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VERNALIZATION SENSITIVITY IN *ARABIDOPSIS THALIANA* (BRASSICACEAE): THE EFFECTS OF LATITUDE AND *FLC* VARIATION[1]

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Latitudinal variation in climate is predicted to select for latitudinal differentiation in sensitivity to the environmental cues that signal plants to flower at the appropriate time for a given climate. In *Arabidopsis thaliana*, flowering is promoted by exposure to cold temperatures (vernalization), and several vernalization pathway loci are known. To test whether natural variation in vernalization sensitivity could account for a previously observed latitudinal cline in flowering time in *A. thaliana*, we exposed 21 European accessions to 0, 10, 20, or 30 d of vernalization and observed leaf number at flowering under short days in a growth chamber. We observed a significant latitudinal cline in vernalization sensitivity: southern accessions were more sensitive to vernalization than northern accessions. In addition, accessions that were late flowering in the absence of vernalization were more sensitive to vernalization cues. Allelic variation at the flowering time regulatory gene *FLC* was not associated with mean vernalization sensitivity, but one allele class exhibited greater variance in vernalization sensitivity.

Key words: Brassicaceae; ecological genomics; *FLC*; flowering time; FRI; latitudinal cline; vernalization.

Timing reproduction to coincide with suitable environmental conditions is a central challenge for organisms, and this challenge is especially important for semelparous organisms, such as annual plants, that reproduce only once in their life cycle. One mechanism by which plants time their reproduction to coincide with suitable conditions is by responding to seasonal changes in temperature and day length (Simpson and Dean, 2002). These cues, and the seasonal environments they predict, vary with latitude and climate. Consequently, the optimal response to seasonal cues is likely to vary geographically, leading to geographic differentiation in flowering responses. For example, since plants in southern latitudes experience milder winters and earlier springs, they may evolve increased vernalization sensitivity—that is, a more rapid acceleration of flowering time due to a given duration of vernalization exposure (e.g., Boudry et al., 2002).

The annual species *Arabidopsis thaliana* is an excellent system for examining geographic differentiation in response to seasonal cues as well as the underlying genetic basis of such variation. In this genetic model species, both a long-day photoperiod and exposure to an extended period of cold temperature (vernalization) accelerate reproduction through separate signaling pathways (Johanson et al., 2000; Sheldon et al., 2000; Michaels and Amasino, 2001; Simpson and Dean, 2002; Michaels et al., 2003). In addition, *Arabidopsis thaliana* inhabits a broad climatic range (Hoffmann, 2002), so it is likely that the selective forces acting on these pathways vary geographically. In fact, this species exhibits significant quantitative genetic variation in vernalization responses (Karlsson et al., 1993; Nordborg and Bergelson, 1999). However, previous studies have not explicitly tested for latitudinal or climatic differentiation in sensitivity to vernalization treatments of different length or tested for effects of natural allelic variation at specific loci on vernalization sensitivity.

Because *A. thaliana* is a genetic model, it offers a useful opportunity to explore how natural genetic variation at specific loci contributes to variation in flowering and vernalization responses. In recent years rapid progress has been made in the determination of the genes involved in the vernalization flowering time pathway of *A. thaliana* (Michaels and Amasino, 2000; Bastow et al., 2004; Sung and Amasino, 2004). In accessions with functional copies of *FRIGIDA* (*FRI*), there is an accumulation of *FLC* mRNA, which inhibits flowering; *FLC* is repressed by vernalization, which makes plants competent to flower (Johanson et al., 2000; Sheldon et al., 2000). Repression of *FLC* by vernalization is stable: *FLC* transcription levels remain low even after plants are no longer exposed to cold temperatures, enabling plants to ''remember'' that winter has passed (Michaels and Amasino, 2000; Sung and Amasino, 2004). Thus *FRI* and *FLC* appear to play a prominent role in mediating vernalization responses, although vernalization can also promote flowering through *FLC*-independent mechanisms (i.e., in *FLC* loss-of-function mutants; Michaels and Amasino, 2001).

We recently observed a latitudinal cline in flowering time in European accessions of *A. thaliana* allowed to experience natural winter conditions in a common garden. However, the cline was detected only in accessions that contained putatively functional copies of *FRI* (Stinchcombe et al., 2004). In particular, we found that accessions from southern latitudes with putatively functional *FRI* alleles flowered significantly earlier than northern accessions with putatively functional *FRI* alleles.

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TABLE 1. Name, stock number (from http://www.arabidopsis.org), latitude and longitude of origin, *FLC* allele class, and vernalization sensitivity of the accessions used in the experiment. Negative longitude values refer to locations west of the prime meridian. Vernalization sensitivity was estimated by regressing rosette leaf number at bolting on days of vernalization using data from the 10-, 20-, and 30-d treatments. Ninety-five percent confidence limits for vernalization sensitivity were estimated by jackknifing. For accessions marked with an asterisk, we sequenced *FRI* to determine whether our previous genotyping approach failed to detect nonfunctional *FRI*. Accessions in FLC^A allele class marked with a dagger (†) were omitted from the ANCOVA test for the effects of *FLC* allele class on vernalization sensitivity because they either contained insertions in *FLC* or we were unable to determine if they contained insertions.

Stock number	Name	Latitude (°N)	Longitude (°E)	*FLC* class	Vernalization sensitivity (95% CL)
CS917*	Da(1)-12	49.8	15.5	A†	−0.150 (−0.265, −0.035)
CS1352*	Lu-1	55.7	13.2	B	−0.643 (−1.420, 0.133)
CS1540	Su-0	53.7	−2.9	B	−0.980 (−1.498, −0.457)
CS6616	Bla-1	41.7	2.8	A†	−1.580 (−2.142, −1.018)
CS6622*	Bla-10	41.7	2.8	A	−4.434 (−5.443, −3.429)
CS6626	Br-0	49.2	16.6	A	−1.647 (−2.438, −0.868)
CS6659*	Cal-0	53.3	−1.6	A	0.102 (−0.413, 0.617)
CS6665*	Chi-1	54	34	A	−0.183 (−0.674, 0.319)
CS6669*	Co-1	40.2	−8.4	A	−0.411 (−0.902, 0.080)
CS6683	Do-0	50.7	8.2	B	−0.630 (−2.036, 0.776)
CS6688	Edi-0	55.9	−3.2	B	−0.890 (−1.391, −0.395)
CS6770	Le-0	52.2	4.5	B	0.169 (−0.347, 0.686)
CS6797	Ms-0	55.8	37.6	B	0.018 (−0.670, 0.702)
CS6807	Nok-0	52.3	4.4	B	−1.397 (−2.286, −0.508)
CS6825	Pa-1	38.1	13.4	A	−0.455 (−0.713, −0.192)
CS6834*	Pla-0	41.9	3.1	A	−0.789 (−1.466, −0.112)
CS6839	Po-0	50.7	7.1	B	−0.406 (−0.806, −0.003)
CS6854*	Sap-0	49.8	14.4	A†	−0.126 (−0.275, 0.024)
CS6855*	Sf-1	42.5	0.5	A	−2.321 (−0.288, −1.759)
CS6867*	Ta-0	49.4	14.7	A†	0.038 (−0.246, 0.327)
CS6918	Ob-2	60.1	23.3	A†	−0.586 (−0.925, −0.248)

A potential mechanism for this cline is that accessions from southern latitudes could have evolved increased vernalization sensitivity, because of shorter vernalizing cues in southern latitudes (Stinchcombe et al., 2004). Exposure to a winter in the common garden in Rhode Island, USA, might have thus produced an inappropriately precocious vernalization response. We subsequently examined whether naturally occurring variation at *FLC* contributed to the observed cline (Caicedo et al., 2004). We found two naturally occurring haplogroups of *FLC* (FLC^A and FLC^B of Caicedo et al. [2004]). Interestingly, we observed that one of the haplogroups (FLC^B) had a predominantly northern distribution in the putatively functional *FRI* background, suggesting that reduced vernalization sensitivity of this allele class could contribute to our observation of late flowering of northern accessions with putatively functional *FRI* (Caicedo et al., 2004).

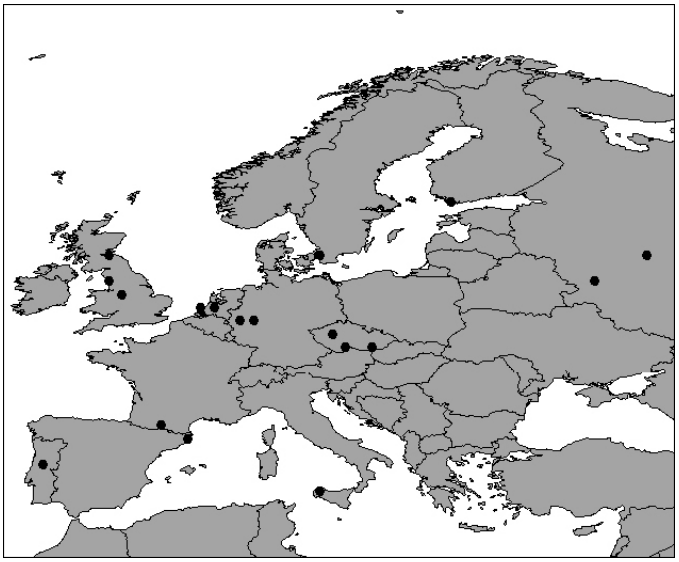

Fig. 1. Map of Europe showing the collection localities for the 21 accessions used in the experiment. Due to several accessions having identical or nearly identical geographic coordinates (Table 1), only 18 dots are shown.

Here we test the hypothesis that there is a latitudinal cline in sensitivity to vernalization length among accessions with putatively functional *FRI* alleles and explore whether variation at *FLC* mediates or contributes to any geographic patterns in vernalization sensitivity. As predicted, we found that accessions collected from southern latitudes were more responsive to vernalization cues than accessions collected from northern latitudes. We find no significant differences in mean vernalization sensitivity between accessions with FLC^A and FLC^B alleles, suggesting that the contribution of *FLC* variation to flowering time determination occurs through some other mechanism. However, we do find that accessions with FLC^A alleles, which exhibit a broader latitudinal distribution, also appear to have greater variation in vernalization responses.

MATERIALS AND METHODS

Study species—*Arabidopsis thaliana* is a primarily self-fertilizing, annual plant that germinates and grows as a rosette prior to bolting (the production of a flowering inflorescence from the apical meristem). Many accessions of *A. thaliana* have a winter annual life history strategy: plants germinate during fall, overwinter as rosettes, and then bolt the following spring (Simpson and Dean, 2002). We obtained from the stock center (http://www.arabidopsis.org) 21 accessions of *A. thaliana* that had been originally collected from Eurasia (accession stock numbers, names, and latitude of origin are given in Table 1; localities are portrayed on a map in Fig. 1). The 21 accessions we used were chosen to maximize overlap with previous studies (e.g., Olsen et al., 2004; Stinchcombe et al., 2004; Caicedo et al., 2004) and because they had been

previously identified as having putatively functional *FRI* alleles (Stinchcombe et al., 2004). Thus the present experiment is limited to investigation of *FRI-FLC*-dependent vernalization responses.

FRI sequencing—To determine whether the genotyping approach we used previously to identify putatively functional *FRI* failed to detect other nonfunctional alleles, we sequenced *FRI* in 10 of the 21 accessions used in the current experiment (Table 1). We extracted genomic DNA from these plants using DNeasy plant mini kits (QIAGEN, Valencia, California, USA) and performed PCR with *Taq* polymerase using standard protocols and published primers (Le Corre et al., 2002). We sequenced gel-purified PCR products directly using BigDye terminator reactions (Applied Biosystems, Foster City, California, USA). Sequences were run on a Prism 3700 capillary automated sequencer (Applied Biosystems). In this sequencing assay, we failed to detect any novel deletions not detected in our original genotyping screen.

FLC genotyping—To address whether accessions with different alleles at *FLC* differed in their vernalization sensitivity, we classified the accessions as belonging to one of the two *FLC* allele classes previously identified (Caicedo et al., 2004). Briefly, the two *FLC* haplogroups differ by 22 single nucleotide polymorphisms (SNPs) and six indels (see Caicedo et al., 2004). By genotyping SNPs that distinguish the two haplogroups, we were able to classify accessions into FLC^A and FLC^B allele classes, respectively (Table 1; see Caicedo et al., 2004).

Vernalization sensitivity experiment—To evaluate vernalization sensitivity in the 21 accessions, we exposed replicates of each accession to four vernalization treatments of differing duration. We planted seeds for the four vernalization treatments in a staggered fashion so that all treatments ended on the same day. For each vernalization treatment, we planted two seeds for each accession into 12 replicate cone-tainers filled with Metro Mix 360 coir (Scott's-Sierra Horticultural Products, Marysville, Ohio, USA). Seeds were then cold-stratified for 3 d in darkness at 4°C to synchronize germination. Cones were then moved to a growth chamber (20°C, 10 : 14 L : D photoperiod) for 3 d to ensure that seeds became metabolically active (after Michaels and Amasino, 1999; Michaels et al., 2003, 2004). Plants were then moved to a cold room (4°C, 10 : 14 L : D photoperiod) for either 0, 10, 20, or 30 d of vernalization, according to the vernalization treatment procedures previously described by Michaels, Amasino, and colleagues (Michaels and Amasino, 2001; Michaels et al., 2003, 2004).

In the cold room, plants were arranged in a randomized, blocked design (2 replicate cones per treatment per accession × 6 blocks). At the end of the vernalization treatment, we thinned germinants to one per cone and returned the plants to growth chambers (20°C, 10 : 14 L : D photoperiod). Each block from the cold room was moved into its own growth chamber compartment, and plants remained in randomized order. We then monitored plants until we observed bolting, and at bolting, we counted the number of rosette leaves for each plant (rosette leaf number is a common index of flowering time in *A. thaliana* (see e.g., Ungerer et al., 2002). For six plants (four of them in the 0-d treatment), we estimated vegetative rosette leaf number at 108 d because of leaf senescence. These six observations represent minimum estimates of leaf number at bolting; all results presented are robust to their inclusion or omission.

Statistical analysis—We first assessed whether there were compartment or chamber effects on rosette leaf number at bolting with a nested ANOVA model with vernalization treatment, growth chamber, compartment nested within chamber, treatment × chamber, and treatment × compartment (chamber) as independent variables. We failed to detect any effects of compartment or chamber on rosette leaf number at bolting ($P > 0.45$ for all terms involving compartment or chamber), and for all subsequent analyses these terms were pooled with the error.

We used data from the control treatment (0 d of vernalization) to estimate rosette leaf number in the absence of vernalization and data from the remaining treatments (10, 20, and 30 d vernalization) to estimate vernalization sensitivity. Using data from the 10-, 20-, and 30-d treatments, we determined whether the accessions differed in vernalization sensitivity with mixed-model ANOVA. In an ANOVA for rosette leaf number, we tested the significance of the accession × treatment interaction in a model that also included the main effects of treatment (fixed effect) and accession (random effect). A significant accession × treatment interaction term indicates that the effects of vernalization on rosette leaf number differed between accessions, i.e., that the accessions differed in vernalization sensitivity. Because a significant accession × treatment interaction can also occur due to heterogeneous variances among treatments, we also tested the significance of this interaction term with log-transformed data.

Vernalization sensitivity was estimated with linear regression by regressing rosette leaf number at bolting on days of vernalization for each accession separately. (We did not explore quadratic regression because we only had three data points for each regression.) To examine the uncertainty in these regression coefficients, we jackknifed 95% confidence limits for each accession (Table 1); however, because our primary interest is not in hypothesis testing for each regression but obtaining the best estimate of an accession's sensitivity, we focus our analysis on the regression coefficients estimated by least squares. We used these regression coefficients as our estimate of vernalization sensitivity. To facilitate interpretation, all estimated regression coefficients were subsequently multiplied by −1, so that more sensitive accessions (i.e., those with steeper slopes, showing greater reduction in flowering time in response to vernalization) would have larger positive vernalization sensitivity estimates, and less sensitive accessions (those with flatter slopes) would have vernalization sensitivity estimates closer to zero. To assess the accuracy of our estimation of vernalization sensitivity with linear regression, we evaluated the correlation between rosette leaf number in the absence of vernalization (0-d control treatment) and the fitted *y*-intercepts from the linear regressions.

After verifying the accuracy of our estimation procedure, we used the estimates of vernalization sensitivity (i.e., the regression coefficients multiplied by −1) to address three questions about vernalization in *Arabidopsis thaliana*: (1) Are accessions that are late-flowering in the absence of vernalization more sensitive to vernalization cues? (2) Is a latitudinal cline in vernalization sensitivity evident in our sample of Eurasian accessions? (3) Does variation at *FLC* significantly affect vernalization sensitivity?

To address the first question, we evaluated the correlation between flowering time in the absence of vernalization and vernalization sensitivity, as estimated by the regression coefficients. Because these traits were estimated independently for each accession with different data, the relationship between flowering in the absence of vernalization and vernalization sensitivity can be rigorously tested. We tested for latitudinal clines in vernalization sensitivity by examining the correlation between the latitude of origin of accessions and their vernalization sensitivity. Support for our hypothesis would be indicated by accessions from southern latitudes exhibiting greater vernalization sensitivity. Because we were testing an a priori directional hypothesis (Caicedo et al., 2004; Stinchcombe et al., 2004), we used a one-tailed significance test, which is appropriate in this context (Rice and Gaines, 1994). In addition to the one-tailed significance test, we also employed jackknifing to estimate confidence limits for the correlation coefficient.

To test for differences in mean vernalization sensitivity between the FLC^A and FLC^B allele classes, we utilized ANCOVA in which vernalization sensitivity was the response variable. In this analysis, *FLC* allele class was the independent variable, and latitude was included as a covariate (following Caicedo et al., 2004; Stinchcombe et al., 2004). Latitude was included as a covariate because there is a significant difference in the mean latitude of origin between accessions with FLC^A and FLC^B allele classes (Caicedo et al., 2004). We omitted five of the accessions (marked with a dagger [†] in Table 1) from this analysis because they either contained insertions that have been shown to affect *FLC* function (Gazzani et al., 2003; Michaels et al., 2003) or because we were unable to determine whether they contained insertions (Caicedo et al., 2004).

RESULTS

Accessions differ in vernalization sensitivity—The 21 accessions differed both in their mean rosette leaf number at

TABLE 2. ANOVA for rosette leaf number at the time of bolting showing significant variation among accessions in both flowering time and responsiveness vernalization treatments.

Source of variation	df	Mean squares	*F*	*P*
Vernalization treatment[a]	2	13 469.23	76.56	<0.0001
Accession[b]	20	6974.88	6.49	<0.0001
Accession × Treatment[a]	40	1115.54	6.34	<0.0001
Error	512	175.93		

[a] Vernalization treatment and Accession × Treatment term tested over mean square error.

[b] Accession term tested over a composite error term of Accession × Treatment and mean square error.

bolting and in vernalization sensitivity, as indicated by the significant accession and accession × treatment interaction for rosette leaf number at bolting in the mixed model ANOVA ($F_{20,40.58} = 6.49$, $P < 0.001$ and $F_{40,512} = 6.34$, $P < 0.001$, respectively; Table 2). Both the accession and accession × treatment interaction terms remain significant even after log transformation of the data ($F_{20,40.58} = 10.65$, $P < 0.001$ and $F_{20,40.58} = 6.30$, $P < 0.001$, respectively). The variation in vernalization sensitivity can be seen in the plot of the reaction norms for each accession (Fig. 2). The mean rosette leaf number for each accession in each treatment is presented in Appendix S1 (see Supplemental Data accompanying the online version of this article).

Accuracy of vernalization sensitivity estimates—We evaluated the accuracy of our model-fitting approach to estimating vernalization sensitivity by comparing the observed rosette leaf number in the unvernalized control treatment to the *y*-intercepts from the regressions. There was a strong correlation between the observed and estimated rosette leaf number in the absence of vernalization ($r = 0.88$, $P < 0.0001$; 95% confidence limits [CL] = 0.76, 0.94), indicating that our linear model-fitting approach was an accurate estimate of vernalization sensitivity.

Late-flowering accessions are more sensitive to vernalization—Accessions that are late-flowering in the absence of vernalization are more sensitive to vernalization cues ($r = 0.76$, $P < 0.0001$; 95% CL = 0.63, 0.81; Fig. 3). Because vernalization sensitivity and flowering in the absence of vernalization were estimated independently, this relationship is not artifactual or due to autocorrelation introduced by the estimation procedure (as it would have been had we included the 0-d treatment in the regressions and evaluated the relationship between the 0-d treatment and the slope or between the slope and intercept of the same regression).

Clinal variation exists in vernalization sensitivity—The accessions show the predicted relationship between latitude and vernalization sensitivity: accessions from northern latitudes were significantly less sensitive to vernalization cues than are accessions from southern latitudes ($r = -0.41$, $P = 0.032$; 95% CL = −0.09, −0.74; Fig. 4). In general, it appears that there is more variation among southern accessions in vernalization sensitivity, a pattern consistent with past observations of increased variation in flowering time among southern accessions (Caicedo et al., 2004; Stinchcombe et al., 2004).

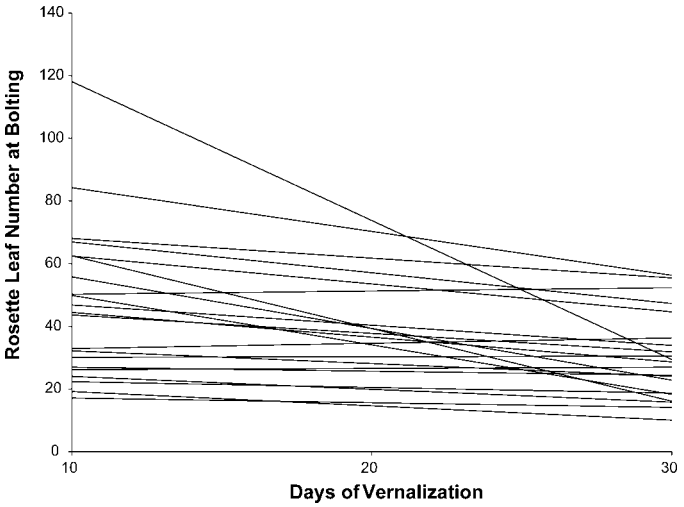

Fig. 2. Reaction norms of vernalization sensitivity for the 21 accessions. Rosette leaf number at bolting is plotted as a function of days of vernalization. Each line represents a different accession and depicts the estimated slope of a regression of rosette leaf number at bolting on days of vernalization.

FLC alleles do not differ in mean vernalization sensitivity—The ANCOVA suggests that there are no strong effects of *FLC* variation on vernalization sensitivity ($F_{1,13} = 0.01$, $P = 0.91$; Fig. 4). Inclusion of *FLC* allele class in the model also reduces the significance of latitude ($F_{1,13} = 1.23$, $P = 0.14$ for one-tailed latitude test). In large part, this likely occurs because *FLC* and latitude are correlated (there is significant latitudinal divergence between the two allele classes [Caicedo et al., 2004]), and because of the limited power of our test ($N = 16$ accessions).

The observation that southern accessions appear to vary more in vernalization sensitivity than do northern accessions is likely due to FLC^A alleles, which occur at higher frequency in southern latitudes, having greater variance than FLC^B alleles in vernalization sensitivity (Fig. 5). These data suggest that while FLC^A alleles may not significantly increase mean ver-

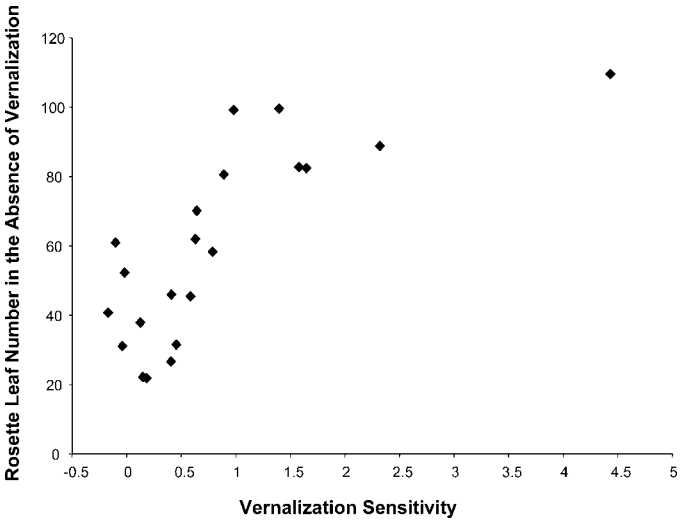

Fig. 3. Relationship between rosette leaf number in the absence of vernalization and sensitivity to vernalization. The correlation between the two variables is highly significant ($P < 0.0001$).

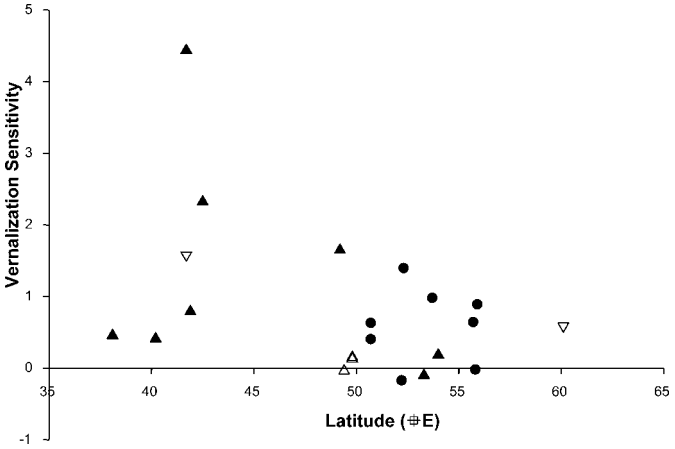

Fig. 4. A latitudinal cline in vernalization sensitivity for the 21 accessions. The correlation between vernalization sensitivity and latitude is significant ($P = 0.029$). All accessions are coded by their *FLC* allele class: filled triangles = FLC^A; filled circles: FLC^B ; open, upward-pointing triangles: FLC^A haplogroup, with insertions; open, downward-pointing triangles: FLC^A haplogroup, presence or absence of insertions unknown. Near 50° latitude, there are two FLC^A accessions with insertions above the *x*-axis with symbols plotted on top of one another because of nearly identical estimates of vernalization sensitivity.

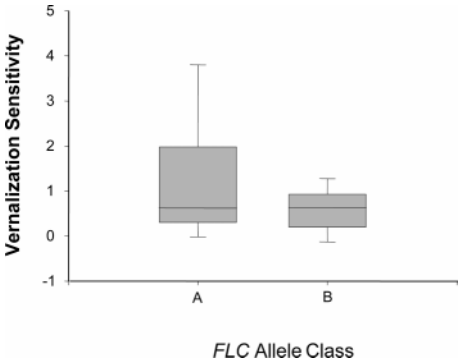

Fig. 5. Box plot depicting the 10th, 25th, 50th, 75th, and 90th percentiles of vernalization sensitivity for the FLC^A and FLC^B allele classes.

nalization sensitivity, they are associated with increased genetic variation in vernalization sensitivity, probably due to variation at other loci.

DISCUSSION

Despite the longstanding observations of variation in flowering time among accessions of *A. thaliana*, until recently there was little evidence of clinal patterns in flowering time that had been predicted based on knowledge of the developmental pathways that led to flowering (Stinchcombe et al., 2004). Our recent observation of a latitudinal cline in flowering time among European accessions grown under ecologically realistic, overwintered conditions suggested that the observed cline could have been due to variation in the responsiveness of accessions to seasonal environmental cues, including plants' responsiveness to changing photoperiods, the strength of vernalizing cues (i.e., how cold vernalization cues are), or the duration of vernalization. Here we specifically sought to test whether variation in sensitivity to vernalization duration existed among accessions of *A. thaliana*, and if so, whether it contributed to the previously observed latitudinal cline.

Although it is well known that vernalization accelerates flowering time in *A. thaliana*, few investigations have characterized quantitative genetic variation in vernalization sensitivity (but see Karlsson et al., 1993; Nordborg and Bergelson, 1999), and no geographic patterns have been detected in past studies. Similar to Karlsson et al. (1993) and Nordborg and Bergelson (1999), we found extensive natural variation in sensitivity to vernalization length; however, in contrast to their results, we found that this variation was geographically structured, such that the southern accessions are more sensitive to vernalization duration (Fig. 4). We also found a trend that suggests that accessions that flower later in the absence of vernalization are more sensitive to vernalization cues (Fig. 3). These data suggest that accessions that have been selected to flower earlier in the absence of vernalization have also evolved decreased sensitivity to vernalization. It would be interesting to determine if this is a general phenomenon for *A. thaliana*—that is, whether constitutively early flowering accessions also have reduced sensitivity to other environmental cues that promote flowering in *A. thaliana*.

Effects of FRI and FLC variation on geographic patterns of flowering time—Together with other recent findings (Caicedo et al., 2004; Stinchcombe et al., 2004), the results of the current study add to the emerging picture of the role of naturally occurring variation in *FRI* and *FLC* in generating clinal variation in flowering time in *A. thaliana*. Specifically, the cline in flowering time appears to be produced by the complex interaction of an underlying cline in vernalization sensitivity and latitudinal differentiation of *FLC*, as well as the effects of other unknown loci.

The latitudinal cline in bolting time described previously (Stinchcombe et al., 2004) was only detected among accessions with a putatively functional copy of *FRI*. Our finding that southern accessions with putatively functional *FRI* alleles flowered significantly earlier than northern accessions with *FRI* alleles suggested that southern accessions had evolved greater vernalization sensitivity. The results of the current study support this hypothesis, because there is a significant latitudinal cline in vernalization sensitivity (Fig. 4).

Our original observation that accessions with nonfunctional *FRI* did not have clinal variation suggested that variation at another gene, dependent on *FRI* functionality, also contributed to the cline. Subsequently, we examined whether variation at *FLC* contributed to variation in flowering time and to the observed latitudinal cline (Caicedo et al., 2004). We found that FLC^B alleles have a predominantly northern distribution in the putatively functional *FRI* background, suggesting that late flowering of northern accessions with putatively functional *FRI* might be due to a lower mean vernalization sensitivity of accessions with FLC^B alleles.

The results of the current study suggest that accessions with FLC^A and FLC^B alleles appear to exhibit the same mean vernalization response (Fig. 5), which does not support our original hypothesis, at least within this sample of *A. thaliana* accessions. However, it is important to note that our study examined sensitivity to vernalization length and not to sensitivity vernalization temperatures; for instance, it is possible that FLC^A and FLC^B alleles differ in their temperature sensitivity to vernalizing cues. In addition, it appears that *FLC* down-

regulates the photoperiod pathway in *A. thaliana* (specifically, *CRY2* expression; El-Assal et al., 2003); thus it also possible that the *FLC* alleles differ significantly in their effectiveness in down-regulating the photoperiod pathway.

Although *FLC* variation did not affect the mean vernalization sensitivity, we did observe increased variance in vernalization sensitivity among accessions with FLC^A alleles (Fig. 5), which is consistent with the broader geographic distribution of FLC^A (see fig. 8 of Caicedo et al., 2004). These results suggest that other polymorphic loci potentially associated with FLC^A alleles are contributing to vernalization sensitivity or, alternatively, that other loci associated with FLC^B alleles are contributing to a lack of vernalization sensitivity in those accessions. These suggestions complement other findings that vernalization can affect flowering through *FLC*-independent mechanisms (Michaels et al., 2001).

Clinal patterns in Arabidopsis thaliana—There have been several recent demonstrations of clinal patterns in a variety of traits in *A. thaliana*, including hypocotyl length (Maloof et al., 2001; Stenoien et al., 2002), growth rate (Li et al., 1998), circadian rhythm (Michael et al., 2003), bolting time, and rosette leaf number at bolting (Caicedo et al., 2004; Stinchcombe et al., 2004). The existence of clinal patterns is considered classic evidence for the action of natural selection (Endler, 1977); the growing abundance of clinal patterns in *A. thaliana* suggests that natural selection on these traits is geographically heterogeneous. Given the climatically complex range inhabited by *Arabidopsis*, it is likely that a combination of climatic factors is responsible for producing some of these clinal patterns (Hoffmann, 2002; Stinchcombe et al., 2004). Disentangling the selective forces responsible for producing these patterns will likely require several approaches. For instance, common garden experiments and climatological data (Hoffmann, 2002; Stinchcombe et al., 2004) can suggest possible selective mechanisms. This approach should ideally be coupled with experimental manipulation of the putative selective forces or reciprocal transplant experiments in the native range in which in situ measurements of natural selection are obtained.

Despite the challenge of determining the selective mechanisms responsible for producing clinal patterns in *Arabidopsis*, these patterns offer several potential resources for understanding the evolutionary genetics of ecologically important traits. Although we have focused on the effects of *FRI* and *FLC* on clinal variation flowering time and vernalization sensitivity, both flowering time and vernalization sensitivity are quantitative traits, and other loci surely contribute to the observed patterns. Fortunately, the existence of clinal variation itself offers some tools for further dissecting these traits.

By crossing accessions from different ends of a cline, it is possible to take advantage of clinal variation to identify new genomic regions (QTL) or loci that contribute to the trait (see Calboli et al. [2003] and Gockel et al. [2002] for examples in *Drosophila melanogaster*). A potential advantage to constructing QTL mapping populations in this manner is that clinal variation allows the inference that at least some of the differentiation between the parental lines has been produced by natural selection, and thus the QTL identified contribute to ecologically relevant adaptive variation. Our results suggest that recombinant inbred lines between northern and southern accessions could be especially useful for identifying and dissecting QTL for vernalization sensitivity.

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APPENDIX S1. Mean rosette leaf number at bolting under 0, 10, 20, and 30 days of vernalization for the 21 accessions used in the experiment and estimates of vernalization sensitivity. Vernalization sensitivity was estimated by regressing leaf number at bolting on days of vernalization using all the phenotypic data from the 10, 20, and 30 day treatments. Confidence limits for vernalization sensitivity were estimated by jackknifing.

		Vernalization treatment (days)				
Stock #	Name	0	10	20	30	Vernalization sensitivity
CS917	Da(1)-12	22.22	15.91	17.75	12.80	−0.150 (−0.265, −0.035)
CS1352	Lu-1	70.17	47.13	39.20	34.22	−0.643 (−1.420, 0.133)
CS1540	Su-0	99.22	68.17	53.13	49.00	−0.980 (−1.498, −0.457)
CS6616	Bla-1	82.73	49.64	34.56	18.00	−1.580 (−2.142, −1.018)
CS6622	Bla-10	109.60	120.75	63.00	31.78	−4.434 (−5.443, −3.429)
CS6626	Br-0	82.43	60.67	31.92	26.50	−1.647 (−2.438, −0.868)
CS6659	Cal-0	60.92	50.70	50.42	52.67	0.102 (−0.413, 0.617)
CS6665	Chi-1	21.83	22.67	19.50	19.33	−0.183 (−0.674, 0.319)
CS6669	Co-1	46.00	24.22	19.50	16.00	−0.411 (−0.902, 0.080)
CS6683	Do-0	62.00	67.75	62.25	55.20	−0.630 (−2.036, 0.776)
CS6688	Edi-0	80.56	60.27	58.20	42.00	−0.890 (−1.391, −0.395)
CS6770	Le-0	40.70	32.45	35.45	35.80	0.169 (−0.347, 0.686)
CS6797	Ms-0	52.30	29.08	33.71	29.11	0.018 (−0.670, 0.702)
CS6807	Nok-0	99.60	84.40	70.00	56.50	−1.397 (−2.286, −0.508)
CS6825	Pa-1	31.50	19.40	12.67	10.50	−0.455 (−0.713, −0.192)
CS6834	Pla-0	58.33	46.11	32.14	30.33	−0.789 (−1.466, −0.112)
CS6839	Po-0	26.64	30.80	30.42	22.92	−0.406 (−0.806, −0.003)
CS6854	Sap-0	37.90	27.25	23.00	24.80	−0.126 (−0.275, 0.024)
CS6855	Sf-1	88.78	63.92	5.00	17.64	−2.321 (−0.288, −1.759)
CS6867	Ta-0	31.10	24.50	28.75	25.60	0.038 (−0.246, 0.327)
CS6918	Ob-2	45.44	44.55	35.44	32.82	−0.586 (−0.925, −0.248)