343 genome-wide SNPs to discard that it derived from seed contamination. *FLC* sequencing identified a transposable element insertion in intron five. Co-segregation of flowering time and *flc*-Don mutation in an F_2(Don-0x*flc*-Don) confirmed *flc*-Don early phenotype. The *flc-3* mutant in Col background (Michaels and Amasino, 1999) was kindly provided by Dr R. M. Amasino (Wisconsin University, USA).

Three pairs of near isogenic lines (NILs) were obtained for *FPL4* locus from heterogeneous inbred families (HIFs). Briefly, F_6 RILs LD-63, DL-311 and DL-331 were

154 selected as heterozygous for a single genomic region overlapping with *FPL4*, in an
155 otherwise homozygous but heterogeneous L*er*/Don-0 genetic background. HIFs
156 derived by selfing of the RILs were genotyped with six PCR markers to select
157 homozygous L*er* and Don-0 plants at *FPL4* region from each family. Pairs of F_8 plants
158 differing in *FPL4* alleles and referred to as HIF-63, HIF-311 and HIF-331 were used for
159 phenotypic analyses.

160 Two introgression lines (ILs) carrying *FPL4*-Don-0 or *FLC*-Don-0 alleles, in a
161 L*er* genetic background, were obtained from RILs LD-60 and DL-266 by recurrent
162 backcrossing and marker assisted selection during three generations. Final ILs
163 homozygous for a single introgression fragment were thoroughly genotyped with the
164 SNP set used to obtain the L*er*/Don-0 genetic map. An IL carrying *FLC* genomic region
165 from Ri-0 accession (*FLC*-Ri-0) in L*er* background was obtained after five backcrosses,
166 following Mendez-Vigo et al*.* (2013). This accession was selected because sequencing
167 and genetic analysis in segregating populations showed that Ri-0 carries a different
168 functional active allele of *FLC*. ILs *FLC*-Col and *FRI*-Sf2 in L*er* background and IL *FRI*-
169 Sf2 in Col background have been previously described (Koornneef *et al.*, 1994; Lee *et*
170 *al*., 1994). *FLC*-Ri-0 and *FLC*-Col alleles were combined with *FRI*-Sf2 by marker
171 assisted selection in F_2 populations.

172

173 **Growth conditions and measurements of flowering related traits**

174 Plants were grown as previously described (Sanchez-Bermejo *et al*. 2012) using a
175 single growth chamber with temperature and photoperiod control. The following
176 environmental conditions were used: 21 °C and long-day (LD) photoperiod (16 h light: 8
177 h darkness) (21LD); 21 °C and LD photoperiod after 4-12 weeks of vernalization
178 treatment (VLD); 21 °C and short-day (SD) photoperiod (8 h light: 16 h darkness)
179 (21SD); and 28 °C and LD photoperiod (28LD). For the vernalization treatment, pots

with one week old seedlings were transferred to a cold room at 4 °C and SD photoperiod.

For evaluation of the RIL population in each environment, all lines and parents were grown in a single experiment organized in a two-complete-blocks design. For evaluation of NILs and ILs, all lines were grown in an experiment organized in three-complete-blocks. Six plants per line were grown in one pot in each block.

For analyses of RNA expression, all genotypes were grown in an experiment organized in three-complete-blocks. Fifty to hundred seeds per genotype were directly sown on soil in a single pot per block. After 4 days of stratification at 4°C, pots were placed in a growth chamber at 21 °C and LD (for the 21LD and 28LD treatment) or SD photoperiod (for the VLD and 21SD treatments). After germination, pots of the 28LD treatment were transferred to 28 °C, whereas pots of VLD were placed in a chamber at 4°C and SD for one month and subsequently moved to a growth chamber at 21 °C and LD.

A total of 12 traits were quantified as follow. Flowering initiation was estimated as flowering time (FT) and total leaf number (LN). FT was measured as days to flowering from the seedling planting date (FT21LD, FT21SD and FT28LD for the corresponding environments) or from the end of the vernalization treatment (FTVLD). LN (LN21LD, LN21SD and LNVLD for the different environments) was the sum of rosette and cauline leaves. In 28LD environment, Don-0 and numerous RILs showed reduced survival and abnormal leaf growth caused by the temperature sensitive gene *ICARUS* (Zhu *et al*., 2015). Consequently, LN could not be determined in these conditions and only 254 out of 377 RILs survived until flowering. The plasticity to photoperiod, vernalization treatment, or ambient temperature for FT (named as FTPP, FTVP, and FTTP, respectively) or for LN (LNPP and LNVP) was estimated as the flowering sensitivity described by Lempe *et al*., (2005). Briefly, FTPP, FTVP and FTTP

were calculated as [log(FT21LD)-log(FT21SD or FTVLD or FT28LD)]/[log(mean FT21LD)-log(mean FT21SD or mean FTVLD or mean FT28LD)] with FT21LD, FT21SD, FTVLD and FT28LD being the mean values of each genotype, and mean FT21LD, mean FT21SD, mean FTVLD and mean FT28LD corresponding to the means of all RILs or parental lines. Similarly, LNPP and LNVP were calculated as [log(LN21LD)-log(LN21SD or LNVLD)]/[log(mean LN21LD)-log(mean LN21SD or LNVLD)].

Genotyping and genetic map construction

DNA was isolated as previously described (Sanchez-Bermejo *et al*. 2012). RILs and ILs were genotyped for a genome-wide set of 343 SNP loci previously selected (Brennan *et al*., 2014) using the SNPlex and Veracode methods through the CEGEN genotyping service (www.cegen.org). A total of 106 SNPs were polymorphic between Don-0 and L*er*, showing an average of 6.8% missing data. Twelve indel and CAPS markers previously described (Mendez-Vigo *et al*. 2010; Sanchez-Bermejo *et al*., 2012) were also included to fill gaps in the genetic map. RIL genotypic data are given in Supporting Information Table S1.

Don-0/L*er* linkage map was constructed using JOINMAP 3.0 software package (Van Ooijen and Voorrips, 2001) with the RI6 mapping population type. 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.

QTL analyses

QTL mapping was carried out separately for each trait using mean RIL values that in the case of FT traits were previously log-transformed to improve the assumptions of the analyses. QTL were located by the multiple-QTL-model method (MQM) implemented in MapQTL v. 4.0 software (Van Ooijen, 2000). QTL were detected using LOD thresholds of 2.5 corresponding to a genome-wide significance α=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. 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 ANOVA using the markers linked to detected QTL. The percentage of variance explained by significant interactions was estimated by ANOVA type III variance components analysis. The total variances explained for each trait by additive plus interaction effects was estimated from general linear models including all significant effects from the detected QTL.

Broad sense heritabilities (h^2_b) were estimated as the variance component among RILs derived from type III ANOVAs. Genotype by environment interactions were tested by two-factor ANOVA using genotypes (RILs) and environments as classifying factors. QTL by environment interactions were tested by general linear models with repeated measurements using trait values in the different environments as the within-subject variables and the QTL marker as the between-subject factor. Statistical tests were performed with SPSS v. 21 and Statistica v. 8 packages.

FLC sequencing and expression analyses

Nine kb of the *FLC* gene from Don-0 and Ri-0 were sequenced using the primers and methods described in Sanchez-Bermejo *et al.*, (2012). This included the complete

coding region, and 2.6 and 0.8 kb fragments located upstream and downstream of the start and stop codons. GenBank accession numbers of DNA sequences generated in this work are KR816688- KR816689.

For analyses of *FLC* expression, seedlings were harvested at the same stage of vegetative development (4-5 leaves) for all treatments. This corresponded to 14-18 day-old seedlings for 21LD, 28LD and 21SD, and to five days after the vernalization treatment. Total RNA was extracted using TRIzol reagent according to manufacturer's protocol (Invitrogen). Potential DNA contamination was removed by DNAse digestion and subsequent RNA purification was carried out using high pure RNA isolation kit (Roche). cDNA was synthesized from 3 micrograms of total RNA using AMV reverse transcriptase (Invitrogen) and dT15 oligonucleotides.

FLC expression was estimated by quantitative RT-PCR using *FLC* oligonucleotides previously described (Helliwell *et al*., 2011) and the *PP2A* gene as endogenous control (Czechowski *et al*., 2005). Both genes were amplified with Power SYBR green mix in a 7300 real time PCR system (Applied Biosystem) and quantified using the standard curve method. All values were relativized with respect to L*er* value in 21LD conditions. Mean and standard errors were derived from three technical replicates.

Results

Plasticity of flowering in Don-0, L*er* and Col grown under multiple environments

To characterize the plasticity of flowering initiation *in A. thaliana*, we selected Don-0 accession and the reference strains L*er* and Col. The three genotypes were grown in nine conditions differing in three environmental factors that are well known to affect flowering: photoperiod (LD and SD), growth ambient temperature (15, 21 and 28 °C) and the length of the vernalization treatment at 4 °C (Figure 1). As expected, the three genotypes showed substantial plasticity for flowering initiation measured as flowering time or leaf number (Table 1). However, the highly significant genotype by environment interactions observed for both traits ($P<10^{-7}$) revealed considerable genetic variation for the plasticity of flowering to the three environmental factors. Overall, Don-0 showed lower plasticity to photoperiod than the reference strains, especially for leaf number, which was very similar in LD and SD (Figure 1). By contrast, Don-0 was more plastic to the vernalization treatment, since it produced more leaves than Col and L*er* without vernalization but fewer after 12 weeks of vernalization treatment. In addition, Don-0 responded differentially to ambient temperature, since L*er* and Col flowered earlier at 28 than at 21 °C, while Don-0 delayed flowering at 28 °C (Figure 1).

Plasticity of flowering in the Don-0/L*er* RIL population

To address the genetic bases of the variation for the plasticity of flowering we developed a RIL population of 377 lines derived from Don-0 and L*er* accessions. Analysis of the RILs in four conditions differing in photoperiod, vernalization treatment and ambient temperature showed substantial genetic variation for FT and LN traits in all conditions, as well as for their plasticities (Figure 2, Table 1). Although SD photoperiod and vernalization delayed and accelerated flowering initiation of most of the RIL population respectively, large amount of quantitative variation was observed

among RILs for the plasticity to both environmental factors. By contrast, 28 °C ambient temperature delayed and accelerated flowering in about half of the RIL population compared to 21 °C. This is shown by the negative and positive values estimated for the plasticity to temperature in RILs, in agreement with the behavior of Don-0 and L*er* parents. The bimodal frequency distributions displayed by several flowering traits and plasticities (Figure 2, Supporting Information Figure S1) suggest the role of a major effect locus. In addition, significant transgression was observed for some flowering traits, especially under the SD photoperiod (Figure 2), which indicates the contribution of other loci with opposite effects than the behavior of parental lines.

Correlation analyses of FT and LN between different environments show high to moderate values ($0.91>r>0.56$;$P<0.001$) indicating the presence of a substantial common genetic basis (Supporting Information Table S2). The signs of correlation coefficients between all flowering traits and plasticities indicate that late flowering plants respond more to vernalization, less to photoperiod and delay flowering at 28 °C. Nevertheless, plasticities to photoperiod and vernalization were highly correlated ($-0.90>r>-0.78$;$P<0.001$), while lower correlations were estimated with the plasticity to ambient temperature ($0.40>r>0.25$;$P<0.001$). Therefore, some genetic components will likely show differential effects at 28 °C than in the rest of environments.

Genetic map of Don-0/L*er* RIL population

A genetic map including 118 markers evenly distributed at an average distance of 3.3 cM was developed. The total map length was 381 cM and the largest genetic interval between adjacent markers reached 12.6 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 two pairs of markers (Supporting Information Figure S2A). The recombination rate was homogeneously distributed along most length of the five chromosomes (average

of 300 kb per cM) and it showed lower values only in some pericentromeric regions. The 377 RILs provided a total of 2670 recombination events on this genetic map, with an average of 7.1 breakpoints per line. As expected for the F_6 generation, heterozygosity of most markers was below 3%, and only markers in two genomic regions presented a higher-than the expected value of 6.5%. Twenty-six markers distributed in multiple regions of all five chromosomes showed distortion from the expected 1:1 segregation of homozygous genotypes (Supporting Information Figure S2B). Thus, this population showed a slightly lower recombination rate and higher segregation distortion than other RIL populations with L*er* reference strain (Mendez-Vigo *et al*., 2010; Sanchez-Bermejo *et al*., 2012). However, the lower mapping power derived from both aspects was compensated by the large population size.

QTL analyses of flowering traits and plasticities in the Don-0/L*er* RIL population

QTL mapping for FT and LN in the four environments identified a total of 10 genomic regions whose additive effects accounted for 52.3-84.2% of the phenotypic variance (Figure 3, Supporting Information Table S3). However, two regions on chromosome 2 and bottom of chromosome 5 likely contain two linked QTLs, as suggested by the small overlapping between confidence intervals and the opposite effects of QTL mapped for the same trait under different environments. Most QTL displayed small effect since only four genomic regions explained more than 5% of the variance. Two of the large effect loci corresponded to *FRI* and *FLC* genes located on top of chromosomes 4 and 5 because L*er*, but not Don-0 parental, carries early loss-of-function alleles of both genes (Shindo *et al*., 2005; Michaels *et al*. 2003; Mendez-Vigo *et al*. 2011). Additive allele effects in these two genomic regions were in agreement with the parental behavior, *FLC* accounting for the bimodal distributions displayed by numerous traits. In addition,

several small effect loci with opposite effects were detected for most traits, which explained the observed phenotypic transgressions.

To identify the loci that determine the genetic variation for the plasticity of FT and LN we first carried out comparative QTL analyses among the four environments (Figure 3). Three to seven QTL were detected for each trait in each condition, seven of the genomic regions showing significant QTLxE interactions (Supporting Information Table S3). These results indicated that a large fraction of QTL displayed differential effects depending on environment and contributed to the genetic variation for the plasticity of flowering. In particular, the effect of the *FLC* locus differed strongly depending on the three environmental factors, despite it was the only QTL detected for all traits and conditions (Figure 4). Overall, the late flowering of *FLC*-Don-0 allele was largely reduced by vernalization, but it was increased by 28 °C compared to 21 °C ambient temperature, and by SD photoperiod with respect to LD. On the contrary, *FLC*-L*er* RILs responded weakly to both temperature components, but strongly to photoperiod, especially for LN (Supporting Information Figure 3S). In addition, two-way genetic interactions were detected between *FLC* and other QTL in four genomic regions of chromosomes 1, 2 and 4. These epistatic effects increased the total explained phenotypic variance between 5 to 12% (Supporting Information Table S3). Specially, *FLC* and *FRI* showed the strongest interactions, *FLC*-Don-0 allele behaving as completely insensitive to *FRI* allele under 21LD conditions (Figure 4A; Supporting Information Table S3). By contrast, under SD photoperiod or at 28 °C, *FRI* showed opposite effect in lines carrying *FLC*-Don-0 alleles than in *FLC*-L*er* RILs, thus explaining the undetectability of *FRI* additive effects at 28 °C by standard QTL mapping. Similarly, *FLC* interactions with the other three QTL depended on the environment (Figure 4 and Supporting Information Figure S3) and were in agreement with the behavior of L*er* and Don-0 parental lines.

379 The genetic variation for the plasticity of flowering was further analysed by
380 carrying out QTL mapping with the five plasticity variables (Figure 3). Three to four QTL
381 were detected for each variable. In total, these loci identified seven genomic regions
382 accounting for the plasticity variation, in accordance with the above comparative
383 analyses. *FLC* affected the plasticity of flowering to the three environmental factors
384 (Figure 3). In addition, *FRI* affected the plasticity to vernalization and ambient
385 temperature, whereas the remaining genomic regions contributed to the plasticity only
386 with respect to a single environmental factor. Interestingly, *FRI* showed opposite effect
387 than *FLC* only on the plasticity to ambient temperature, in agreement with the distinct
388 *FRI*/*FLC* interaction patterns observed for FT at 21 and 28 °C (Figure 4A). Finally,
389 analysis of two-way genetic interactions showed that *FLC* interacts significantly with
390 *FRI* and with the chromosome 2 locus for some plasticity traits (Supporting Information
391 Table S3).

392

393 **Validation and characterization of *FPL4* locus**

394 The genomic region located in the middle of chromosome 4 was named as *F*lowering
395 *Pl*asticity 4 (*FPL4*) because it showed moderate to large effects on FT and LN in
396 several conditions, as well as on the plasticity to photoperiod. To characterize this
397 locus we developed the introgression line IL-*FPL4*-Don-0 in L*er* genetic background,
398 and three pairs of NILs derived from HIFs. Phenotypic analyses in four environmental
399 conditions (Figure 5) showed that all pairs of NILs differ in FT and/or LN in SD
400 photoperiod. In addition, *FPL4* displayed a smaller effect in the remaining conditions
401 but only in some pairs of NILs. Mapping the introgression segments of these lines
402 located *FPL4* in the genomic interval 9.5-10.6 Mb (data not shown), which does not
403 overlap with the *PHYD* gene previously identified as contributing to flowering natural

variation (Aukerman *et al*., 1994). Thus, *FPL4* appears as a new QTL affecting mainly the plasticity of flowering to photoperiod.

Effects of different natural *FLC* and *FRI* alleles on the plasticity of flowering

To verify that Don-0 accession carried an active *FLC* allele, we first sequenced its coding and promoter regions. Comparison with L*er FLC* sequence showed that Don-0 differs in 20 silent SNPs and 13 indels in non-coding regions (Figure 6A). In particular, Don-0 was characterized by a large deletion of 2.2 kb located in the first intron, which spans the promoter and the full sequence encoding the long non-coding RNA known as COLDAIR (Heo and Sung, 2011). Second, we isolated a γ-radiation mutant of *FLC* in Don-0 background (*flc*-Don). This mutant showed early flowering and very reduced *FLC* expression, indicating that *FLC*-Don-0 allele is functional as flowering repressor (Figure 6D). Genotyping of the 2.2 kb deletion of *FLC*-Don-0 in a collection of 182 Iberian populations (Mendez-Vigo *et al*., 2011) showed that this allele is only present in Don and Bon populations located 10 km apart. This deletion was present in all individuals analysed from Bon and Don populations, which were sampled two times over six years. Thus, *FLC*-Don-0 allele shows very low frequency at regional scale, but it is fixed and is temporally stable at local scale.

To validate the specific effects of *FLC*-Don-0 on the plasticity of flowering we generated an introgression line (IL) of this allele in L*er* genetic background, and compared this with L*er* ILs carrying *FLC* active alleles from Col and Ri-0, or *FLC*-L*er* inactive alleles (Figure 6B). All four *FLC* alleles did not differ in protein structural mutations indicating that functional differences are determined by *cis*-regulatory polymorphisms. Since L*er* carries *FRI* inactive alleles, we also developed ILs of *FLC*-L*er*, *FLC*-Col and *FLC*-Ri-0 in the same L*er* background but with active *FRI*-Sf2 alleles (Figure 6C). Flowering and *FLC* expression analyses in four environmental conditions

showed that flowering time strongly correlated with *FLC* expression (Figure 6F). However, lines differed largely in their behaviours, since in the absence of *FRI* active alleles, *FLC*-Col and *FLC*-Ri-0 ILs flowered slightly later and showed three to 30-fold higher *FLC* expression than L*er* (Figure 6B). As expected, both *FLC*-Col and *FLC*-Ri-0 alleles interacted synergistically with *FRI*, because they flowered much later and showed up to 30-fold higher expression in the presence of *FRI*-Sf2 alleles. By contrast, *FLC*-Don-0 IL, in the absence of *FRI* active allele, displayed comparable flowering times and *FLC* expression levels than *FLC*-Col and *FLC*-Ri-0 in combination with *FRI*-Sf2 alleles. Therefore, *FLC*-Don-0 appeared as a distinct gain-of-function allele that behaves like a *FRI* activated allele.

Analyses of plasticities showed that vernalization treatments accelerated flowering and reduced *FLC* expression in all genotypes but L*er*. The level of *FLC* expression at 21 °C strongly correlated with the plasticity of flowering to vernalization (Figure 6G). In particular, *FLC*-Don-0 IL, in the absence of *FRI* active alleles, showed similar vernalization response than lines with other *FLC* active alleles in the presence of *FRI*-Sf2 (Figure 6B and 6C). On the contrary, the sign of the plasticity of flowering to ambient temperature differed among ILs (Figure 6G and slopes in Figures 6B and 6C). Lines with low *FLC* expression (*FLC*-L*er*; *FLC*-Col or *FLC*-Ri-0 in the presence of *FRI*-L*er*) flowered earlier at 28 than 21 °C (positive plasticity), whereas lines with high *FLC* expression (*FLC*-Don-0; *FLC*-Col or *FLC*-Ri-0 with *FRI*-Sf2) flowered later at high ambient temperature (negative plasticity). Overall, *FLC* expression was not affected significantly by ambient temperature, but the basal level of *FLC* expression at 21 °C correlated with the sign of the plasticity to temperature (Figure 6G). This behaviour was also observed in Col genetic background (Figure 6E) since Col *FLC*-Col lines, with and without *FRI*-Sf2, displayed negative and positive flowering plasticities respectively. In addition, genotypes also differed in the plasticity to photoperiod. As observed for ambient temperature, *FLC* expression was not affected significantly by photoperiod in

most genotypes, but *FLC* expression at 21 °C negatively correlated with the plasticity to photoperiod (Figure 6G). All lines flowered later in SD than LD conditions, but those with low *FLC* expression displayed higher plasticity to photoperiod than lines with high *FLC* level. These results show that distinct functional alleles of *FLC* affect differentially the plasticity of flowering, and that *FLC*-Don-0 responds to environmental factors like a *FRI* activated *FLC* allele.

Discussion

Interactions among environmentally sensitive and specific loci account for the genetic variation for the plasticity of flowering

As shown by accessions L*er* and Don-0 originating from Northern and Southern Europe, *A. thaliana* shows substantial natural variation for the plasticity of flowering initiation to photoperiod, vernalization treatment and growth ambient temperature. In particular, both accessions display opposite behavior when grown at high ambient temperature, L*er* flowering earlier and Don-0 later, than at standard 21 °C. This is agreement with the delay of flowering observed in some other *A. thaliana* accessions (Balasubramanian *et al*., 2006) but contrasts with the similar behavior of the laboratory strains used for genetic and molecular analyses of temperature responses (Verhage *et al*., 2014). In addition, this intraspecific plasticity variation is in accordance with the reported divergence among flowering responses to warming changes, since many plant species accelerate flowering, but some do not respond or show delayed flowering (Cook *et al*., 2012).

Our quantitative genetic study of a large RIL population analysed in four controlled environmental conditions shows that the plasticity of flowering in *A. thaliana* is determined by a combination of two genetic mechanisms involving a large number of QTL. First, environmentally specific and sensitive loci account for this plasticity. Only the *FLC* region shows large flowering effects, although quantitatively different, in all environmental conditions, hence indicating that *FLC* alleles are environment-sensitive. By contrast, most QTL display smaller flowering effects, and mainly in some but not all conditions, indicating that these loci have certain environmental specificity. This is best illustrated with the characterization of *FPL4*, which appears as a new flowering QTL acting mostly under SD photoperiod. The rest of loci likely correspond to new alleles of previously isolated QTL since they overlapped with well-known flowering genes (Figure

3). Second, analysis of genetic interactions shows that epistasis is a major component contributing to the genetic variation for the plasticity of flowering, *FLC* interacting with several QTL. Genetic interactions also depend on the environment, as illustrated with the *FRI*x*FLC* interaction leading to an opposite *FRI* effect depending on *FLC* allele in some but not all conditions.

Overall, *A. thaliana* variation for the plasticity of flowering is mainly determined by multiple environment-specific loci that interact with the *FLC* gene. Accordingly, *FLC* appears as a central modulator of plasticity whose sensitivity to the environment involves genetic interactions with environment-specific loci. In agreement with this result, *FLC* has been previously shown to encode a MADS transcription factor that integrates information from the vernalization, photoperiod or temperature pathways and that interacts with genes like *FRI*, *CRY2*, *FLM*, *FT* or *SVP* affecting the natural variation for flowering initiation (El-Assal *et al*., 2003; Scortecci *et al*., 2003; Li *et al*., 2008; Huang *et al*., 2013). Interestingly, another flowering integrator with large effect, *FT*, has been recently found in a related species, *Boechera stricta*, contributing to the natural variation for the plasticity of flowering through genetic interactions with the genomic background (Lee *et al*., 2014). Therefore, several flowering integrator genes, also referred to as network hubs, probably share similar genetic mechanisms to account for the flowering plasticity variation in different plant species. Thus, multi-environment studies of large segregating populations are now revealing the genetic mechanisms underlying adaptive quantitative variation in a changing environment.

Natural *cis*-regulatory polymorphisms at *FLC* modulate differentially the plasticity of flowering to multiple environmental factors

Numerous natural *FLC* alleles have been previously described in *A. thaliana*, which are mostly differentiated by *cis*-regulatory polymorphisms with different effects on gene function (reviewed in Alonso-Blanco and Mendez-Vigo, 2014). These include strong loss-of-function alleles caused by transposon intronic insertions, such as the L*er* allele (Michaels *et al*., 2003), as well as alleles showing reduced or increased *FLC* response to vernalization produced by SNPs or deletions in the 5' *FLC* nucleation region (Sanchez-Bermejo *et al*., 2012; Li *et al*., 2014), or by a SNP affecting the splicing of the long non-coding antisense RNA *COOLAIR* (Li *et al*., 2015). The study of Don-0 accession has identified a novel *FLC* allele charactericed by a deletion of the complete genomic region encoding the long non-coding RNA *COLDAIR*, which has been hypothesized to participate in the regulation of *FLC* silencing (Heo and Sung, 2011). Expression and phenotypic analysis of introgression lines indicates that *FLC*-Don-0 is a gain-of-function allele displaying a high basal expression that is likely caused by this or other closely linked *cis*-regulatory polymorphism.

The comparative analysis of four *FLC* alleles provides relevant information on several functional aspects of *FLC*. First, in contrast to previous studies (Heo and Sung, 2011; Helliwell *et al*., 2011), our results indicate that the sense non-coding RNA *COLDAIR* is not essential for the *FLC* repression mediated by vernalization, since *FLC* expression was strongly reduced by low-temperature treatment in Don-0 and *FLC*-Don introgression line. Second, the behavior of *FLC*-Don-0 as a *FRI* activated *FLC* allele, suggests that *FRI* and the *COLDAIR* genomic region share a common mechanism to regulate *FLC* expression. In agreement with the *COLDAIR* deletion and the phenotypes of *FLC*-Don-0 lines, it has been shown that reduction of *COLDAIR* RNA expression leads to increased *FLC* expression and late flowering in the absence of vernalization (Heo and Sung, 2011; Hu *et al*., 2014). In addition, *FRI* degradation during vernalization is accompanied with increased levels of *COLDAIR* (Hu *et al*., 2014) although the functional links between *FRI* and the various long non-codding RNAs of

FLC remain unknown (Hu *et al*., 2014; Li *et al*., 2015). Third, *FLC* not only affects the flowering response to vernalization but also the plasticity to ambient temperature and photoperiod. In agreement with previous studies (Balasubramanian *et al*., 2006) *FLC* strongly determines the response to high ambient temperature, since genotypes with low or high basal *FLC* expression show positive or negative flowering response to high ambient temperature, respectively. However, in contrast to the vernalization treatment, these environmental cues do not affect *FLC* expression, suggesting that the level of *FLC* protein negatively regulates the flowering promotive effects of high ambient temperature and long-day photoperiod. Correspondingly, it has been shown that *FLC* binds to the promoter of the photoperiod sensitive gene *FT* and that it interacts at the protein level with SVP, another MADS transcription factor involved in the regulation of flowering by ambient temperature and photoperiod (Li *et al*., 2008; Mendez-Vigo *et al*., 2013). Therefore, *FLC* affects the plasticity of flowering to multiple environmental factors through its epigenetic transcriptional regulation and through its role as transcriptional regulatory protein.

Currently, *FLC* is likely to be the plant gene with the largest functional diversity described in nature (Alonso-Blanco and Mendez-Vigo, 2014). In addition, nucleotide *cis*-regulatory polymorphisms in *FLC* often display restricted geographical and climatic patterns, suggesting that *FLC* natural variation is involved in precise climatic adaptation (Mendez-Vigo *et al*., 2011; Sanchez-Bermejo *et al* 2012; Li *et al*., 2014, 2015). The local and temporally stable distribution of *FLC*-Don-0 allele in the southern limit of the species also supports that this allele might be involved in local adaptation. Even though, we do not know the environmental factors that could maintain this variation, and if selection might act on flowering or other traits also affected by *FLC*, such as vegetative growth or germination (Chiang *et al*., 2009; Mendez-Vigo *et al*., 2010). Our results reveal that *FLC* displays a complex function in the regulation of flowering as a major plasticity modulator that depends on multiple environmental factors, and that

interacts with the genetic background. We have shown that different natural *FLC* alleles present distinct environmental sensitivities and different genetic interactions. However, it remains unknown if all effects of natural *FLC* alleles on the plasticity of flowering are adaptive, and/or if the dependence of *FLC* effects on environment and background contribute to maintain the large amount of functional variation at *FLC*. Further studies addressing the broad effects of *FLC* on the plasticity of flowering, beyond its known function on the regulation of flowering by vernalization, will shed light on the ecological and evolutionary relevance of *FLC* natural variation.

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Tables

Table1. Flowering traits and plasticities in L*er*, Don-0 and the RIL population.

Values are mean ± SE of 10-20 plants. FTVLD, LNVLD, FTVP and LNVP were estimated after 12 weeks of vernalization treatment. ND: non-determined. h^2_b: broad sense heritability. FT21LD, FT21SD, FTVLD and FT28LD: flowering times in the four environmental conditions; LN21LD, LN21SD, and LNVLD: total leaf numbers in the same environments; FTPP, LNPP, FTVP, LNVP and FTTP: FT or LN plasticities of FT or LN to photoperiod, vernalization or ambient growth temperature, respectively.

[1]: Lines grown in the same experiment than the Don-0/L*er* RIL population.

[2]: Positive values of the plasticity of flowering indicate acceleration by vernalization (FTVP and LNVP) or high ambient temperature (FTTP), but delay by SD photoperiod (FTPP and LNPP).

Trait	L*er*	Don-0	L*er*[1]	Don-0[1]	RIL mean	Min-max RIL	h^2_b
FT21LD	28.9±0.2	55.4±0.8	33.0±0.6	63.4±1.5	57.4±27.8	22–150	0.91
LN21LD	11.1±0.3	35.7±1.9	12.6±0.3	43.7±1.0	36.7±23.5	7–108	0.93
FT21SD	57.9±0.7	70.2±1.2	86.2±0.9	102.7±2.2	107.9±34.0	56–215	0.84
LN21SD	39.1±0.9	37.0±1.7	44.2±1.0	57.6±1.2	51.4±17.7	15–92	0.85
FTVLD	20.2±0.2	18.4±1.2	23.4±0.5	23.6±0.4	24.9±3.8	17–39	0.69
LNVLD	13.7±0.2	9.8±0.5	13.4±0.3	10.7±0.2	13.4±2.3	9–21	0.69
FT28LD	24.2±0.3	68.4±2.4	26.1±0.5	76.0±2.5	65.3±40.2	19–150	0.88
FTPP[2]	1.66	0.57	1.57	0.79	1.11±0.45	0–2	ND
LNPP[2]	2.59	0.07	3.72	0.82	1.56±1.39	-2–5	ND
FTVP[2]	0.58	1.33	0.42	1.19	0.87±0.50	0–2	ND
LNVP[2]	0.05	1.61	-0.06	1.4	0.76±0.66	-1–2	ND
FTTP[2]	4.66	-0.5	1.77	-0.87	0.74±2.39	-5–7	ND

Figure Legends

Figure 1. Plasticity of flowering traits in *A. thaliana*. A) Flowering time and B) Total leaf number of Don-0, L*er* and Col accessions grown in conditions differing in photoperiod (upper panel), vernalization treatment (middle panel) or growth temperature (lower panel). Each panel shows mean±SE of 10-20 plants per genotype.

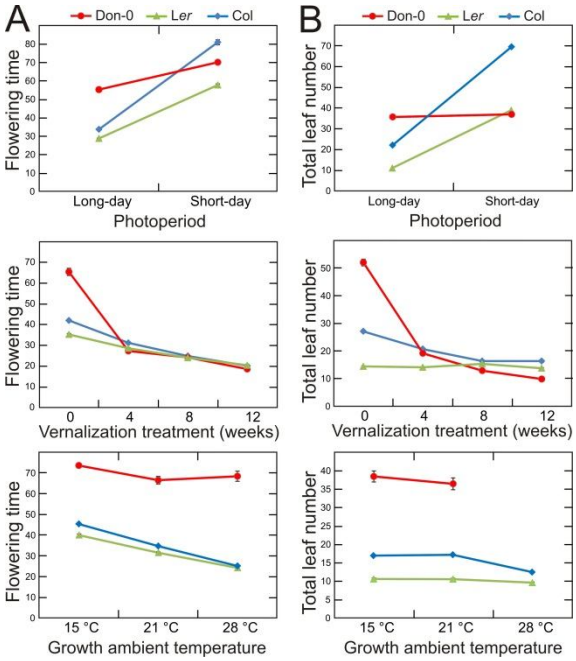


Figure 2. Frequency distributions of flowering time and plasticity in the Don-0/L*er* RIL population. A) Flowering time in four environmental conditions shown in the upper right corner. B) Plasticity of flowering time to photoperiod, vernalization treatment and growth ambient temperature. Arrows and 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. Vernalization treatment was 12 weeks at 4 °C.

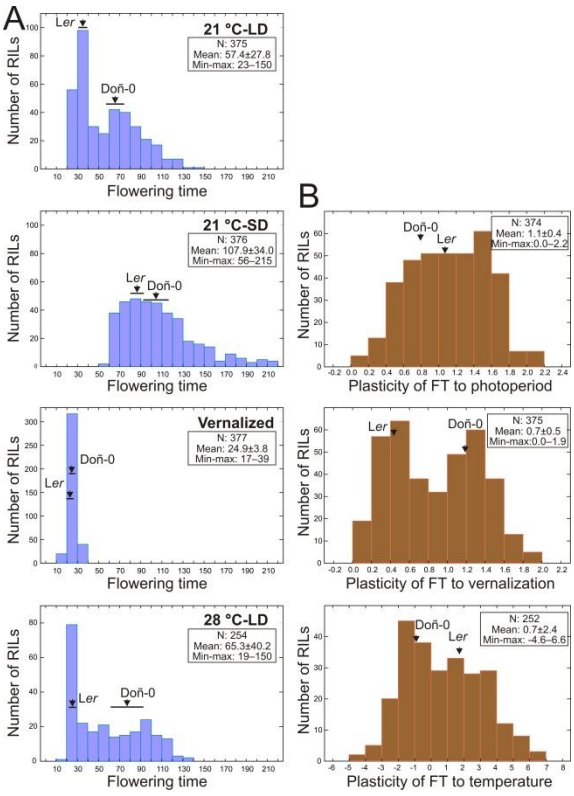
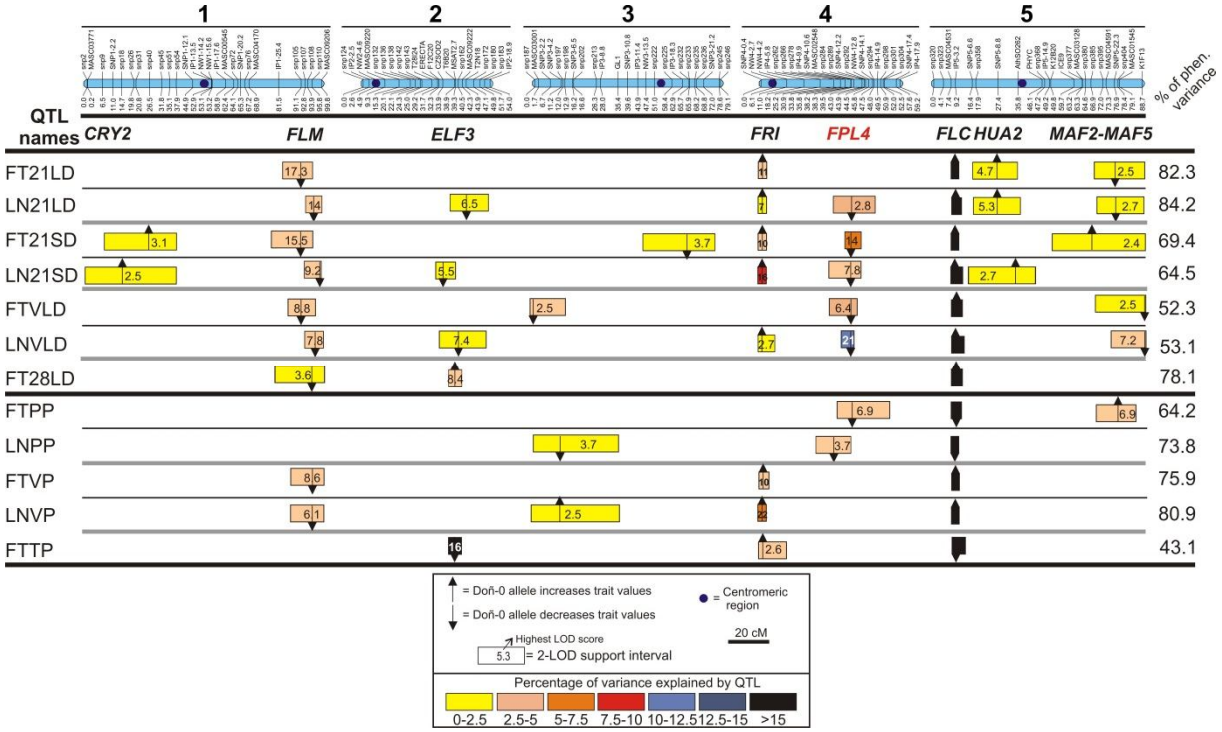


Figure 3. QTL mapping of flowering traits and plasticities in Don-0/L*er* RIL population. Bars on the top represent the genetic map of the five linkage groups. Black thick horizontal lines separate flowering initiation (FT and LN) traits and the plasticities of flowering in relation to different environmental factors. Grey thick horizontal lines separate different environments. Numbers in the right column show the percentage of phenotypic variance explained by the additive effects of all detected QTL. For each trait, the locations of QTL are shown as 2-LOD support intervals; the position of arrows correspond to maximum LOD score values, which are shown inside the boxes except those higher than 40. Colors of QTL boxes correspond to ranges of QTL explained variances according to the legend. Upper and lower arrows indicate that the additive effect of Don-0 alleles increase or decrease the trait values, respectively, in comparison to L*er* allele. QTL regions are named below the genetic map according to overlapping flowering QTL previously cloned. QTL that do not overlap with cloned QTL are named in red colour text. FT21LD, FT21SD, FTVLD and FT28LD: flowering time in the four environmental conditions; LN21LD, LN21SD, and LNVLD: total leaf number in the same environments; FTPP, LNPP, FTVP, LNVP and FTTP: plasticity of FT or LN to photoperiod, vernalization or ambient temperature.

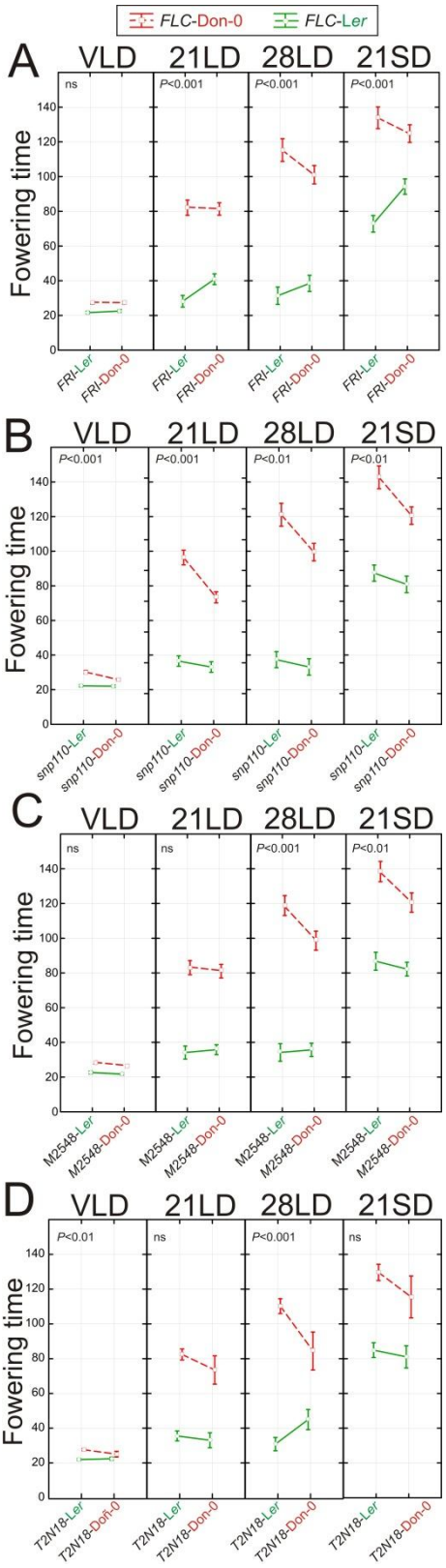

1
2
3
4
5
% of phen. variance
QTL names
CRY2
FLM
ELF3
FRI
FPL4
FLC
HUA2
MAF2-MAF5
FT21LD 82.3
LN21LD 84.2
FT21SD 69.4
LN21SD 64.5
FTVLD 52.3
LNVLD 53.1
FT28LD 78.1
FTPP 64.2
LNPP 73.8
FTVP 75.9
LNVP 80.9
FTTP 43.1
= Doň-0 allele increases trait values
= Doň-0 allele decreases trait values
= Centromeric region
20 cM
Highest LOD score
5.3 = 2-LOD support interval
Percentage of variance explained by QTL
0-2.5 2.5-5 5-7.5 7.5-10 10-12.5 12.5-15 >15

836

837

838 **Figure 4.** Genetic and environmental interactions of *FLC* on flowering time.

839 Each panel shows the genetic interactions between *FLC* and the QTL around *FRI* (A),
840 snp110 (B), MASC02548 (C) and T2N18 (D) in four environmental conditions.
841 Subpanels display the mean flowering time and the 95% confidence interval of the four
842 RIL genotypic classes (*N*=225 lines shared in all environments). In the left-upper corner
843 of each subpanel is shown the statistical significance of the QTL by QTL interaction.
844 Environments are arranged according to their flowering promotive effect, from the
845 strongest to the weakest strength. VLD: 21 °C and LD after three months vernalization
846 treatment; 21LD: 21 °C and LD; 28LD: 28 °C and LD; 21SD: 21 °C and SD. *FRI*,
847 snp110, MASC02548 and T2N18 are located around positions 4-0.0, 1-95.8, 4-38.3
848 and 2-43.9, respectively.


FLC-Don-0
FLC-Ler
A
VLD
21LD
28LD
21SD
ns
P<0.001
P<0.001
P<0.001
Fowering time
FRI-Ler
FRI-Don-0
B
P<0.001
P<0.001
P<0.01
P<0.01
snp110-Ler
snp110-Don-0
C
ns
ns
P<0.001
P<0.01
M2548-Ler
M2548-Don-0
D
P<0.01
ns
P<0.001
ns
T2N18-Ler
T2N18-Don-0

849

850

Figure 5. Reaction norms of *FPL4* NILs for flowering traits. A) flowering time and B) leaf number of IL-*FPL4*-Don-0 and the parental lines L*er* and Don-0. C, E and G) flowering time, and D, F and H) leaf number, of HIF-331, HIF-311 and HIF-63, respectively. Differences between L*er* and Don-0 lines from each pair of NILs were tested by Student *t* test. *: *P*< 0.05; **: *P*< 0.01; ***: *P*< 0.001. VLD: 21 °C and LD after three months vernalization treatment; 21LD: 21 °C and LD; 28LD: 28 °C and LD; 21SD: 21 °C and SD.

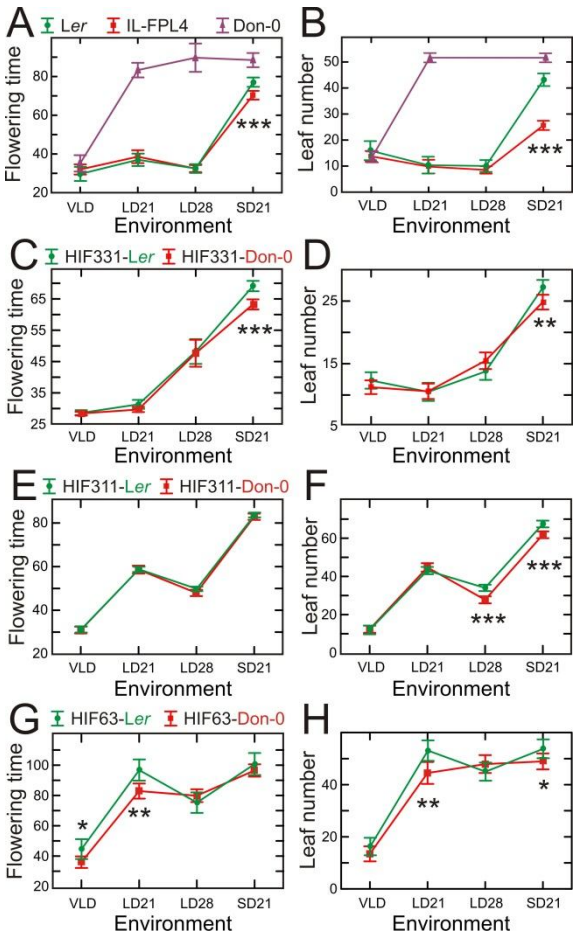
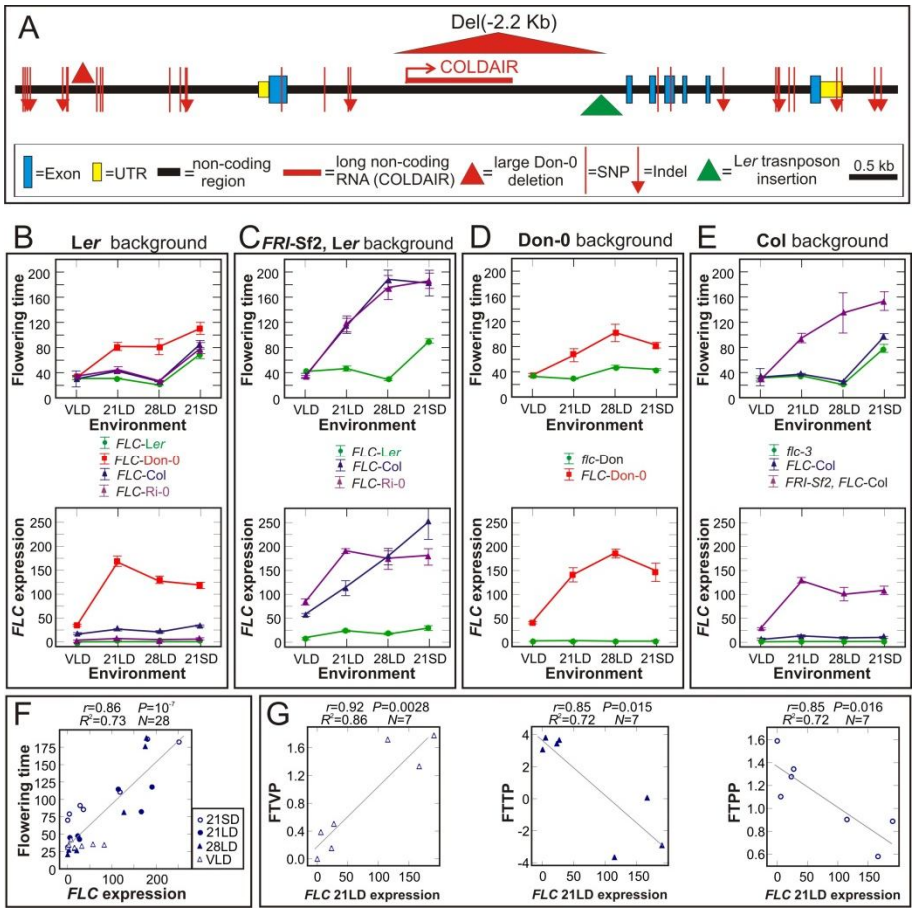


Figure 6. Plasticity of flowering and *FLC* expression in genotypes with different natural or artificial *FLC* alleles. A) Genomic structure of *FLC* gene showing Don-0/L*er* nucleotide polymorphisms. B-C) Reaction norms for flowering time and *FLC* expression in L*er* and in introgression lines carrying *FLC* alleles from Don-0, Col, or Ri-0 in a L*er* genetic background with *FRI*-L*er* (B) or with *FRI*-Sf2 (C). D) Reaction norms of Don-0 and *flc-Don* mutant in Don-0 background. E) Reaction norms of Col, a *FRI*-Sf2 introgression line and *flc-3* mutant in Col background. F) Correlation between *FLC* expression and flowering time. G) Correlation between *FLC* expression in 21LD environment and flowering plasticity to vernalization (FTVP), to high ambient temperature (FTTP) or to photoperiod (FTPP). In F-G, only the seven genotypes in L*er* genetic background (described in B and C) are included. VLD: 21 °C and LD after one (*FLC* expression) or three (FT) months vernalization treatment; 21LD: 21 °C and LD; 28LD: 28 °C and LD; 21SD: 21 °C and SD.



875 **Supporting Information**

876 **Table S1.** Genotypic data of Don-0/L*er* RILs.

877 **Table S2.** Correlations among flowering and plasticity traits in Don-0/L*er* RIL
878 population.

879 **Table S3.** QTL affecting flowering traits and plasticities in Don-0/L*er* RIL population.

880 **Figure S1.** Frequency distributions of leaf number and plasticity in Don-0/L*er* RIL
881 population.

882 **Figure S2.** Don-0/L*er* linkage map.

883 **Figure S3.** Genetic and environmental interactions of *FLC* on leaf number.

884

885

886

SUPPORTING INFORMATION

Figure S1. Frequency distributions of leaf number and plasticity in the Don-0/L*er* RIL population. A) Leaf number in three environmental conditions shown in the upper right corner. B) Plasticity of leaf number to photoperiod and vernalization treatment. 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. Vernalization treatment was given by 12 weeks at 4 °C.

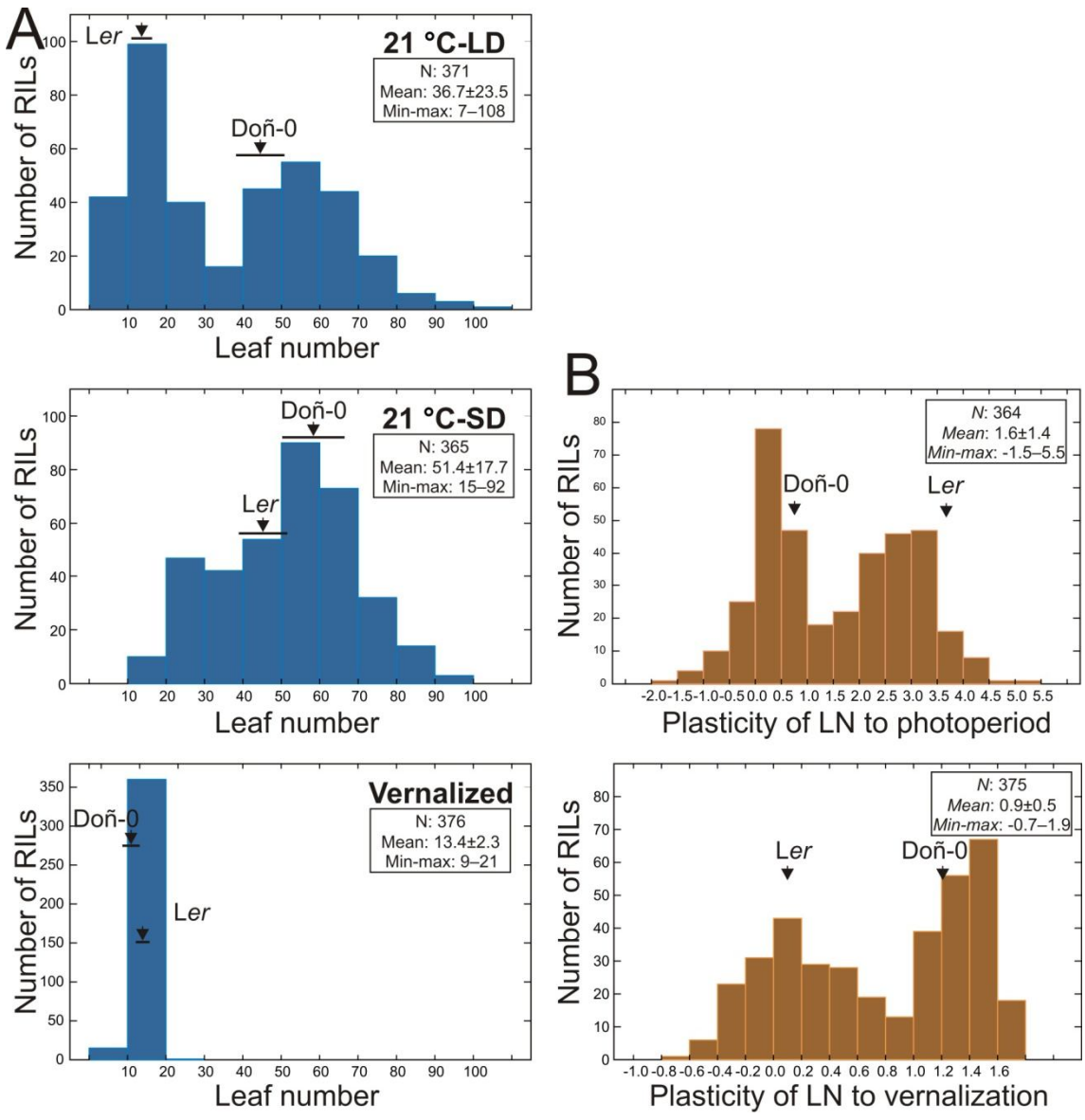


Figure S2. Don-0/L*er* linkage map. A) Comparison of physical and genetic maps along the five linkage groups. B) Segregation distortion in the five linkage groups. Linkage group numbers are shown in the left-top corner of each panel. Large circles depict centromeric regions, whereas arrows indicate the two pairs of inverted markers (see text for details). Dotted lines correspond to homozygote frequency thresholds (*P*=0.01); asterisks indicate markers with higher than expected heterozygosity (*P*<0.01).

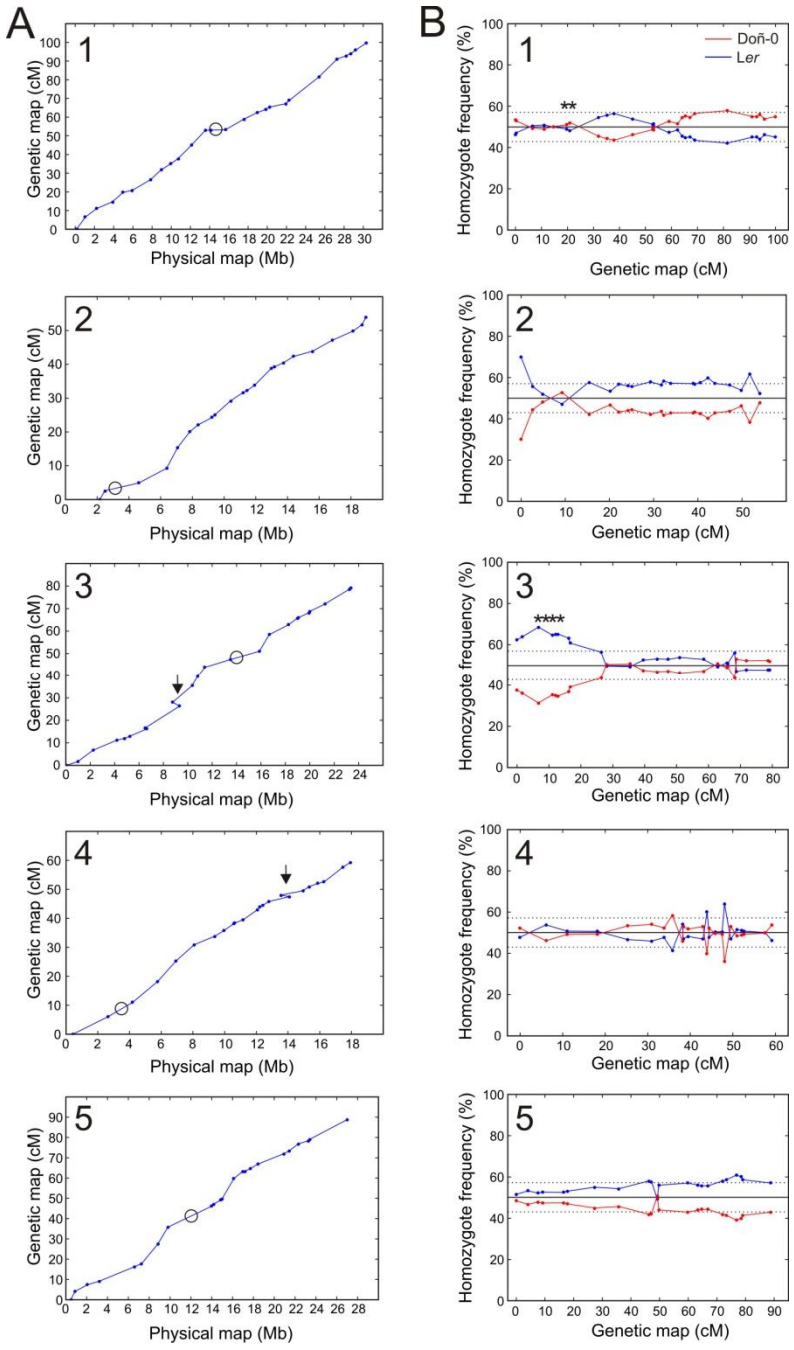


Figure S3. Genetic and environmental interactions of *FLC* on leaf number. Each panel shows the genetic interactions between *FLC* and a QTL around *FRI* (A), snp110 (B), MASC02548 (C) and T2N18 (D) in three environmental conditions. Subpanels display the mean leaf number and the 95% confidence interval of the four RIL genotypic classes (*N*=225 lines shared in all environments). In the left-upper corner of each subpanel is shown the statistical significance of the QTL by QTL interaction. Environments are arranged according to their flowering promotive effect, from the strongest to the weakest strength, as follow: VLD: 21 °C and LD photoperiod after three months vernalization treatment; 21LD: 21 °C under LD photoperiod; 28LD: 28 °C under LD photoperiod; 21SD: 21 °C under SD photoperiod. *FRI*, snp110, MASC02548 and T2N18 are located around positions 4-0.0, 1-95.8, 4-38.3 and 2-43.9, respectively.