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Adaptive divergence in flowering time among natural populations of *Arabidopsis thaliana*: Estimates of selection and QTL mapping

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To identify the ecological and genetic mechanisms of local adaptation requires estimating selection on traits, identifying their genetic basis, and evaluating whether divergence in adaptive traits is due to conditional neutrality or genetic trade-offs. To this end, we conducted field experiments for three years using recombinant inbred lines (RILs) derived from two ecotypes of *Arabidopsis thaliana* (Italy, Sweden), and at each parental site examined selection on flowering time and mapped quantitative trait loci (QTL). There was strong selection for early flowering in Italy, but weak selection in Sweden. Eleven distinct flowering time QTL were detected, and for each the Italian genotype caused earlier flowering. Twenty-seven candidate genes were identified, two of which (*FLC* and *VIN3*) appear under major flowering time QTL in Italy. Seven of eight QTL in Italy with narrow credible intervals colocalized with previously reported fitness QTL, in comparison to three of four in Sweden. The results demonstrate that the magnitude of selection on flowering time differs strikingly between our study populations, that the genetic basis of flowering time variation is multigenic with some QTL of large effect, and suggest that divergence in flowering time between ecotypes is due mainly to conditional neutrality.

KEY WORDS: **Adaptation, *Arabidopsis thaliana*, phenology, recombinant inbred lines, selection, trade-off.**

Geographic variation in the pattern and magnitude of selection often results in the evolution of local adaptation, where populations achieve higher fitness in their "home" environments than populations from other environments (Lenormand 2002; Kawecki and Ebert 2004; Yeaman and Whitlock 2011; Savolainen et al. 2013). Studies of local adaptation employ a variety of approaches (Savolainen et al. 2013), including indirect tests using genomic information often in combination with environmental data and/or common-garden experiments in the field (Coop et al. 2010; Fournier-Level et al. 2011; De Mita et al. 2013; Savolainen et al. 2013; Lotterhos and Whitlock 2015), or direct tests such as reciprocal transplant experiments where different populations or species are planted in "home" and "away" sites (Clausen et al. 1940; Schemske 1984; Kawecki and Ebert 2004; Angert and Schemske 2005; Lowry et al. 2008; Ågren and Schemske 2012; Chen and Schemske 2015; Toräng et al. 2015). These approaches have provided evidence of local adaptation in a wide range of taxa (Hereford 2009; Savolainen et al. 2013), including plants (Leimu

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and Fischer 2008), flies (Turner et al. 2008; Wittkopp et al. 2011), fish (Fraser et al. 2011), and humans (Fraser 2013; Qian et al. 2013), yet important questions remain.

Understanding the ecological and evolutionary mechanisms that contribute to local adaptation requires identifying patterns of selection on traits that contribute to local adaptation and the genetic basis of these traits (Kawecki and Ebert 2004; Olson-Manning et al. 2012; Anderson et al. 2013; Savolainen et al. 2013; Richardson et al. 2014; Tiffin and Ross-Ibarra 2014). However, despite the large number of studies that have demonstrated local adaptation, surprisingly few have also identified the traits involved, or the patterns of selection on adaptive traits in the native habitats of locally adapted populations (Hereford 2009). This is a challenging problem, in that populations often differ in a variety of traits and it is difficult to distinguish differentially adapted traits from those that might have evolved by random genetic drift. Moreover, a history of strong divergent selection leading to the evolution of local adaptation will reduce phenotypic variation within populations, making it difficult to employ standard phenotypic selection approaches such as those proposed by Lande and Arnold (1983) for estimating patterns of selection. Thus, the ecological requirement for the evolution of local adaptation, that is, spatial variation in the pattern of selection on adaptive traits, is often poorly understood.

The genetic basis of local adaptation is also largely unknown despite recent breakthroughs in molecular genetic techniques (Savolainen et al. 2013). Two questions are of particular interest. First, what is the genetic basis of adaptive traits? Specifically, are adaptive traits controlled by few or many loci, and do the loci that underlie adaptive traits have large or small phenotypic effects (Remington 2015; Dittmar et al. 2016)? To answer these questions requires estimating the number and effect sizes of genes that contribute to phenotypic divergence in locally adapted populations (Anderson et al. 2013; Savolainen et al. 2013). Second, what are the genetic mechanisms that underlie fitness trade-offs? A genetic trade-off occurs when an allele at a single locus is favored in one environment but has reduced fitness in other environments. Alternatively there may be conditional neutrality, where genotypes favored in one environment are neutral elsewhere. This requires genetic divergence at two or more loci to produce a pattern of local adaptation. The importance of conditional neutrality versus genetic trade-offs in the evolution of local adaption is not well understood (Hall et al. 2010; Anderson et al. 2011b, Anderson et al. 2011b, 2013; Ågren et al. 2013).

The challenges outlined above can be overcome in large part by conducting genetic crosses between locally adapted populations to produce F_2, backcross, or RIL mapping populations that segregate for putatively adaptive traits. These experimental populations will display a wider range of phenotypic variation than the parental populations, increasing the opportunity for estimating the strength and form of selection on individual traits (Huang et al. 2010). Moreover, through the application of quantitative trait locus (QTL) mapping approaches, segregating populations also allow an analysis of the genetic architecture of adaptive traits and a direct test of the relative importance of conditional neutrality and genetic trade-offs (Anderson et al. 2013). These approaches thus provide a powerful means of identifying the ecological and genetic mechanisms that contribute to local adaptation in nature.

Here, we present the results of long-term field studies using RILs produced from locally adapted populations of the model plant *A. thaliana* (hereafter *Arabidopsis*) to investigate the role of flowering time in the evolution of local adaptation. Flowering time is widely recognized as an important adaptive trait in many plant species (e.g., Olsson and Ågren 2002; Hall and Willis 2006; Elzinga et al. 2007; Franks et al. 2007; Wadgymar et al. 2015). Numerous studies have concluded that population differentiation for flowering time in *Arabidopsis* is adaptive. These include the analysis of latitudinal clines for flowering time (Stinchcombe et al. 2004) and genome-wide association mapping (e.g., Brachi et al. 2013; Fournier-Level et al. 2013). Although these and related studies consistently find that flowering time in *Arabidopsis* is adaptive, they were not designed to specifically investigate the role of flowering time in local adaptation. To do so requires direct evidence that the studied populations are locally adapted, and that the fitness consequences of differences in flowering time are investigated in the native environments.

In the present study, we used RILs derived from a cross between one population located near the northern limit of the native geographic range (Sweden) and one population located near the southern limit (Italy) in Europe (Ågren et al. 2013). Multiyear reciprocal transplant studies demonstrated that the parental populations are adapted to their local environments (Ågren and Schemske 2012). The RILs were used in field experiments carried out over three years at each of the parental sites, and QTL mapping identified 15 distinct QTL for overall fitness, six of which showed evidence of genetic trade-offs (Ågren et al. 2013). Previously, we conducted a QTL mapping study of flowering time using RILs grown in growth chambers that mimicked the parental environments (temperature and photoperiod), and identified 10 QTL, five of which colocalized with fitness QTL in the Italian conditions and two in the Swedish conditions (Dittmar et al. 2014), providing indirect evidence that these flowering time QTL contribute to local adaptation. The present study expands on these results to investigate both the patterns of selection on flowering time and the genetic basis of flowering time at each of the parental sites. We collected data on flowering time of RILs and parental genotypes in the field experiments described by Ågren et al. (2013), and combined these with previously published fitness data.

First, we quantified selection on flowering time by monitoring >40,000 plants in total, testing the hypotheses that (1)

flowering time is subject to stabilizing selection with an optimum flowering time corresponding to that of the local genotype, and (2) differences in flowering time can explain the difference in fitness between the local and nonlocal genotype at each of the two sites. We also examined whether flowering time influences fitness because of effects on survival, fecundity, or both. Next, we mapped QTL for flowering time, estimated their effect sizes, and determined if the same QTL are found in both environments. For each flowering time QTL, we identified known flowering time genes. Finally, we determined whether flowering time QTL found in these experiments colocalize with QTL identified previously for overall fitness and for fitness trade-offs (Ågren et al. 2013). Taken together, our study of the ecological genetics of flowering time provides a comprehensive picture of how this adaptive trait contributes to the evolution of local adaptation in natural *Arabidopsis* populations.

Material and Methods

STUDY SYSTEM

We used a RIL population derived from a cross between two locally adapted populations of *A. thaliana*, one from the northern (Sweden: 62°48′N, 18°12′E) and one from the southern (Italy: 42°07′N, 12°29′E) margin of its native range (Koornneef et al. 2004). Both source population are winter annuals: seeds germinate in the fall (October–November in Italy, and August–September in Sweden), the plants overwinter as a vegetative rosette, and flower and mature fruits in spring (February–April in Italy, and May–June in Sweden). The difference in timing of different life-history transitions is related to the drastically different climatic conditions at the two sites. Overwintering plants in Sweden are typically exposed to several months of subfreezing soil temperatures, whereas soil temperatures below freezing are very rare in Italy (one day with −0.1°C in eight years of recording; Ågren and Schemske 2012). At both sites, soils dry out during flowering and fruit maturation, but after that the water potential stays very low for markedly longer in Italy compared to Sweden (Postma et al. 2016). The RILs are genotyped for 348 SNP markers evenly spaced across the five nuclear chromosomes of the Columbia physical map. Detailed information about the RIL genotyping and linkage map construction can be found in Ågren et al. (2013).

FIELD EXPERIMENT

To quantify selection and map QTL for flowering time and fitness, in three consecutive years (2009–2011), we planted seedlings of 398 randomly selected RILs and the two parents in experimental gardens established in natural vegetation at the sites of the source populations (43,964 experimental plants in total). Across the three years, 404 RILs were included in the experiment; 390 RILs were represented in all site × year combinations. Planting procedures are described in Ågren et al. (2013) and are briefly summarized below.

Seeds were planted in Petri dishes on agar, cold stratified in the dark at +4°C for one week, and then moved to a growth room (22°C/16°C, 16 h day at 150 μE/m^2/s PAR (photosynthetically active radiation), 8 h dark) where the seeds germinated. Nine days after germination, seedlings were transplanted to randomized positions in plug trays composed of 299 cells (cell size: 20 mm × 20 mm × 40 mm) filled with local soil in Italy, and with an equal mixture of local sand, gravel, and unfertilized peat in Sweden. In 2009, 20 seedlings of each RIL and 184 seedlings of each parent were transplanted. To reduce edge effects in this year, we excluded plants in the outer three rows of the array, giving a final sample size of 12–20 (median 17) plants per site × RIL combination, and about 150 plants per site × parental line combination (Ågren and Schemske 2012). In 2010 and 2011, we established three rows of "border" plants (all RILs contributed equally) that were not considered in subsequent analyses. In these years, we transplanted 18 seedlings of each RIL and 180 seedlings of each parent to positions inside the border.

During transplantation, plug trays were kept in a greenhouse at about 18°C/12°C and 16-h day/8-h night. Within six days, the trays were transported to the field sites where they were sunk into the ground (on 16 September 2009, 10 September 2010, and 8 September 2011 in Sweden, and on 7 November 2009, 30 October 2010, and 7 November 2011 in Italy). The transplanted seedlings were at approximately the same stage of development as naturally germinating plants in the source population.

We scored survival to reproduction and number of fruits per reproducing plant, and quantified total fitness as the number of fruits produced per seedling planted (Ågren et al. 2013). Within a week of transplanting, we recorded the survival of transplanted seedlings. Seedling mortality during this first week was attributed to transplant shock and these seedlings were excluded from subsequent analyses. After the initial census, the status of all transplanted plants (alive/dead) was checked at least once before the end of the year, and the date of first flowering (hereafter, flowering time) of individual plants was determined from censuses conducted regularly during the flowering period (typically biweekly in Italy where flowering start varies widely, and every to every second day in Sweden where the period of flowering start is more compressed). Plants that had one or more developing fruits when first recorded as flowering were assigned a day of first flowering intermediate to the census date and the date of the preceding census. At fruit maturation, we recorded survival and the number of fruits produced by reproducing plants. Fruit production is strongly correlated with total seed production in both parental lines (Ågren and Schemske 2012), and was used as an estimate of fecundity. The mapping of fitness QTL was reported in Ågren et al. (2013).

To examine genotype × environment effects in the RILs, we compared the flowering time distributions across years and sites and calculated correlations between the mean flowering time of the RILs among years within each site and between sites within years. In addition, we used the data in Dittmar et al. (2014) to calculate the correlations between the mean flowering time of the RILs grown in growth chambers that mimicked field environments of the two sites and the mean flowering time of RILs grown in the field in the present study.

SELECTION ON FLOWERING TIME

We estimated genotypic selection on flowering time following the methods of Rausher (1992) using regression analyses with relative fitness (genotypic mean fitness divided by the grand mean fitness) as the response variable and the standardized mean flowering time of each RIL as the explanatory variable. For each site-by-year combination, we estimated RIL fitness as the least-square mean number of fruits per seedling from mixed-model analysis of variance (ANOVA) that included RIL (random effect) and two variables controlling for position effects (tray and row; both treated as fixed effects). For each RIL, we also calculated the proportion of plants surviving to reproduction, mean flowering time, and mean fecundity (mean number of fruits per surviving plant). Relative fitness and standardized flowering time were calculated separately for each site × year combination. We estimated the directional selection differential S_i from a regression model including only the linear term, and the quadratic (selection differential C_{ii}) from the quadratic term of the full regression model separately for each site × year combination. The quadratic selection differential was obtained by doubling the coefficient extracted from the regression model (Stinchcombe et al. 2008). To examine whether mean flowering time was related to survival, fecundity, or both, we also estimated selection differentials from models in which RIL relative fitness was calculated based on survival probability and fecundity, respectively. To determine whether selection differentials differed in sign and/or magnitude between sites, we also analyzed models that included site and its interaction with standardized mean flowering time (separately by year).

QTL MAPPING METHODS

We mapped QTL for RIL mean time to first flower using R/qtl (Broman & Sen 2009), following the previously published protocol for this population (Ågren et al. 2013; Dittmar et al. 2014; Oakley et al. 2014; Postma and Ågren 2015; 2016). To summarize briefly, we first quantile normalized the data (Broman & Sen 2009) to improve normality. We determined LOD (logarithm of odds) thresholds for both additive QTL and pairwise epistatic interactions (experiment wise $\alpha = 0.05$) using 10,000 permutations. The best multiple QTL model was found using Haley–Knott regression, employing the automated stepwise model selection procedure (Manichaikul et al. 2009). For each QTL, we produced Bayesian 95% credible intervals in R/qtl (Broman & Sen 2009). We used ANOVA (fitqtl procedure; Broman & Sen 2009) to calculate the LOD score and percent variance explained for each QTL using quantile normalized data, and then refit this model using the nonnormalized data to generate genotypic effect sizes in units of days to first flowering.

We used 95% Bayesian credible intervals and point estimates of QTL to establish criteria for evaluating whether flowering time QTL observed in different years and/or sites were located within the same or different genomic regions. For simplicity, these categories are hereafter referred to, respectively, as "same" versus "distinct." Flowering time QTL were classified as the same when their 95% Bayesian credible intervals overlapped, or if their point estimates were very similar (i.e., within 2 cM). This latter criterion was needed in cases where point estimates were very similar, but individual credible intervals were smaller than the distance between the two closest markers.

SPATIOTEMPORAL VARIATION IN QTL EFFECTS

To further explore differences in QTL effects between sites and years, we used ANOVA to estimate the effects of genotype (Italy or Sweden) at each of the distinct flowering time QTL, site, year, and the interactions of QTL genotype with site and year on RIL mean flowering time. QTL genotype was the marker locus closest to the mean map positions (weighted by LOD score). When significant interactions involving site or year were detected, we used contrasts to test for QTL effects on flowering time for each site × year combination. Variance inflation factors were all lower than 3, suggesting that collinearity of independent variables was not a serious problem.

CANDIDATE GENES

Candidate genes within the 95% credible intervals of flowering time QTL were identified using datasets of gene ontology (GO) annotations and locations (the GOSLIM and the version 9 GFF file, respectively) downloaded from the Arabidopsis Information Resource (TAIR; www.Arabidopsis.org) following previous approaches (Dittmar et al. 2014; Oakley et al. 2014). We filtered the GOSLIM file for genes containing "vernalization" or "flowering" in the GO terms. This list was then filtered to include only annotations based on experimental evidence (direct assay, mutant phenotypes, expression patterns, or genetic or physical interactions). Finally, using the TAIR version 9 GFF file, we filtered this list of genes to include only those in which the start position occurred within 300 Kb (~1 cM, the average distance between markers) beyond either end of the 95% credible intervals of our flowering time QTL. We excluded QTL where the credible interval of the QTL was greater than 15.2 cM (one-fourth of the smallest chromosome). This cutoff conservatively reduces the

potentially large number of genes found under those QTL for which the location is most uncertain.

COMPARING LOCATIONS OF FLOWERING TIME QTL AND FITNESS QTL

To determine if flowering time QTL colocalized with fitness QTL, we compared the genomic positions of each distinct flowering time QTL with that of fitness QTL identified in the same RIL population in the same sites and years (Ågren et al. 2013). We considered that distinct flowering time QTL colocalized with fitness QTL when at least one flowering time QTL point estimate fell within the range of point estimates for fitness QTL. To be conservative, we restricted this comparison to flowering time QTL with a credible interval <15.2 cM (corresponding to <1/4 of the length of the smallest chromosome) following previous approaches (Ågren et al. 2013; Dittmar et al. 2014; Oakley et al. 2014; Postma and Ågren, 2015, 2016). Although we feel that the criteria we have established to determine colocalization are conservative, there is a clear need for more sophisticated methods that can be applied to datasets such as those presented here.

Results

FLOWERING TIME OF PARENTS AND RILS

The Italian parent flowered earlier than the Swedish parent at both sites, and the difference in flowering time was markedly larger in Italy (significant site × population interaction in two-way ANOVA in the third year $F_{1,469} = 218.1$, $P < 0.0001$; see Ågren and Schemske 2012 for analyses of flowering time of the parental genotypes the first two years; Table S1). In Italy, the difference in mean flowering time between parents was 33, 50, and 43 days, in 2009, 2010, and 2011, respectively, and in Sweden the difference was three, three, and nine days (Fig. 1 and Table S1). RIL mean flowering times in Italy were intermediate to those of the parental lines and varied widely (range 30, 56, and 49 days, in the 2009, 2010, and 2011 experiments; SD of grand mean flowering time based on RIL means, 6.4–11.1 days; Table S1). By comparison, in Sweden, considerable transgressive variation was observed in the second and third year, and the range of RIL mean flowering times was narrower (seven, 14, and 18 days; SD of grand mean 1.1–2.8 days; Fig. 1 and Table S1) than that observed in Italy. Flowering time varied significantly among RILs, and in the three years of study, genotype (RIL) accounted for 18.1, 9.9, and 14.7% of the variance in flowering time among individual plants in Italy, and 9.5, 1.4, and 5.8% of that in Sweden, respectively. The proportion of the phenotypic variance explained by RIL was particularly low in Sweden in 2010, when native vole populations peaked in abundance and nonselectively destroyed about half of the plants in the experiment (cf. Ågren et al. 2013).

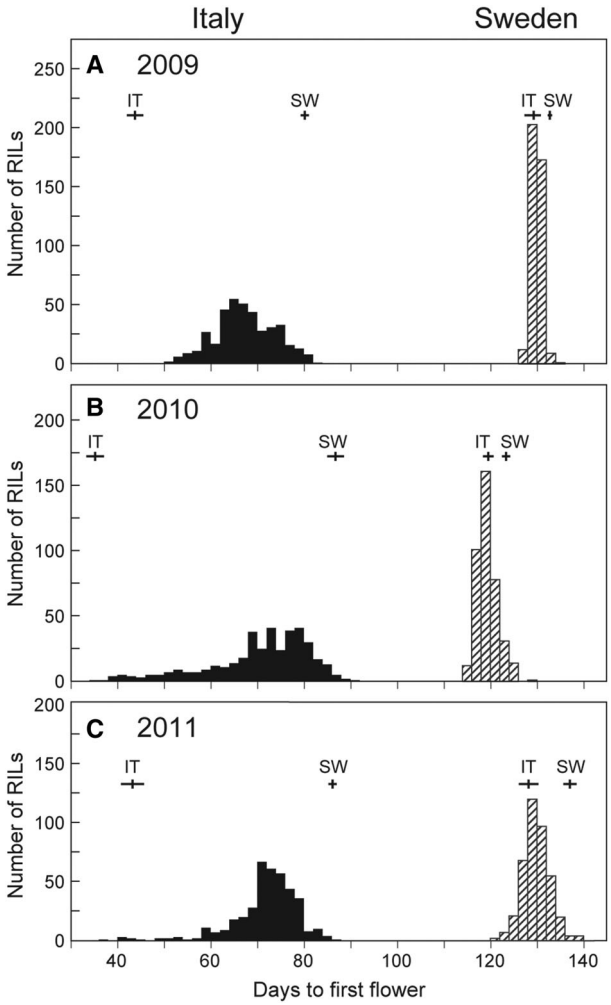

Figure 1. Distribution of recombinant inbred line means for flowering time (day of the year) in field experiments established in Italy and Sweden in 2009 (A), 2010 (B), and 2011 (C). Means for parental lines and associated 95% confidence intervals are indicated.

The correlations between years for flowering time of the RILs were markedly higher in Italy (mean [range], 0.86 [0.83–0.90]) than in Sweden (0.25 [0.14–0.38]) and the correlations between sites, within years were also low (Table S2). The correlations in mean flowering time of the RILs between the previously published growth chamber experiment (Dittmar et al. 2014) and the present field experiments was very high for "Italy" (Italy chamber × Italy field, 0.84 [0.83–0.85]), but low for "Sweden" (Sweden chamber × Sweden field, 0.16 [0.10–0.21]; Table S2).

SELECTION ON FLOWERING TIME

Selection on flowering time differed between the two environments (statistically significant difference in directional selection on flowering time in all three years; Table S3). In Italy, there was strong directional selection for earlier flowering in all three years

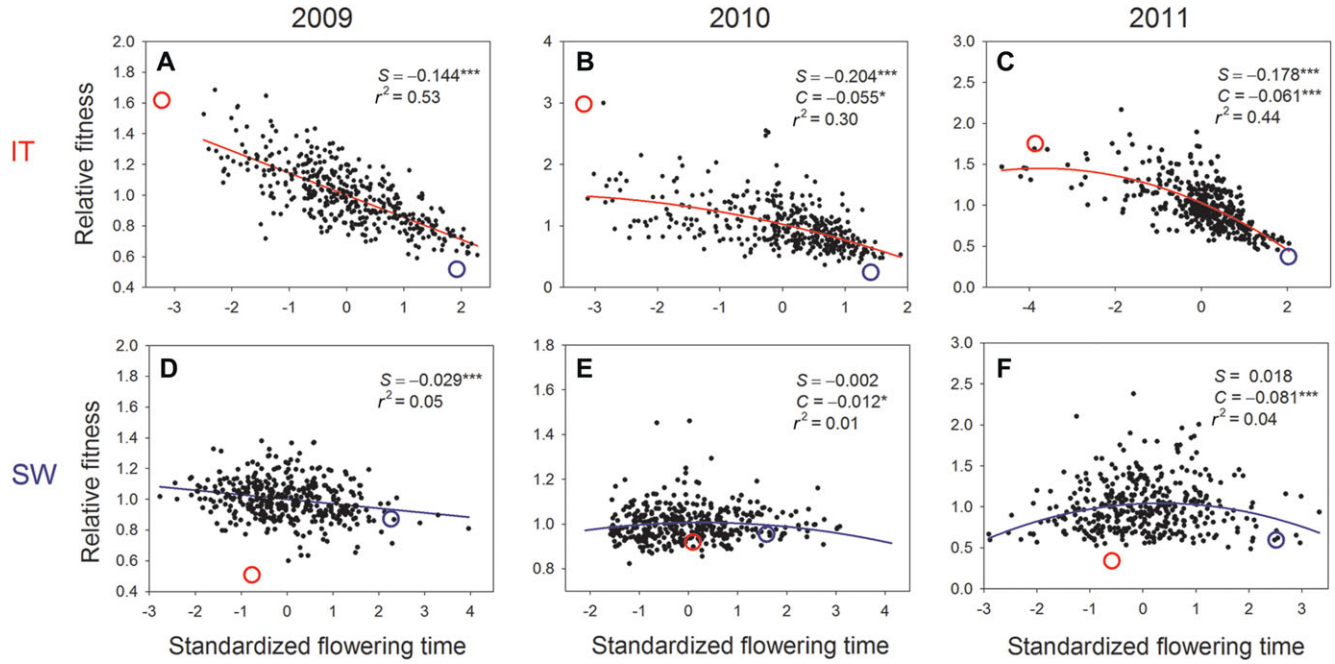

Figure 2. Standardized genotypic selection on flowering time ($n = 398$ recombinant inbred lines) in Italy (A–C) and Sweden (D–F) in 2009, 2010, and 2011. Linear (S) and quadratic (C) selection differentials are indicated when statistically significant (Table S4). Standardized flowering time and fitness of the two parental genotypes relative to RIL mean fitness are indicated with red (Italy) and blue (Sweden) circles.

(Fig. 2A–C; Table S4). In 2010 and 2011, the quadratic term was negative and statistically significant, but the fitness function had no clear intermediate optimum. Instead, the highest fitness was recorded for the earliest flowering RILs, that is, those that had a flowering time phenotype closest to that of the Italy ecotype (Fig. 2B and C). In Sweden, there was weak directional selection for earlier flowering in 2009 and weak stabilizing selection for an intermediate flowering time in 2010 and 2011 with the optimum closer to that of the Italy ecotype than to that of the Sweden ecotype in both years (Fig. 2E and F; Table S4). In 2009, the negative quadratic selection differential was not statistically significant (Fig. 2D; Table S4). These estimates represent total selection on flowering time, that is, direct selection on flowering time plus indirect selection due to selection on genetically correlated traits.

Both survival and fecundity were correlated with RIL mean flowering time (Table S4, Figs. S1 and S2). In Italy, early flowering was generally associated with high survival, although in 2011, the earliest flowering genotypes had somewhat reduced survival (negative quadratic differential with an intermediate optimum; Fig. S1). In Sweden in 2011, selection through survival favored an intermediate flowering time (negative quadratic selection differential), with the optimum flowering time somewhat later than the overall RIL mean (directional selection for later flowering), but still earlier than that of the Sweden ecotype (Fig. S1). In 2009, flowering time was not related to RIL survival in Sweden. Selection differentials based on fecundity largely mirrored those observed for total fitness at both sites, except that no stabilizing selection on flowering time was recorded in Sweden in 2010 (Table S4 and Fig. S2).

Flowering time explained 30–53% of the variance in relative total fitness in Italy, but only 1–5% in Sweden (Table S4, Fig. 2). Corresponding differences between Italy and Sweden in proportion of variance explained by flowering time were observed for relative fitness based on survival and fecundity, respectively (Table S4, Figs. S1 and S2).

ADAPTIVE SIGNIFICANCE OF DIFFERENCES IN FLOWERING TIME BETWEEN THE PARENTAL GENOTYPES

In Italy, the fitness function based on variation in RIL means predicted the fitness difference between the Italian and Swedish genotype very well in two of three years (Fig. 2A and C). In the third year (2010), traits other than flowering time apparently contributed to the fitness advantage of the local genotype (Fig. 2B). By contrast, in Sweden, selection on flowering time was weak, and optimal flowering time was closer to that of the foreign than to that of the local genotype. Still, the local genotype had higher fitness, demonstrating that differences in other adaptive traits more than compensated for the suboptimal flowering time of the Swedish genotype (Fig. 2D–F).

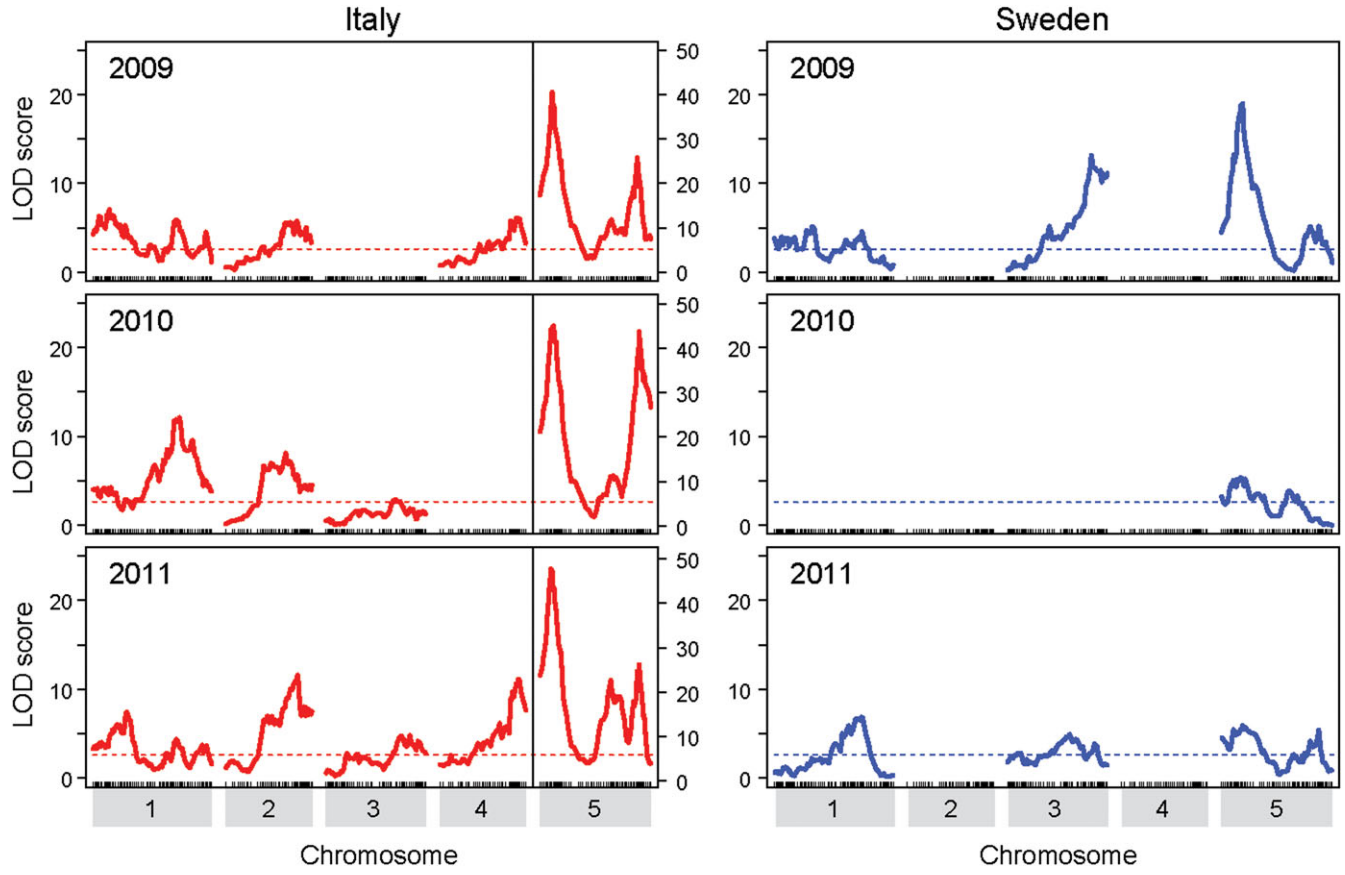

Figure 3. LOD profiles from multiple-QTL models of flowering time (quantile normalized) in the 2009, 2010, and 2011 experiments conducted in Italy and Sweden. Tick marks on *x*-axes indicate marker positions. Note different scale on *y*-axes for chromosome 5 in Italy.

NUMBER AND EFFECT SIZE OF QTL FOR FLOWERING TIME

In the three years of study, we identified nine, seven, and 10 flowering time QTL in Italy, together explaining on average 79% (range 74–82%, $N = 3$ years) of the variance in mean flowering time among RILs (Figs. 3 and 4, Tables 1 and S5). In Sweden, we identified five, one, and four flowering time QTL, together explaining on average 23% (range 6–41%) of the variance in mean flowering time among RILs (Figs. 3 and 4, Tables 1 and S5). Ten distinct QTLs (with nonoverlapping 95% Bayesian credible intervals; see Methods) were found in Italy and five in Sweden.

The point estimates of FlrT 5:1 and FlrT 5:2 formed two distinct groups (Fig. 4). In Italy, QTL were found at 5:1 in all three years, and in Sweden, QTL were found at 5:2 in all three years. Despite the consistent locations of point estimates for these QTL, in two years the credible interval of FlrT 5:2 overlapped those of FlrT 5:1. At present, there is no established procedure for determining colocalization between QTL from different multivariate QTL models. To investigate if these QTL are distinct, we first created a single LOD profile for each site by summing the LOD profiles for this region of the genome (from the full multivariate QTL models in each site and year) over the three years. Visual inspection of these LOD profiles indicated two distinct peaks (Fig. S3). Second, we employed a likelihood ratio test of a two-QTL model versus a single-QTL model (Jiang and Zeng 1995; Leinonen et al. 2013; Oakley et al. 2014) using the composite LOD profiles for each site. We compared the maximum peak of the summed LOD profile of the two site-level profiles (single-QTL model) to the maximum calculated by adding the LOD peaks of the individual site-level LOD profiles (two-QTL model). The two-QTL model was more likely than the single-QTL model (LOD of 160.92 vs. 155.07, respectively), and this difference was statistically significant ($\chi^2 = 5.85$, df $= 1$, $P = 0.016$).

For both sites and all years taken together, 11 distinct flowering time QTL were identified, of which six were observed only in Italy, one only in Sweden, and four were shared across sites (Fig. 4, Table 1). No significant epistatic interactions were detected among flowering time QTL.

For all flowering time QTL, the Italian genotype was associated with earlier flowering, and effect sizes, estimated in absolute number of days, were generally larger in Italy (mean = 3.4 days; range, 1.3–10.5 days) than in Sweden (mean = 0.9 days; range 0.4–1.5 days; Fig. 5). The effect sizes of nine of the 11 flowering

Table 1. QTL for flowering time from field experiments with recombinant inbred lines grown in Italy and Sweden in 2009, 2010, and 2011.

	2009					2010					2011						
Flowering time QTL	Pos	Bayesian 95% CI	LOD	Effect size	PVE	Pos	Bayesian 95% CI	LOD	Effect size	PVE	Pos	Bayesian 95% CI	LOD	Effect size	PVE	Fitness[1]	Number of candidate genes
Italy																	
1:1	11.5	3.3–18.9	7.0	1.7	1.7	12.6	0.0–16.1	4.3	2.7	1.3	23.7	6.0–26.5	7.5	2.0	1.6	NA	NA
1:2	58.8	57.3–61.1	5.9	1.7	1.4	61.1	57.3–61.8	12.1	4.1	3.9	58.8	57.3–63.1	4.4	1.3	0.9	Yes	2
1:3	79.6	77.0–80.9	4.5	1.4	1.1						77.2	72.0–80.9	3.7	1.7	0.8	Yes	1
2:1	50.4	39.8–56.9	5.8	1.5	1.4	42.3	26.8–46.9	8.1	3.2	2.6	51.0	48.0–51.0	11.6	2.5	2.6	Yes	3
3:1						49.1	19.7–69.6	2.9	2.1	0.9	59.2	50.1–66.8	4.8	1.6	1.0	NA	NA
4:1	54.1	50.0–57.7	6.1	1.7	1.4						55.5	52.8–56.6	11.2	2.6	2.5	Yes	4
5:1	8.5	8.5–8.5	40.5	5.0	11.8	9.4	7.7–9.4	44.9	10.1	17.8	7.7	7.7–8.5	47.2	5.7	13.0	Yes	1
5:3	50.5	49.9–51.6	11.9	2.5	2.9	51.6	49.9–54.3	11.1	4.7	3.6	49.9	49.9–49.9	22.0	3.5	5.2	No	4
5:4	68.7	68.7–68.7	25.9	5.3	6.9	70.1	70.1–70.1	43.6	10.5	17.2	70.1	70.1–70.1	25.6	5.1	6.2	Yes	1
5:5	77.4	74.5–78.2	8.3	2.6	2.0						78.2	72.1–78.2	3.3	2.1	0.7	Yes	6
Sweden																	
1:1	26.5	0.0–28.1	5.1	0.42	3.6											NA	NA
1:2	61.8	49.8–63.1	4.6	0.41	3.2						61.8	52.5–63.1	6.9	1.49	6.4	*Yes*	2
3:1	59.2	58.6–65.5	13.2	0.66	9.7						43.8	3.4–63.1	4.9	1.26	4.5	Yes	2
5:2	15.0	13.1–15.0	19.0	0.80	14.5	13.6	5.7–18.5	23.2	0.90	6.0	14.7	8.5–23.7	5.9	1.29	5.5	No	4
5:4	68.7	59.6–70.1	5.2	0.42	3.7						68.7	62.6–69.4	5.4	1.26	5.0	Yes	1

Entries include: map positions in centi-Morgan (Pos), 95% Bayesian credible intervals, LOD scores, effect sizes, and proportion of variance explained (PVE). Effect sizes are quantified as the difference in mean flowering time (days) between genotypes homozygous for the Swedish and Italian allele, respectively; positive values indicate that the Swedish genotype is associated with later flowering. Flowering time QTL that mapped to the same region as QTL for fitness in at least one site × year combination (see Fig. 4), and number of candidate genes for flowering time identified (see Methods) are also given. Colocalization with fitness QTL and possible candidate genes were not examined for QTL × site combinations where the credible interval of the flowering time QTL was large, >15.2 cM, designated as NA. QTL shared between sites are in bold.

[1]Instances where the flowering time QTL colocalizes with trade-off QTL are underlined. Colocalizaton with a fitness QTL with a maladaptive local allele are indicated with italics.

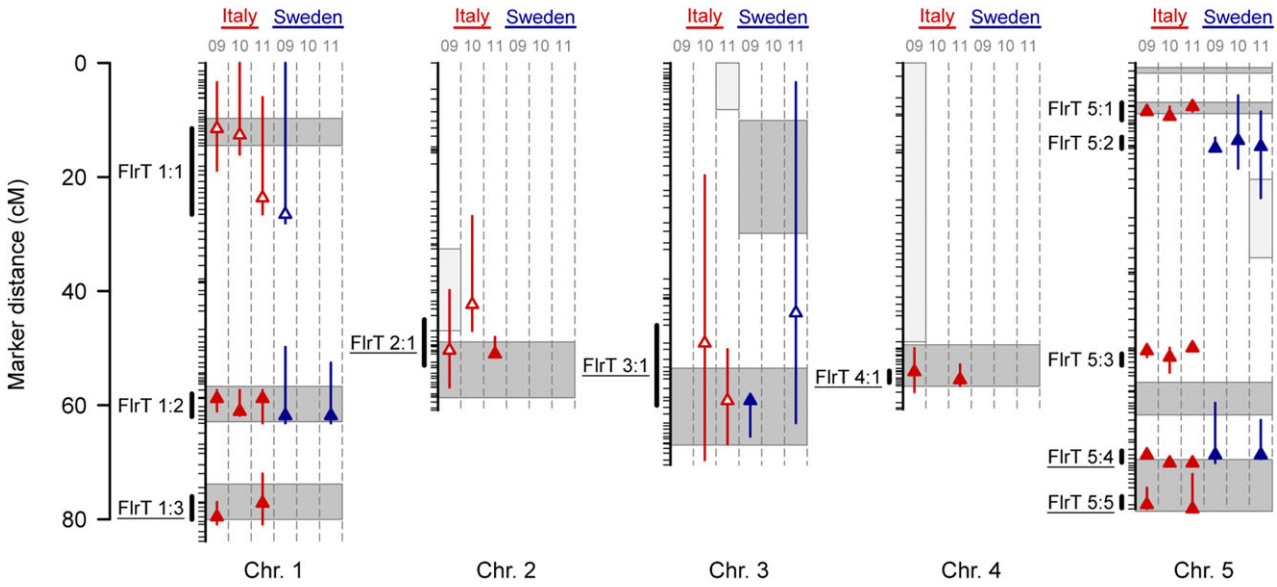

Figure 4. Flowering time QTL detected in field experiments in Italy and Sweden in 2009, 2010, and 2011. Arrows indicate QTL position and the effect of the Swedish genotype (upward = later flowering time). The vertical range of each arrow gives the 95% Bayesian credible interval. The vertical black line adjacent to each distinct flowering time QTL specifies the range of point estimates for that QTL. The shaded boxes indicate the range of point estimates for fitness QTL detected in more than one site × year combination (dark gray boxes), or the 95% credible interval for fitness QTL observed at just one site in one year (light gray boxes). Unfilled arrow heads identify flowering time QTL not considered in our analysis of colocalization of flowering time and fitness QTL because their credible intervals were considered too wide (>15.2 cM, 1/4 of the smallest chromosome) to provide an accurate point estimate of QTL position. Flowering time QTL colocalizing with trade-off QTL are underlined. Tick marks on *y*-axis by each chromosome indicate marker positions.

Table 2. Difference in mean flowering time (day of the year) between the Italian and Swedish ecotype of *Arabidopsis thaliana*, number of flowering-time QTL detected (see Table 1 for details), and the cumulative effect of flowering time QTL (number of days and proportion of difference between parents, respectively) in reciprocal transplant experiments conducted in three years.

Site	Year	Difference (SW-IT)	Number of QTL detected	Cumulative QTL effect (days)	Cumulative QTL effect (proportion)
Italy	2009	33.2[1]	9	23.3	0.70
Italy	2010	50.5[1]	7	37.5	0.74
Italy	2011	43.0	10	28.0	0.65
Sweden	2009	3.2[1]	5	2.7	0.84
Sweden	2010	3.2[1]	1	0.9	0.28
Sweden	2011	8.9	4	5.3	0.60

[1]Data from Ågren and Schemske (2012).

time QTL differed between sites (significant QTL × site interaction in ANOVA; Table S6). In all nine cases, the effect was stronger in Italy, and for six of the nine QTL, no significant effect on flowering time was recorded in Sweden (Table 1). However, if effect sizes are estimated as proportions of the difference between parents, the sum of effect sizes was similar between sites except in Sweden 2010 when it was particularly low (range, Italy 65–74%, Sweden 28–84%; Table 2). For five flowering time QTL the effect varied both among years and sites (FlrT 1:2, 2:1, 5:1, 5:3, 5:4; significant three-way interactions in ANOVA; Table S6).

CANDIDATE GENES

For 10 of the 11 distinct flowering time QTL, at least one of the 95% credible intervals documented for individual site × year combinations was less than our cutoff of 15.2 cM. Twenty-seven candidate genes were identified under these 10 QTL (one to six per QTL) based on GO annotations with experimental evidence (Table S7). Several of the candidate genes identified are known to influence flowering time, and have been identified in QTL studies of flowering time in *Arabidopsis*, including *FT* (florigen), *FLC*, *VIN3*, *MAF1* (*FLM*), *MAF2-5*, *COP1*, *AGL24*, *LHP1* (reviewed in

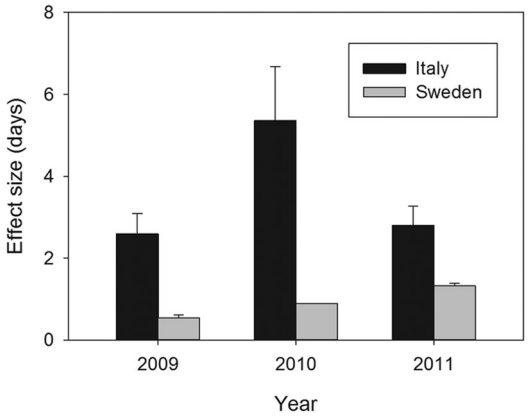

Figure 5. Mean absolute effect size (days to first flower; +SE) of flowering time QTL detected in field experiments established in Italy and Sweden in 2009, 2010, and 2011. Effect size was quantified as the difference in mean flowering time (days) between genotypes homozygous for the Swedish and Italian allele, respectively. See Table 1 for number of flowering time QTL detected in each site × year combination.

Amasino 2010; Grillo et al. 2013; Kim and Sung 2013; Suter et al. 2014). The two largest effect QTL at the Italian site (FlrT 5:1 and FlrT 5:4), each explained on average about 15% (seven days) of the difference in flowering time between the parents. These two QTL each contained only a single strong candidate gene (*FLC* and *VIN3*, respectively). As noted in previous QTL studies in this mapping population, we found no evidence that *FRIGIDA* (*FRI*) contributes to differences in flowering time between the ecotypes (Grillo et al. 2013; Dittmar et al. 2014).

OVERLAP BETWEEN FLOWERING TIME AND FITNESS QTL

Eight of the eleven distinct flowering time QTL colocalized with fitness QTL identified in our previous experiments (Ågren et al. 2013; Fig. 4; Table 1). In Italy, colocalization with fitness QTL was observed for seven flowering time QTL, and for all of these fitness QTL, the local allele was favored (Table 1). In Sweden, three flowering time QTL colocalized with fitness QTL, but while two were near a fitness QTL for which the local allele had a selective advantage (FlrT 3:1 and 5:4), the third was near a fitness QTL for which the local allele was maladaptive (FlrT 1:2; Table 1). Six flowering time QTL (FlrT 1:3, 2:1, 3:1, 4:1, 5:4, and 5:5) had point estimates within the genomic regions associated with five of the six fitness QTL that displayed evidence of trade-offs between Italy and Sweden (Ågren et al. 2013). In Italy, all six flowering time QTL significantly affected flowering time (Fig. 4), whereas in Sweden this was true for only two of these QTL (FlrT 3:1 and 5:4).

Discussion

Genetically based variation in phenology has been documented along environmental gradients in many plants and animals (Bradshaw and Holzapfel 2001; Savolainen et al. 2007), but the extent to which phenological differences contribute to adaptive differentiation among populations is typically not known. By growing RILs derived from a cross between two locally adapted populations of *A. thaliana* at the native sites of the parental genotypes, we have shown that the adaptive significance of flowering time differs between sites. In Italy, the difference in flowering time between the Swedish and Italian ecotypes was large and could explain much of the advantage of the local genotype, but in Sweden the parental difference was small and could not explain the advantage of the local population (Fig. 2). Similarly, colocalization of flowering time and fitness QTL was more common in Italy. Tests of the functional and genetic basis of local adaptation should ideally be conducted at the native sites, and the present study illustrates that this is particularly important for traits showing G × E interactions.

The Italian genotype flowered earlier than the Swedish genotype at both experimental sites, which is consistent with latitudinal variation in flowering time previously documented in *A. thaliana* across Europe (Stinchcombe et al. 2004). In Italy, there was strong selection for earlier flowering. The highest fitness was recorded for RILs that had a flowering time close to that of the local genotype. However, because of very limited transgressive variation in Italy, it was not possible to determine whether an even earlier flowering would be associated with lower fitness, as expected if the flowering time of the local genotype corresponds to a fitness optimum. In contrast, at the Swedish site, the optimal flowering time in all years was closer to that of the foreign genotype than to that of the native genotype. Several factors may contribute to this mismatch between optimal and observed flowering time of the Swedish genotype. First, the magnitude of the difference in flowering time between the two parental genotypes was markedly smaller in Sweden compared to Italy, and the variation in flowering time observed only weakly affected plant relative fitness. Weak selection on flowering time in Sweden should increase the potential for genetic drift to influence the evolution of flowering time. Second, current flowering time may not be optimal because of recent climatic warming. Our previous mapping of fitness QTL identified several maladaptive QTL in the Swedish population, that is, QTL for which the nonnative allele was associated with higher fitness in Sweden (Ågren et al. 2013), and one QTL affecting flowering time in Sweden (FlrT 1:2) overlapped with such fitness QTL. Similarly, in a set of four common gardens distributed across Europe, Wilczek et al. (2014) found that genotypes originating from historically warmer sites than the planting site had higher mean relative fitness than local genotypes, which would be consistent with lagging adaptation to warming climate.

Alternatively, the apparent mismatch between observed and optimal flowering time in Sweden may reflect intermittent strong selection against early flowering not captured during the three years of our field experiments.

In Italy, flowering time was subject to markedly stronger selection than in Sweden, and explained a much larger proportion of fitness variation among RILs (30–53% vs. 1–5%), suggesting that flowering time is more important for fitness variation in the southern environment. One caveat is that our genotypic selection analyses considered only variation in flowering time, and estimates of selection on a single trait may be influenced (strengthened or weakened) by selection on genetically correlated traits (Mitchell-Olds and Shaw 1987; Kingsolver and Diamond 2011). However, the results of the selection analyses were qualitatively similar whether other traits such as freezing tolerance (quantified under controlled conditions; Oakley et al. 2014) and leaf trichome density were included in the models (data not shown). This suggests that the effects of flowering time on fitness are not simply the product of genetic correlations between flowering time and these two other putatively adaptive traits.

Selection generally favored early flowering at both sites. However, in one of the three years (2011), the earliest flowering genotypes tended to have reduced survival at both sites, resulting in stabilizing selection through survival (Fig. S1). In Sweden, stabilizing selection on flowering time was also observed through fecundity in the same year (Fig. S2). Rapid development after snow melt in Sweden increases the risk that plants are subject to inclement weather, and in years with cold springs, this may favor genotypes that flower later. Damage from late frosts is not uncommon among early-flowering plants in the temperate and boreal zones (Schemske et al. 1978; Ågren 1988; Inouye 2008), and should constrain the evolution of earlier flowering among species flowering in spring.

Similar to previous studies in *Arabidopsis* (Wilczek et al. 2009; Brachi et al. 2010; Ågren and Schemske 2012) and other species (Anderson et al. 2011a), we documented considerable G × E interactions for flowering time. Both the overall phenotypic variance in flowering time and the proportion of variance explained by genotype was lower when the RIL population was planted in Sweden compared to when planted in Italy. Moreover, correlations between RIL mean flowering time in Italy and in Sweden were weak in comparison to correlations among flowering time in different years in Italy. Ten flowering QTL were documented in Italy, whereas only five were detected in Sweden, of which four were shared between sites. For all QTL, the Swedish genotype was associated with later flowering, but effect sizes for most QTL were smaller in Sweden than in Italy. Still, the proportion of the difference between parental lines that could be explained by the sum of QTL effects was high at both sites (≥60% except in one year in Sweden). The results demonstrate that the genetic architecture of flowering time differed between the two environments, and suggest that mutations in several of the genes underlying flowering time QTL in Italy would have little or no effect on flowering time in Sweden. At the northern site, they would thus be subject to selection only if they had pleiotropic effects on some other trait influencing fitness.

Moreover, not all flowering time QTL were observed in all years. In Italy, the number of flowering time QTL detected in each year was nine (2009), seven (2010), and 10 (2011), as compared to five (2009), one (2010), and four (2011) in Sweden. In Italy, six flowering time QTL were observed in all three years, whereas four were observed in two years. In Sweden, one flowering time QTL was detected in all years, three were observed in two years, and one was observed only once. These results illustrate the importance of multiyear studies for assessing the genetic basis of traits that display G × E interactions.

We identified several candidate genes within the genomic regions associated with flowering time QTL. Many of the flowering time candidate genes near our QTL are involved in the vernalization pathway, including the polycomb repressor complex (PCR2) affecting epigenetic modification of *FLC* during vernalization (Amasino 2010; Mozgova and Hennig 2015). One of the largest effect QTL (FlrT 5:4) contains *VIN3*, which has been described as a master regulator of vernalization (Kim and Sung 2013). With vernalization, *VIN3* is involved in the epigenetic modification and repression of *FLC* via PCR2, which leads to earlier flowering (Amasino 2010; Mozgova and Hennig 2015) by reducing repression of *FT* (which likely underlies FlrT 1:2). Grillo et al. (2013) and Dittmar et al. (2014) found that *VIN3* colocalized with flowering time QTL in this mapping population, and Grillo et al. (2013) reported that the coding sequence of *VIN3* differed between the parents (four nonsynonymous substitutions and a 3 bp deletion), and suggested that *VIN3* was the strongest candidate gene underlying parental differences in flowering time.

Our results indicating that *VIN3* is a strong flowering time candidate are in contrast with many previous QTL studies (reviewed in Grillo et al. 2013, but see Strange et al. 2011). We note that in many of the mapping populations used in these studies, at least one parent contained a nonfunctional *FRI* allele (e.g., Shindo et al. 2006). *VIN3* was first identified in a mutagenized line with a functional *FRI* allele (Sung and Amasino 2004), and it may be that natural variation in *VIN3* will be most easily observable in functional *FRI* backgrounds such as our cross. The other large-effect QTL in Italy (FlrT 5:1) contains *FLC*, which has also been identified in this (Dittmar et al. 2014) and several other mapping populations (reviewed in Grillo et al. 2013). There are no nonsynonymous differences in *FLC* between the parents of our mapping population (Grillo et al. 2013), but the functional importance of changes in noncoding regions of this gene is well known (Shindo et al. 2006; Li et al. 2014; Li et al. 2015). In addition to the

candidate genes identified, other genes may also affect flowering time in the regions of these QTL. Functional experiments will be required to determine conclusively the role of individual genes for differences in flowering time between the parental genotypes.

Unlike a previous growth chamber study in this system (Dittmar et al. 2014), we did not detect a QTL at *FLC* in the Swedish environment. Instead, we found a nearby but distinct QTL (FlrT 5:2) containing *LHP1*. This gene is thought to play an important role in epigenetic modification of *FLC* (Mylne et al. 2006; Sung et al. 2006; Amasino 2010; Kim et al. 2010; Mozgova and Hennig 2015). Although *LHP1* has been identified as a candidate underlying flowering time QTL (Shindo et al 2006; Strange et al. 2011, reviewed in Grillo et al. 2013), to our knowledge ours is the first study to report distinct QTL at both *FLC* and *LHP1* in the same mapping population. It is further intriguing that only the QTL at *FLC* was detected in a previous growth chamber experiment simulating temperature and photoperiod at the two sites (Ditmar et al. 2014). It thus seems possible that differences at both *FLC* and *LHP1* can affect flowering time, but their relative importance depends on the environment. Future growth chamber and field experiments with near isogenic lines for these and other genes individually and in combination should yield important insight into the interplay between the environment and the gene networks underlying flowering time variation in this mapping population.

Similar to the results of the genotypic selection analysis, an examination of colocalization of QTL for flowering time and previously mapped fitness QTL (Ågren et al. 2013) indicated that differences in flowering time are more important for the advantage of the local genotype in Italy than in Sweden. Because of selection for earlier flowering in Italy, the Italian alleles at flowering time QTL are expected to be favored in Italy. In Sweden, however, weak stabilizing selection for a phenotype with intermediate flowering time should reduce the likelihood of colocalization of flowering time and fitness QTL, and should potentially favor a combination of Italian and Swedish alleles at different flowering time QTL. Consistent with these predictions, flowering time QTL with narrow credible intervals had point estimates that fell within the ranges of point estimates for six of 13 previously detected fitness QTL in Italy, but with only three of 12 fitness QTL in Sweden. For all of these fitness QTL, the local allele was favored in Italy (Fig. 4; Table 1). By comparison, in Sweden, two of the three flowering time QTL colocalized with fitness QTL for which the local allele was favored (FlrT 3:1 and FlrT 5:4), whereas the third colocalized with a fitness QTL for which the Italian allele was favored at both sites (FlrT 1:2; Table 1).

Moreover, four of the six flowering time QTL that overlapped with trade-off QTL (FlrT 1:3, 2:1, 4:1 and 5:5) did not significantly affect flowering time in Sweden, suggesting that their associations with fitness in Sweden were caused by tight linkage to other genes influencing fitness, or by pleiotropic effects on some other adaptive trait. Of these, both FlrT 4:1 and FlrT 5:5 are located close to freezing tolerance QTL for which the Swedish allele strongly increases freezing tolerance compared to the Italian allele (see Oakley et al. 2014). The results thus suggest that most flowering time QTL are conditionally neutral: positively affecting fitness in Italy, but selectively neutral in Sweden. Near isogenic and CRISPR lines are currently being developed to determine the independent and combined effects of these QTL on flowering time, local adaptation, and fitness trade-offs across environments.

The present study provides an example of how experiments conducted at native sites and employing mapping populations segregating for putatively adaptive traits can provide critical insight into the functional and genetic basis of local adaptation. The general approach can readily be extended to other mapping populations (Savolainen et al. 2013), and to the study of additional traits (Anderson et al. 2011a) and life-history stages (Postma and Ågren 2016). Moreover, as genomic information becomes available for more species, similar approaches can be employed in studies of local adaptation in a wide range of nonmodel organisms.

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DATA ARCHIVING

RIL seeds and genotype data are available at the Arabidopsis Biological Resource Center, ABRC (CS98760).

Raw data and formatted genotype and phenotype files for QTL mapping and selection analyses are available at Dryad http://dx.doi.org/10.5061/dryad.77971.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Flowering time (day of the year; mean ± SE) of the Italian and Swedish ecotype of *Arabidopsis thaliana* in reciprocal transplant experiments conducted in three years.

Table S2. Correlations among RIL mean flowering time in Italy and Sweden in the 2009–2011 field experiments and in growth chambers simulating Italy and Sweden climates (growth chamber data from Dittmar et al. 2014).

Table S3. Effect of site on linear (S) and quadratic (C) genotypic selection differentials for flowering time in an experimental RIL population grown in Italy and Sweden 2009, 2010, and 2011, respectively ($n = 398$ recombinant inbred lines), analyzed with analysis of covariance (ANCOVA) separately by year.

Table S4. Linear (S) and quadratic (C) genotypic selection differentials for flowering time in an experimental RIL population grown in Italy and Sweden 2009, 2010, and 2011, respectively ($n = 398$ recombinant inbred lines).

Table S5. Multiple-QTL models fitted to data on RIL mean flowering time (day of the year) with the stepwise function in R/qtl.

Table S6. Effects of site (Italy vs. Sweden), year (2009, 2010 or 2011), and genotype at marker loci closest to mean positions of the detected 11 flowering time QTL on RIL mean flowering time examined with ANOVA.

Table S7. Candidate genes underlying flowering time QTL, annotated with "flowering" or "vernalization" in the GO terms using experimental evidence (TAIR; www.Arabidopsis.org).

Figure S1. Standardized genotypic selection on flowering time ($n = 398$ recombinant inbred lines) in Italy (A, B, C) and Sweden (D, E, F) in 2009, 2010, and 2011. Relative fitness based on RIL survival.

Figure S2. Standardized genotypic selection on flowering time ($n = 398$ recombinant inbred lines) in Italy (A, B, C) and Sweden (D, E, F) in 2009, 2010, and 2011. Relative fitness based on RIL mean fecundity.

Figure S3. Summed LOD profile plots by site for the genomic region including FlrT 5:1 and FlrT 5:2.