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DISCORDANT LONGITUDINAL CLINES IN FLOWERING TIME AND *PHYTOCHROME C* IN *ARABIDOPSIS THALIANA*

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Using seasonal cues to time reproduction appropriately is crucial for many organisms. Plants in particular often use photoperiod to signal the time to transition to flowering. Because seasonality varies latitudinally, adaptation to local climate is expected to result in corresponding clines in photoperiod-related traits. By experimentally manipulating photoperiod cues and measuring the flowering responses and photoperiod plasticity of 138 Eurasian accessions of *Arabidopsis thaliana,* we detected strong longitudinal but not latitudinal clines in flowering responses. The presence of longitudinal clines suggests that critical photoperiod cues vary among populations occurring at similar latitudes. Haplotypes at *PHYC,* a locus hypothesized to play a role in adaptation to light cues, were also longitudinally differentiated. Controlling for neutral population structure revealed that *PHYC* haplotype influenced flowering time; however, the distribution of *PHYC* haplotypes occurred in the opposite direction to the phenotypic cline, suggesting that loci other than *PHYC* are responsible for the longitudinal pattern in photoperiod response. Our results provide previously missing empirical support for the importance of *PHYC* in mediating photoperiod sensitivity in natural populations of *A. thaliana*. However, they also suggest that other loci and epistatic interactions likely play a role in the determination of flowering time and that the environmental factors influencing photoperiod in plants vary longitudinally as well as latitudinally.

KEY WORDS: **Association mapping, clines, ecological genomics, flowering time, FRI, phenotypic plasticity, photoperiod, PHYC.**

For organisms living in seasonal environments, the timing of reproduction to coincide with favorable environmental conditions is a major determinant of fitness (e.g., Bradshaw and Holzapfel 2001; Weinig et al. 2003; Putterill et al. 2004). As such, selection on traits that allow organisms to match their reproductive timing with seasonal conditions is expected to be strong. At temperate latitudes, changes in day length act as accurate and reliable cues of oncoming changes in seasonality and temperature (Bradshaw et al. 2004; Matthias et al. 2007). As a consequence, organisms have evolved a variety of mechanisms that use photoperiod cues to appropriately time reproduction, migration, and diapause (see e.g., Vaartaja 1959; Withrow 1959; Ray and Alexander 1966; Bradshaw 1976; Bradshaw and Holzapfel 2001; Schmidt et al. 2005; Schmidt and Conde 2006; Ingvarsson et al. 2006; Hall et al. 2007).

The ability to alter the timing of development and reproduction in response to the duration and photoperiod of light is particularly important in plants, especially in semelparous life histories (Chintraruck and Ketellapper 1969; Westerman 1971; Thomas and Vince-Prue 1997; van Dijk and Hautekeete 2007). The genetic model plant *Arabidopsis thaliana* is a facultative long-day plant, and has been subject to intensive quantitative and molecular genetic investigation to characterize its photoperiod response (e.g., Westerman 1971; Karlsson et al. 1993; Lee

and Amasino 1995; Alonso-Blanco et al. 1998; Onouchi and Coupland 1998; Reeves and Coupland 2001; Simpson and Dean 2002; El-Assal et al. 2003; Mockler et el. 2003; Pigliucci et al. 2003; Ungerer et al. 2003; Valverde et al. 2004; Corbesier and Coupland 2005). Intensive research into the mechanisms controlling flowering time under long and short days has provided an unmatched understanding of the molecular and physiological basis of photoperiod responses in *A. thaliana*.

Despite the tremendous volume of information about the genetics, physiology, and molecular basis of *A. thaliana* flowering under long and short days, we still lack basic knowledge on natural variation in the photoperiod response, as well as the adaptive significance of that variation. Are genotypes that are more plastic in response to photoperiod cues (i.e., those that show greater differences between their flowering times under long and short days) at a selective advantage or disadvantage in some environments, habitats, or latitudes? In perhaps the most direct test for photoperiod adaptation in *A. thaliana*, Banta et al. (2007) showed that 21 accessions gathered from several regions of Europe did not have highest fitness in chambers set to photoperiods corresponding to their latitudes of origin. However, as Banta et al. (2007) note, such an approach does not eliminate the possibility that accessions collected from widely different latitudes and climates use photoperiod as a proxy for other sources of environmental variation, such as temperature and precipitation. In northern latitudes, where seasonality is more pronounced, the fitness cost of flowering at the wrong time might be particularly severe. Therefore, if photoperiod serves as a cue for seasonal regimes, it may be predicted that accessions from northern latitudes will show greater plasticity, or sensitivity ('sensitivity' sensu Falconer 1990; Falconer and MacKay 1996), in their response to photoperiod than accessions from more southern latitudes. However, whether patterns of natural variation in *A. thaliana* correspond to this prediction is currently unknown.

Recent advances in the molecular genetics of photoperiod-induced flowering in *A. thaliana* (Monte et al. 2003; Balasubramanian et al. 2006) allow a test of the role of specific candidate genes likely to influence photoperiod sensitivity and contribute to clinal variation. The phytochrome gene, *PHYC*, has opposing effects on flowering time under short and long days, with functional *PHYC* alleles inhibiting flowering under short days, but promoting flowering under long days (Monte et al. 2003). Further support for the potential role of *PHYC* comes from three important findings described by Balasubramanian et al. (2006), who noted that: (1) Naturally occurring genetic variation at *PHYC* significantly affects flowering time in F_2 crosses, as well as hypocotyl length in natural accessions, but primarily under short day and red-light conditions; (2) Two common haplotypes for *PHYC* occur in natural accessions and differ in gene expression and phytochrome activity; and (3) Significant latitudinal differentiation, above and beyond what is found in neutral markers, occurs among *PHYC* haplotypes. Using crosses, QTL mapping, and null mutants, both studies have shown an effect of *PHYC* on flowering under short days but not long days (Monte et al. 2003; Balasubramanian et al. 2006). However, despite strong contributions from these previous studies, it still remains unknown whether natural variation in *PHYC* affects a genotype's sensitivity to photoperiod—that is, to what extent flowering is accelerated by changes in day length. Both the differential effects of *PHYC* on flowering time under short and long days and its latitudinally biased distribution suggest that it is a strong candidate for affecting both photoperiod sensitivity and latitudinal clines in photoperiod sensitivity.

Here we test for adaptive variation in the photoperiod response in *A. thaliana* by evaluating whether photoperiod sensitivity follows a latitudinal cline in European and Asian accessions. Because seasonal variation in temperature and climate (i.e., seasonality) increases with latitude, we predicted that accessions gathered from northern latitudes would be more sensitive to changes in photoperiod cues than accessions collected from southern latitudes. To evaluate the role of genetic variation at *PHYC* in the geographic distribution of photoperiod sensitivity, we paired these analyses of clinal variation with a candidate gene mapping approach (e.g., Aranzana et al. 2005; Brock et al. 2007; Weber et al. 2007; Zhao et al. 2007; see Stinchcombe and Hoekstra 2008 for a review). For our analyses of geographic clines in both quantitative phenotypes and haplotype distributions, we control for the effects of cryptic population structure using previously published genome-wide SNP data (Schmid et al. 2005, 2006). Although clinal variation is commonly considered a hallmark of adaptive evolution, it can also be produced by a wide variety of nonadaptive processes, especially in traits with a simple genetic basis (Endler 1977; Vasemagi 2006). However, by controlling for cryptic population structure using information from neutral loci, clinal patterns above and beyond what is expected under nonadaptive scenarios may be detected (e.g., Korves et al. 2007).

Specifically, we sought to answer the following questions: (1) Is there clinal variation in photoperiod sensitivity in *A. thaliana*? (2) Does genetic variation at the phytochrome gene *PHYC* affect the plastic response of flowering time to photoperiod, or flowering time under short and long days? (3) Does the geographic distribution of *PHYC* haplotypes reflect the observed geographic pattern in flowering phenotypes? We find longitudinal clines in photoperiod sensitivity and flowering time under short days, but no evidence for a latitudinal cline in photoperiod sensitivity. Variation at *PHYC* affects flowering time under short days, but not photoperiod sensitivity, even when controlling for the effects of population genetic structure. In addition, the geographic distribution of *PHYC* haplotypes and the longitudinal cline in flowering phenotypes were discordant, suggesting that the combined effects

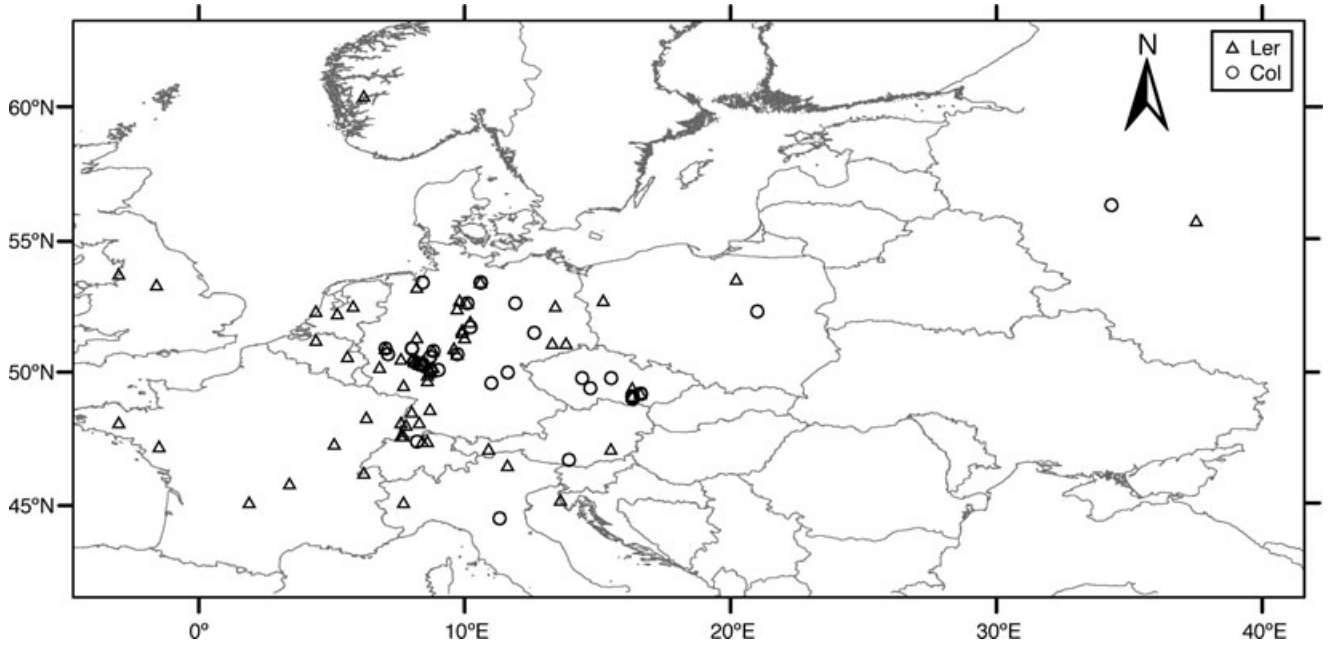

Figure 1. Geographic distribution of *PHYC* haplotypes in natural *Arabidopsis thaliana* accessions ($N = 135$). Two allele classes are distinguished by a large indel in the *PHYC* promoter and are denoted as "Ler" and "Col" to reflect the differing *PHYC* haplotypes of these widely used accessions (*Landsberg erecta*, *Columbia*). Three additional accessions (Is-1: 50.5°N/7.6°E; Li-6: 50.4°N/8.1°E and Tu-0: 45.1°N/7.7°E), were not included in the genotyping assays, but were included in photoperiod response assays (see Supporting Table S1).

of other loci on these phenotypes are of greater importance than variation at *PHYC* alone.

Material and Methods

STUDY SPECIES AND PLANT MATERIAL

Arabidopsis thaliana, commonly known as mouse-ear cress or thale cress, is an annual, rosette plant native to Eurasia and introduced to North America. As a result of the intense genetic, physiological, and developmental research on *A. thaliana*, a large number of accessions or samples have been collected across a range of habitats and latitudes (from the Mediterranean to the Arctic Circle) and are available from the stock center (*Arabidopsis* Biological Resource center, www.arabidopsis.org). The 138 Eurasian accessions used here originate from a range of approximately 15° latitude and 40° longitude (Fig. 1) and were selected to maximize overlap with Korves et al. (2007).

EXPERIMENTAL DESIGN

Seeds from 138 accessions were planted into $2^1/_4''$ round pots with Sunshine growth mix #3 (Sun Gro Horticulture Canada CM Ltd., Vancouver, B.C.), and cold stratified in the dark at 4°C for 4 days. Plants were then randomly assigned to either the short-day (SD) or long-day (LD) photoperiod treatment in separate growth chambers, with the restriction that accessions were equally represented among shelves within chambers. Growth conditions were 16:8 h light:dark under LD, and 10:14 h light:dark under SD. To ensure that photoperiod was the only difference between chambers, both treatments experienced the same light intensity (200 $\mu m/m^2/s$), and temperature regime (24°C for 16 h and 18°C for 8 h, corresponding to the LD photoperiod regime). We used six replicates per accession for the LD treatment ($N = 826$ plants). Because of limited chamber space in the SD treatment, in this treatment we used four replicates per 114 randomly selected accessions of the 138 and 5 replicates per the remaining 24 accessions ($N = 576$ plants). The SD treatment was terminated after 121 days due to a chamber failure, at which point 25 plants had failed to flower.

For each plant that germinated, we measured flowering phenology daily using two common measures of flowering time in *A. thaliana*: rosette leaf number at bolting, and the number of days between germination and the appearance of the first open flower. For the 25 plants that had failed to flower in the SD treatment, we assigned a flowering time of 121 days, and counted the number of rosette leaves as an estimate of the minimum number of leaves that would have been produced before flowering. All results presented below are robust to the inclusion or exclusion of these 25 plants.

PHYC GENOTYPING

To characterize the relative contribution of *PHYC* to variation in flowering time and in photoperiod sensitivity, we genotyped a large subset of accessions (128) for their *PHYC* haplotype. Balasubramnian et al. (2006) present convincing evidence that

PHYC is commonly found in two haplotype classes (denoted Ler and Col, because these two widely used accessions differ in their *PHYC* haplotype) that differ by a total of 40 SNPs or eight amino acid changes (in complete linkage disequilibrium between the two groups). These haplotypes are also distinguished by a large indel polymorphism 500 bp upstream from the start codon for *PHYC*.

We used this length polymorphism to distinguish between Ler and Col haplotypes in our accessions by designing primers that flanked the indel. DNA was extracted from newly germinated plants using the DNeasy Plant Kit (Qiagen, Valencia, CA). PCR conditions were 94°C 2 min, 30 cycles (94°C 15 sec, 57°C 15 sec, 72°C 30 sec), 72°C 10 min with 2 mM $MgCl_2$, 1× PCR buffer, 0.2 mM dNTPs, 0.02 U Taq polymerase (Fermentas, Burlington, Ontario, Canada) and 1μM each primer (forward 5′TTGGTGTTTCGGTCTTTTCC3′, reverse 5′TGGAACGTTCCTCCTTAGTGG3′). To confirm that we were genotyping the *PHYC* promoter, we sequenced PCR products for eight accessions and aligned them to published sequences for this region (Balasubramanian et al. 2006). The genotype of a small number of accessions (10/95) contrasted (Ler vs. Col) with those assigned by Balasubramanian et al. (2006). However, our amplification results were subsequently confirmed in two to three additional genotyping assays each (i.e., 3–4 total amplifications) and based on consistency with sequencing results (Supporting Table S1). We used our genotyping results for all subsequent analyses.

DATA ANALYSIS

Genetic variation for photoperiod sensitivity

We assessed the occurrence of genetic variation for photoperiod sensitivity using mixed-model analysis of variance (ANOVA) (Proc Mixed SAS ver. 9.1.3). We evaluated the importance of accession, photoperiod treatment (LD versus SD), growth chamber shelf, and the accession × photoperiod treatment interaction on flowering time and rosette leaf number. In these models, accession and its interaction with photoperiod treatment were random effects. A significant accession effect indicates genetic variation in the trait in the experiment as whole, whereas a significant accession × treatment effect indicates genetic variation for the plastic response to photoperiod (i.e., genetic variation for photoperiod sensitivity). The significance of random effects was tested with a likelihood-ratio test (one-tailed chi-square test with one degree of freedom) by comparing the −2·log likelihoods among models with and without the random effect of interest (Littell et al. 1996; p. 44).

Estimation of photoperiod sensitivity

We estimated photoperiod sensitivity as the difference between an accession's mean phenotype (flowering time and rosette leaf number) under SD and LD ($\Delta = \overline{X}_{SD} - \overline{X}_{LD}$, where Δ is the change in the traits, such that larger values indicate a greater values under LD). Accession means for flowering time were calculated as least-square means from the mixed model described above, which included shelf effects.

Clinal variation in photoperiod sensitivity

As a preliminary assessment of the presence of clinal variation in photoperiod sensitivity, we used multiple regression. We regressed each measure of photoperiod sensitivity (Δ rosette leaf number and Δ days until flowering) on the latitude and longitude of accession origin. In these models, a significant effect of latitude or longitude indicates geographic differentiation in photoperiod sensitivity. Because a preliminary analysis suggested that quadratic and cross-product terms were nonsignificant, here we present only linear analyses.

Contribution of PHYC to flowering phenotypes and photoperiod responses

To examine the relative contributions of *PHYC* and neutral population structure to the geographic patterns described by our preliminary analysis, we used association mapping techniques (Pritchard et al. 2000a; Thornsberry et al. 2001; Yu et al. 2005; Stinchcombe and Hoekstra 2008). We regressed phenotypes (flowering time, rosette leaf number at bolting, and plasticities) on latitude, longitude, *PHYC* haplotype, and the latitude × *PHYC* and longitude × *PHYC* interactions. We included latitude and longitude as covariates in this model for two reasons: (1) To account for geographically based genetic differentiation in flowering phenotypes due to loci other than *PHYC* in our geographically broad range (spanning approx. 15° latitude and 40° longitude) of sampled accessions, and (2) for detection of potential epistatic interactions between *PHYC* and any (unknown) loci that are latitudinally or longitudinally differentiated, which are suggested by the presence of latitude × *PHYC* or longitude × *PHYC* interactions.

Because population structure can lead to spurious associations between genes and phenotypes (see e.g., Cardon and Palmer 2003; Aranzana et al. 2005; Campbell et al. 2005; Zhao et al. 2007), we included coefficients that statistically describe an accession's inferred ancestry as covariates in our models ("inferred-ancestry estimates" from Korves et al. 2007). Briefly, the program *structure 2.0* (Pritchard et al. 2000a,b) was used to estimate a hypothetical number of ancestral populations, based on SNP data originally described by Schmid et al. (2005, 2006). Ancestry coefficients, which are continuous covariates ranging from 0 to 1, describe the proportion of an accession's genome that comes from each of these inferred ancestral populations (i.e., for each accession, the sum of its coefficients equals 1). Therefore, a significant effect of an ancestry coefficient in our analyses would suggest that variation in the traits of interest are due, at least in part, to population structure. The coefficients we analyzed were from a

model with $K = 6$ populations, and a burn-in of 30,000 runs, 40,000 repetitions for parameter estimation, and with admixture and correlated gene frequencies between populations (see Korves et al. 2007 for details). In contrast to the analysis approach used by Korves et al. (2007), we used a log-contrasts transformation of the six ancestry coefficients, in which the first five proportions were divided by the sixth, and the resulting values were subsequently log-transformed (Aitchison 1986; Blows et al. 2004). The log-contrasts transformation, designed for this type of compositional data, is necessary because all six proportions would be colinear (as they must sum to 1), but the results are robust to choice of which proportion is used as the divisor (Aitchison 1986, p. 78). In addition, as opposed to omitting a particular untransformed ancestry coefficient (the choice of which would be arbitrary) to avoid colinearity, the log-contrasts transformation uses all of the available data.

We tested whether *PHYC* haplotypes in our sample showed a latitudinally biased distribution similar to previous studies (Balasubramanian et al. 2006) using a logistic regression to model *PHYC* haplotypes as a function of latitude and longitude. We included longitude in the model because latitude and longitude are correlated throughout continental Europe and in our sample of localities. To determine if any differentiation in *PHYC* haplotypes was above and beyond what might be expected based on neutral population structure, we also performed these analyses with the estimated ancestry coefficients as covariates.

Because our sample and that of Balasubramanian et al. (2006) were not completely overlapping (95 of the 221 accessions they analyzed were also included in our sample), we repeated our analyses of latitudinal and longitudinal differentiation in two additional ways: first, by analyzing only the accessions in common between both studies, and second by analyzing a comprehensive dataset that included all unique accessions from both studies. For the comprehensive dataset, we added longitude values to the Balasubramanian et al. (2006) dataset based on published literature (preferentially, in order, using data from Korves et al. 2007; Lempe et al. 2005; Nordborg et al. 2005; and Michael et al. 2003), supplemented by searching atlases for the longitude of the locations noted by the stock centers. We were unable to determine the exact locality and hence longitudes of a subset (4/264) of the accessions. Ancestry coefficients were not included in our comprehensive dataset analysis, as these data are only available for the 138 that overlapped with Korves et al. (2007). In addition, because the geographic distribution of *PHYC* haplotypes is potentially dependent on allelic variation at the *FRIGIDA* (*FRI*) locus (Balasubramanian et al. 2006), we repeated all of the logistic regression analyses described above including *FRI* genotypes for each of the 138 accessions (Stinchcombe et al. 2004; Caicedo et al. 2004; Balasubramanian et al. 2006; Korves et al. 2007).

Table 1. Mixed model ANOVAs for flowering time (A) and rosette leaf number at bolting (B) for the growth chamber experiment. For fixed effects *F* tests are presented, whereas for random effects we present the likelihood-ratio test based on models with and without the random effect of interest.

Source	F or χ^2 Statistic	P-value
A. Flowering time		
Shelf (photoperiod treatment)	$F_{2,769}=12.12$	<0.0001
Photoperiod treatment	$F_{1,132}=843.46$	<0.0001
Accession	$\chi^2=192.3$	<0.0001
Accession × Treatment	$\chi^2=359.1$	<0.0001
B. Rosette leaf number at flowering		
Shelf (photoperiod treatment)	$F_{2,787}=6.81$	0.0012
Photoperiod treatment	$F_{1,134}=850.13$	<0.0001
Accession	$\chi^2=99.6$	<0.0001
Accession × Treatment	$\chi^2=507.0$	<0.0001

Results

Genetic variation in photoperiod sensitivity

The 138 accessions showed tremendous variation in photoperiod sensitivity (Table 1, Fig. 2A,B). As is commonly observed for *A. thaliana*, we detected more rapid flowering under LD than SD photoperiods. Under LD conditions, plants had a mean flowering time 25.7 ± 0.9 days with 19.7 ± 0.7 rosette leaves at bolting, whereas under SD conditions mean flowering time was 58.0 ± 0.9 days, and plants had 39.0 ± 0.7 rosette leaves (lsmeans ± 1 SE). In addition to these differences in the overall flowering phenotypes between the treatments, the accessions exhibited genetic variation in their flowering phenotypes and in their plasticity to photoperiod (the accession and accession × photoperiod treatment interaction terms, Table 1). The presence of accession × treatment interactions (i.e., G × E) for flowering time and rosette leaf number at bolting suggests that genetic variation exists for photoperiod sensitivity in this sample of *A. thaliana*.

Although there was a significant effect of chamber shelf, the magnitude of these differences was small (< 0.5 days and 0.5 rosette leaves in LD, and < 2.5 days and 1.9 rosette leaves in SD). However, to account for this in subsequent analyses, we estimated the mean rosette leaf number and flowering time of each accession as least-square means from a fixed effects model containing accession and chamber shelf.

Clinal variation in photoperiod sensitivity

For each accession, we estimated its sensitivity by calculating the photoperiod-induced plasticity in flowering time and rosette leaf number. These sensitivities failed to show significant latitudinal trends ($P > 0.17$), regardless of the phenotype used to estimate photoperiod sensitivity. However, in contrast to our expectations, we did detect a significant longitudinal trend in

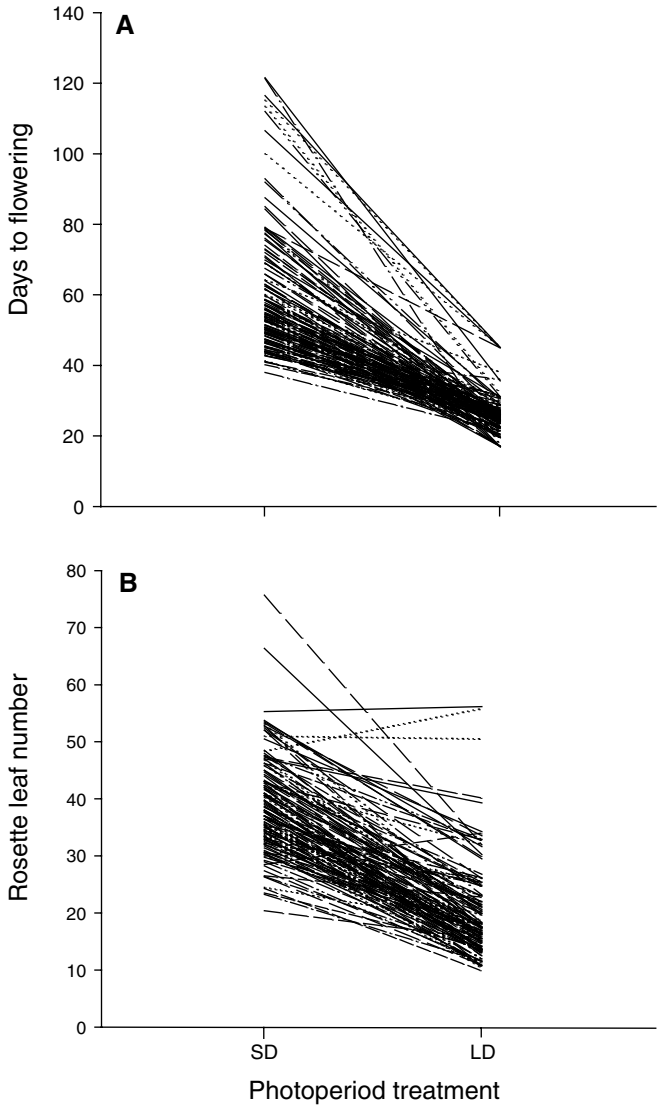

Figure 2. **Reaction norms for photoperiod response in natural *Arabidopsis thaliana* accessions. Each line connects the mean response for *n* replicates for each of 138 accessions (SD: *n* = 4 or 5; LD: *n* = 6; see text for details) between photoperiod treatments. The two treatments differed only in photoperiod length; short-days (SD) with 10:14 h light:dark versus long-days (LD) with 16:8 h light:dark. (A) Plasticity measured as days from germination until the first flower opened (days to flowering). (B) Plasticity measured as number of rosette leaves at bolting (rosette leaf number).**

photoperiod sensitivity, measured both as rosette leaf number plasticity and flowering time plasticity (Table 2). These analyses suggested that accessions collected from more eastern locations showed less photoperiod sensitivity than did accessions collected from western locations.

Clinal differentiation in PHYC haplotypes

In contrast to the results reported by Balasubramanian et al. (2006), we failed to find a latitudinal trend in the haplotype distribution of *PHYC* in our sample (Fig. 3A). Logistic regression of *PHYC* haplotype on latitude and longitude showed a nonsignificant effect of latitude (logistic regression coefficient ± 1 SE = 0.0296 ± 0.09, Wald $\chi^2 = 0.11$, $P = 0.74$), although we did detect a significant effect of longitude (logistic regression coefficient ± 1 SE = 0.1056 ± 0.04, Wald $\chi^2 = 5.65$, $P = 0.0174$). These analyses indicate that the Ler haplotype has a more western distribution than the Col haplotype (Fig. 3A,B). Moreover, the inclusion of ancestry coefficients from *structure 2.0* in the logistic regression did not affect the overall pattern: latitude remained nonsignificant ($P = 0.76$), and the effect of longitude remained significant and of similar magnitude (logistic regression coefficient ± 1 SE = 0.1171 ± 0.05, $P = 0.0181$), suggesting that the Ler and Col haplotypes were longitudinally differentiated beyond what would be expected due to population structure.

Table 2. **Multiple regressions testing for clinal variation for photoperiod sensitivity measured as the plasticity in rosette leaves (A) and in flowering time in days (B).**

Parameter	Estimate (± 1 SE)	*F*-value	*P*
A. Rosette leaf number plasticity (Δ Rosette leaves)			
Latitude of origin	0.42 (0.30)	1.94	0.17
Longitude of origin	−0.32 (0.14)	5.13	0.0251
B. Flowering time plasticity (Δ days)			
Latitude of origin	−0.39 (0.63)	0.39	0.53
Longitude of origin	−0.62 (0.30)	4.33	0.0394

To determine the potential reasons for the discrepancy between the results presented above and those of Balasubramanian et al. (2006), we repeated these analyses on two other subsets of the data: (A) Only those accessions that overlapped between studies, and (B) a comprehensive list of accessions across both studies. In the first, overlapping subset of accessions ($N = 95$), we also failed to detect a latitudinal trend in *PHYC* distribution (logistic regression coefficient ± 1 SE = 0.06081 ± 0.10, Wald $\chi^2 = 0.36$, $P = 0.55$). In this subset, we also detected a significant longitudinal trend in *PHYC* distribution (logistic regression coefficient ± 1 SE = 0.147 ± 0.06, Wald $\chi^2 = 5.84$, $P = 0.0156$). Similar to our full sample, in this subset, the Ler haplotype had a more western distribution.

Analysis of the comprehensive dataset ($N = 257$ accessions with complete data) indicated that *PHYC* shows both significant latitudinal and longitudinal differentiation ($P < 0.01$). In particular, we found that the Col haplotype showed a significantly more northern distribution (logistic regression coefficient ± 1 SE = 0.0795 ± 0.0315, Wald $\chi^2 = 6.37$, $P = 0.0116$), as previously reported. Similar to our sample of 138, we also detected significant longitudinal differentiation in the comprehensive dataset similar to our sample of 138, although of lower magnitude (logistic

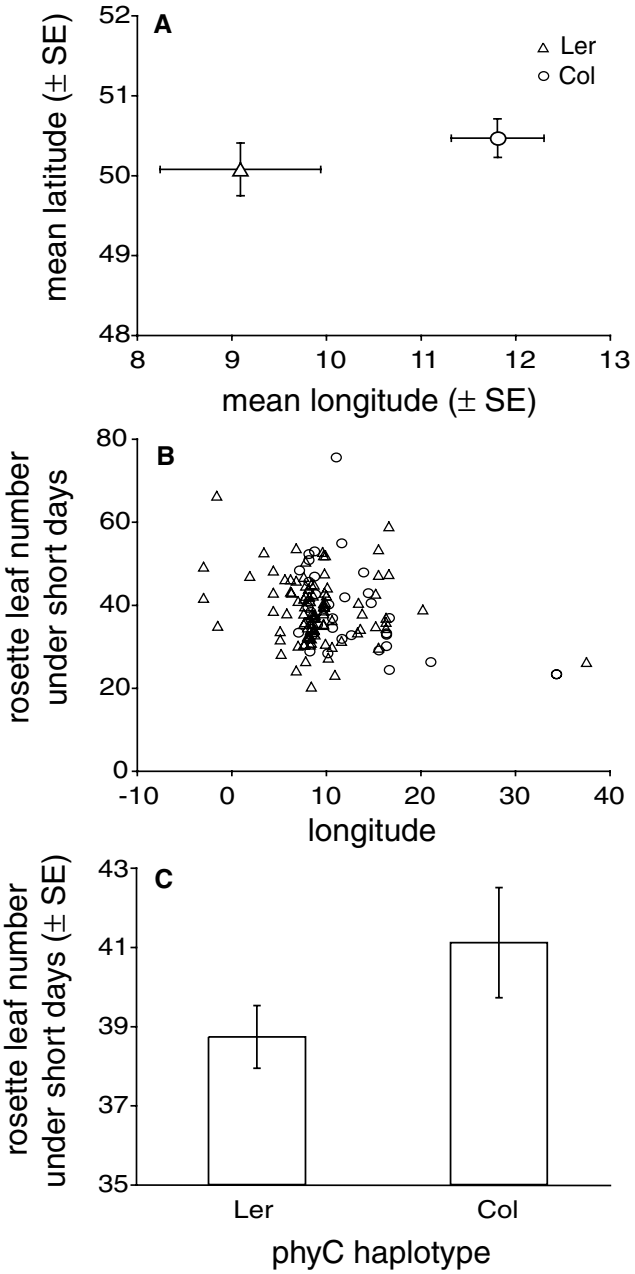

Figure 3. The distribution and effects of *PHYC* haplotype for 135 natural *Arabidopsis thaliana* accessions. (A) Mean latitudinal and longitudinal distribution of *PHYC* haplotypes (Ler *N* = 98; Col *N* = 38). (B) The association between accession longitude of origin and rosette leaf number phenotype, with *PHYC* haplotypes noted (Pearson correlation $r = -0.26$, $P = 0.0023$). Note the difference in y-axis scale from (A). (C) Variation in rosette leaf number at bolting between the two *PHYC* haplotypes (lsmeans ± SE).

regression coefficient ± 1 SE = 0.0281 ± 0.01, Wald $\chi^2 = 6.65$, $P = 0.0099$).

Finally, the inclusion of *FRI* in these models affected neither the pattern nor significance of the previous analyses; these results are presented in Supporting Table S2.

Effects of PHYC on flowering phenotypes

Using a structured association mapping approach, we failed to detect any effects of *PHYC*, latitude, longitude, or ancestry coefficients on flowering time and rosette leaf number at bolting under LD, so here we focus on the SD treatment only. There were significant effects of *PHYC* haplotype, longitude, the ancestry coefficients, and the latitude × *PHYC* interaction on rosette leaf number under SD (Table 3). The presence of a significant longitude effect indicates that other unknown loci that have differentiated longitudinally also affect rosette leaf number. For *PHYC*, the Col haplotype was associated with a significantly greater number of rosette leaves at flowering than the Ler haplotype (41.1 ± 1.4 leaves versus 38.7 ± 0.8; Fig. 3C), similar to results reported by Balasubramanian et al. (2006).

The effects of latitude on rosette leaf number under SD were nonsignificant overall, although there was a significant latitude × *PHYC* interaction. The latitude × *PHYC* interaction suggests an epistatic interaction between an unknown locus or loci that have differentiated latitudinally and *PHYC*. Closer inspection of this interaction revealed that opposite associations of haplotype with latitude caused the interaction and cancelled the main effect of latitude on the phenotype. Although rosette leaf number at bolting of the Col haplotype exhibited a nonsignificant, negative association with latitude, rosette leaf number at bolting of the Ler haplotype exhibited a contrasting and significant positive association with latitude.

Longitude had a negative effect on rosette leaf number at flowering and days to flowering under SD ($F > 5.16$, $P < 0.025$ for both), with accessions from eastern locations flowering earlier and with fewer rosette leaves (Fig. 3B). The effect of longitude on rosette leaf number under SD was similar across *PHYC* haplotypes, as indicated by the absence of a *PHYC* × longitude interaction.

Effects of PHYC on photoperiod sensitivity

Despite the significant effects of *PHYC* on one of our flowering phenotypes (rosette leaf number under SD), we failed to detect significant effects of *PHYC* on photoperiod sensitivity. These results held whether photoperiod sensitivity was estimated as the change in rosette leaf number or the change in the number of days until flowering (both $F < 2.50$, $P > 0.11$). In addition, although we found a *PHYC* × latitude interaction for rosette leaf number under SD but not LD, we failed to detect a significant *PHYC* × latitude interaction on photoperiod sensitivity as estimated by the change in rosette leaf number ($F_{1,121} = 2.04$, $P = 0.16$; Table 3). Consistent with the longitudinal trends documented in rosette leaf number at flowering, and *PHYC* haplotype (see above), we detected a significant effect of longitude on photoperiod sensitivity, as estimated by Δ rosette leaf number. More eastern accessions were less photoperiod-sensitive (i.e., a similar number of rosette

Table 3. Analysis of covariance for rosette leaf number at flowering under short days and photoperiod sensitivity, as estimated by the change in rosette leaf number between short and long day treatments (Δ Rosette leaf number). Significant effects are shown in bold.

Source	Rosette leaf number (SD)			Δ Rosette leaf number		
	df	*F*	*P*	df	*F*	*P*
Latitude	1	0.49	0.49	1	0.09	0.76
Longitude	**1**	**9.43**	**0.0026**	**1**	**4.50**	**0.0359**
PhyC	**1**	**6.89**	**0.0098**	1	2.50	0.12
Latitude × *PhyC*	**1**	**5.81**	**0.0174**	1	2.04	0.16
Longitude × *PhyC*	**1**	2.08	0.15	1	1.21	0.27
Ancestry coefficient 1	1	**6.73**	**0.0106**	**1**	0.37	0.55
Ancestry coefficient 2	1	0.02	0.88	1	1.03	0.31
Ancestry coefficient 3	1	2.20	0.14	1	1.37	0.25
Ancestry coefficient 4	1	1.51	0.22	1	1.43	0.23
Ancestry coefficient 5	**1**	**11.80**	**0.0008**	1	0.09	0.76
Error	124			121		

leaves at bolting under SD and LD). These results on photoperiod sensitivity are consistent with our findings for rosette leaf number at bolting in SD having a longitudinal trend: more eastern accessions flower earlier (i.e., in a manner more similar to LD), and hence have a smaller difference between LD and SD phenotypes.

Consequences of geographic outliers

Two of the accessions used in our experiment (CS913, CS919) have widely divergent longitudes (34.3 and 37.5°E, approx. 10° longitude or 2 standard deviations from the closest longitude in the dataset, see Fig. 1). Accordingly, we evaluated whether any of the results presented above—especially the longitudinal clines, and the effects of *PHYC* on flowering phenotypes and photoperiod sensitivity—were driven by these two accessions. The exclusion of these two outlying accessions did not affect our findings on latitudinal and longitudinal differentiation in *PHYC* haplotypes. Moreover, even after excluding these two accessions, we still detected significant longitudinal clines in flowering time and rosette leaf number under SD, and rosette leaf number plasticity (Supporting Table S3). For rosette leaf number under SD, we also detected significant effects of *PHYC,* as well as latitude × *PHYC* and longitude × *PHYC* interactions. The only qualitative difference between these results and the results including the outlying accessions was the presence of the longitude × *PHYC* interaction. Inspection of this interaction revealed that longitude always had a negative effect on rosette leaf number in SD, but that this relationship was stronger (i.e., more negative) in accessions with the Col haplotype. As noted above, significant latitude or longitude effects in these analyses suggest that other unknown loci that have differentiated latitudinally (or longitudinally) affect the traits, and latitude × *PHYC* and longitude × *PHYC* interactions suggest epistasis between *PHYC* and other loci.

The exclusion of the two eastern-most accessions also revealed a marginally significant effect of *PHYC* on rosette leaf number plasticity ($P = 0.068$). In particular, without the two Russian accessions, there is suggestive evidence that accessions with the Col haplotype showed greater photoperiod sensitivity, as measured by rosette leaf number plasticity (Col: 21.40 ± 1.50, Ler: 19.35 ± 0.81; recall that larger numbers indicate greater sensitivity).

Discussion

Despite our understanding of the molecular networks that mediate flowering time under different day lengths in *A. thaliana*, there is still little information on the adaptive significance of candidate loci affecting light response traits. We used a multivariate approach that combined information from quantitative genetic and molecular genetic analysis with genome-wide nucleotide variation to address geographic patterns in flowering time. Our results suggest a cline in flowering time in Eurasian accessions of *A. thaliana* that is consistent with longitudinal adaptation, as well as a geographically biased distribution of genetic variation at our candidate locus *PHYC,* although these patterns were not concordant. Below, we discuss possible explanations for the longitudinal gradients in flowering time and *PHYC* haplotype differentiation, their discordance, and the potential implications of the geographical patterns observed.

LONGITUDINAL TRENDS IN FLOWERING TIME AND PHYC

Environmental factors that vary clinally are expected to generate similar patterns in selection and, therefore, in ecologically important traits. The relationship we observed between plasticity and longitude suggests that selection has favored a more plastic response to photoperiod in western Eurasia. It is generally expected

that major environmental cues for flowering, such as photoperiod and temperature, vary with latitude. In contrast, the observed cline indicates that the agents determining selection on flowering time in *A. thaliana* also vary longitudinally. Although plants at the same latitude experience similar photoperiod regimes, selection may favor different photoperiod optima depending on longitude. In other words, if climate varies from west to east across the Eurasian continent, the photoperiod that signals the optimal time to flower is also likely to vary longitudinally across *A. thaliana*'s native range. Although our approach implicates clinally varying selection, ideally these relationships should be confirmed with in situ experiments that measure selection on these traits and plastic responses in ecologically realistic settings.

The cline in photoperiod plasticity appears to be driven by genetic variation in the response under short days. In *A. thaliana,* short days cue the onset of fall and cold temperatures, suggesting that the longitudinal pattern we observe is the result of geographic variation in fall cue optima. Indeed, we find that eastern accessions of *A. thaliana* flower earlier under short days, consistent with recent work suggesting that fall arrives earlier in the east (Menzel et al. 2005). In addition, longitudinal gradients in the seasonal phenology of multiple plant species suggest that selection on life-history traits might vary accordingly. For example, a recent study of long-term (1879–1998) plant phenological data (e.g., bud burst, first flower, leaf coloring, fruit ripening, etc.) found that longitudinal variation was at least as significant as latitudinal variation for determining seasonal onset (Menzel et al. 2005). Menzel et al. (2005) suggest that longitudinal variation may result from the climatic effects of oceanic current oscillations, which vary depending on the degree to which populations are inland versus coastal (i.e., "continentality"). A similar pattern was also observed in European beech populations (*Fagus sylvatica* L.) at a finer spatial scale ($\sim 8^\circ$ longitude) in Poland: more easterly populations were found to cease growth earlier in fall (Chmura and Rozkowski 2002). Moreover, earlier senescence resulted in increased fitness, suggesting that longitudinal variation in the timing of fall onset has generated adaptive responses to seasonal cues in beech. Combined, these results indicate that a full understanding of the evolution of flowering phenology will require knowledge of the environmental factors that vary longitudinally as well as latitudinally.

Although it is formally possible that the longitudinal cline we have detected in flowering time is due solely to drift or historical forces rather than selection, we think this possibility is less likely. First, the longitudinal cline in flowering time remained significant even after accounting for neutral population structure inferred from SNP data, the latter of which should reflect primarily historical forces. Second, it less likely that neutral processes could create a cline in flowering time, a quantitative trait influenced by several loci than in traits determined by one or a few loci, where stochastic forces may predominate (Endler 1977; Vasemagi 2006). In addition, flowering time in *A. thaliana* is likely to affect fitness in a range of settings: past studies have shown that temperature-based plasticity in flowering time is under selection in laboratory environments (Stinchcombe et al. 2004), that there are genetic correlations between flowering time and other ecologically important traits (resistance to mammalian herbivory: Weinig et al. 2003; dehydration avoidance: McKay et al. 2003), and that there are associations between variation at flowering time genes and fitness in the field (Korves et al. 2007).

We also found evidence that, after accounting for population structure, variation at the candidate locus *PHYC* influenced flowering time in *A. thaliana* with Ler haplotypes flowering earlier than Col haplotypes. This is the first broad-scale demonstration of an association between *PHYC* genotype and quantitative genetic variation for flowering time across a wide sample of natural accessions of *A. thaliana*. In light of previous studies using QTL mapping, F_2 crosses, and mutant analysis that suggested a role for *PHYC* in this trait (Monte et al. 2003; Balasubramanian et al. 2006), we now have further evidence that *PHYC* plays an important role in natural variation in flowering time. However, our results also suggest that other loci clearly influence flowering time, and the possibility that epistatic interactions between *PHYC* and other unknown loci also contribute. Accordingly, the association of *PHYC* with flowering time is geographically, genetically, and evolutionarily complex.

Although one would predict that the distribution of *PHYC* haplotypes should reflect the observed cline in phenotype (Fig. 3B), instead we see the opposite pattern: Ler haplotypes are common in the west (Fig. 3A and 3B) and are earlier flowering (Fig. 3C). Thus, the longitudinal clines in phenotypes and genotypes appear to be conflicting (Fig. 3A–3C). At minimum, these data suggest that the effects of other genes on flowering under SD are of greater combined magnitude than the effects of *PHYC* alone. Furthermore, the geographic pattern at *PHYC* remained after accounting for neutral population structure, raising the possibility that the longitudinal differentiation in *PHYC* haplotypes is adaptive, although disentangling whether the geographic distribution of *PHYC* represents purely historical factors or a combination of historical and selective forces is likely to be quite challenging (see below). One hypothesis for the discordance between haplotype distribution (whatever its mechanistic origin) and the cline might be that *PHYC* has pleiotropic effects on both flowering time and another trait that has been the target of selection; however, this particular trait(s) remains a mystery. *PHYC* has been suggested to influence other phenotypes, including hypocotyl growth (Monte et al. 2003; Balasubramanian et al. 2006), seedling de-etiolation, petiole elongation, and cotyledon expansion (Monte et al. 2003), and possibly germination timing (Poppe and Schäfer 1997). More work on how such traits vary in natural populations or in response

to photoperiod could shed light on why selection might act on these or other traits in a longitudinal manner.

One caveat of a candidate gene mapping approach is the potential for residual neutral population structure to remain unexplained, therefore biasing inferences of the causal effects of a locus (Zhao et al. 2007), or geographically adaptive patterns. Indeed, longitudinal differentiation at neutral marker loci has been documented for several species across Eurasia, including *A. thaliana* (Skrede et al. 2006; Marmi et al. 2006; Beck et al. 2008). As the longitudinal distribution of *PHYC* mirrors the longitudinal distribution of other neutral markers, it is also remains possible that the geographic differentiation of *PHYC* is due to nonselective reasons, or more likely, a mixture of historical and selective forces (given the effects of *PHYC* on ecologically important traits). To address this, we performed an additional analysis to determine whether latitudinal or longitudinal patterns were evident in the neutral population structure in our sample. Regressions of our inferred ancestry coefficients (which are based on SNPs, and should be more reflective of historical and demographic forces) on latitude and longitude indicated significant latitudinally and longitudinally distributed population structure (data not shown). Although we cannot ascertain definitively that we have accounted for all neutral population structure in our sample by including these ancestry coefficients in our flowering time model, this result increases our confidence in the longitudinal cline detected in flowering time and haplotype distribution.

LATITUDINAL VARIATION IN PHYC AND FLOWERING TIME

There is considerable support for latitudinal clines in flowering time in *A. thaliana* from experiments under both controlled and under natural conditions (Stinchcombe et al. 2004; Lempe et al. 2005). Because temperature and photoperiod both vary latitudinally and are known to promote flowering in *A. thaliana*, it has been hypothesized that these factors are the environmental cues to which plants have adapted. Although there is support for latitudinal clines in vernalization responses (i.e., responses to prolonged winter cold; Stinchcombe et al. 2005; Lempe et al. 2005), there has been comparatively little investigation of geographic trends in photoperiod responses. However, two lines of evidence suggested that it is possible that *A. thaliana* may have adapted to photoperiod: (1) Accessions in a common garden study exhibited a significant effect of latitude of origin on flowering time, even after controlling for site of origin winter and summer temperature effects with multiple regression (Stinchcombe et al. 2004), and (2) There is abundant variation in photoperiod responses among natural accessions, even after accounting for the effects of genetic variation at major flowering time genes such as *FRI* and *FLC* (Lempe et al. 2005).

In the sample of 138 accessions used in the photoperiod experiment, we observed neither a latitudinal cline in the candidate gene nor the phenotypic response. However, we did detect latitudinal differentiation in *PHYC* in the comprehensive dataset (i.e., unique samples from our experiment + those of Balasubramanian et al. [2006]), which mirrors the patterns previously described by Balasubramanian et al. (2006), even when controlling for the effects of longitude. These results suggest that our failure to detect latitudinal differentiation in *PHYC* in our sample was due more to its particular composition, rather than the absence of this pattern in Eurasian samples of *A. thaliana* more generally. The potential sensitivity of accession studies to the geographic composition of the sample has been observed before. For instance, Shindo et al. (2005) failed to detect a latitudinal cline in flowering time in a set of accessions that included samples from both Europe and the invasive range (North America) in single analysis, whereas both Stinchcombe et al. (2004) and Lempe et al. (2005) detected latitudinal clines in flowering time while analyzing accessions solely from Eurasia. Likewise, Korves et al. (2007) concluded that the effects of the *FRI* and *FLC* genes on flowering time and fitness in a field study were sensitive to the geographic composition of the sample. These examples point to the need for caution in accession studies, because the material used will almost certainly represent different (and incomplete) portions of the geographic range. As a consequence, these studies will include accessions from potentially very different climates, ecological settings, and demographic and selective histories.

Apart from the composition of the sample, our failure to detect clinal variation in flowering phenotypes and photoperiod plasticity also has several potential biological explanations. First, as described above, the *PHYC* locus failed to show latitudinal differentiation in our sample. As *PHYC* variation affected flowering time under SD but not LD, it is possible that we failed to detect latitudinal trends in photoperiod plasticity because of *PHYC's* lack of latitudinal differentiation in our sample. Two lines of evidence suggest support for this interpretation. In the comprehensive dataset, in which *PHYC* is latitudinally differentiated, the Col *PHYC* haplotype shows a more northern distribution. The nonsignificant trend in our set of 138 accessions was for accessions with the Col *PHYC* haplotype to show greater photoperiod plasticity. Second, removal of the two outlying Russian accessions revealed a marginally significant ($P = 0.068$) effect of *PHYC* on photoperiod plasticity, again with the Col haplotype exhibiting greater plasticity. Given the statistically marginal support for these interpretations, however, this hypothesis requires further testing.

Another potential reason for the absence of latitudinal clines in our data may be due to the experimental growth conditions. For instance, we equalized the temperatures between SD and LD treatments to isolate the effects of day length per se, even though this approach does not account for the fact that SD photoperiods are

often accompanied by colder temperatures. As such, if *A. thaliana* has adapted to both photoperiod and temperature cues, it may be possible to detect evidence of the former only in experimental environments that accurately mimic natural temperature fluctuations. Past studies finding latitudinal clines in flowering responses have either provided vernalization cues experimentally or naturally (Stinchcombe et al. 2004, 2005; Lempe et al. 2005). Recent studies manipulating photoperiod and temperature simultaneously (Li et al. 2006; Scarcelli et al. 2007) have successfully detected epistatic interactions involving the vernalization pathway (*FRI*, *FLC*) and other flowering time genes (*FLM*) using this approach, suggesting that it would also be useful for dissecting photoperiod responses.

CONCLUSIONS

A current challenge in evolutionary biology is to understand the contribution of individual genomic regions to adaptive patterns in complex, ecologically important traits. With respect to plant adaptation to light cues, variation at the *PHYC* locus represents one piece of the puzzle. However, our results suggest that environmental cues other than photoperiod and genes other than *PHYC* are both crucial components of an integrated response to seasonal cues. Fully understanding the genetic basis of adaptation to the ecological complexity experienced by natural populations of *A. thaliana*, and any other species, will require an integration of ecological, molecular, and quantitative genetic approaches (Mitchell-Olds and Schmitt 2006). Addressing this challenge will require pairing ecological approaches such as reciprocal transplant and common garden experiments (e.g., Callahan and Pigliucci 2002; Griffith et al. 2004; Stinchcombe et al. 2004; Korves et al. 2007; Rutter and Fenster 2007) with molecular, genomic, and quantitative assessment of ecologically important traits.

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Supporting Information

The following supporting information is available for this article:

Table S1. Complete listing of Arabidopsis accession stock numbers, accession abbreviations, latitude and longitude of origin, mean flowering phenotypes, and *PHYC, FRI,* and *FLC* genotypes.

Table S2. Effects of latitude, longitude, and *FRI* genotype on the distribution of *PHYC* haplotypes. (A) Analysis of 138 accessions used in the photoperiod experiment ($N = 135$), (B) Analysis of a subset of accessions common to the photoperiod experiment and Balasubramanian et al. (2006) ($N = 95$), and (C) Analysis of the comprehensive dataset including data from both studies ($N = 255$). For A–C, all parameters have 1 df.

Table S3. Analysis of rosette leaf number at bolting under SD, flowering time under SD, and rosette leaf number plasticity excluding two accessions (CS913, CS919) with widely divergent longitudes.

This material is available as part of the online article.