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Indirect effects of *FRIGIDA*: floral trait (co)variances are altered by seasonally variable abiotic factors associated with flowering time

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Abstract

Reproductive timing is a critical life-history event that could influence the (co)variation of traits developing later in ontogeny by regulating exposure to seasonally variable factors. In a field experiment with *Arabidopsis thaliana*, we explore whether allelic variation at a flowering-time gene of major effect (*FRIGIDA*) affects (co)variation of floral traits by regulating exposure to photoperiod, temperature, and moisture levels. We detect a positive latitudinal cline in floral organ size among plants with putatively functional *FRI* alleles. Statistically controlling for bolting day removes the cline, suggesting that seasonal abiotic variation affects floral morphology. Both photoperiod and precipitation at bolting correlate positively with the length of petals, stamens, and pistils. Additionally, floral (co)variances differ significantly across *FRI* backgrounds, such that the sign of some floral-trait correlations reverses. Subsequent experimental manipulations of photoperiod and water availability demonstrate direct effects of these abiotic factors on floral traits. In sum, these results highlight how the timing of life-history events can affect the expression of traits developing later in ontogeny, and provide some of the first empirical evidence for the effects of major genes on evolutionary potential.

Introduction

The timing of reproduction is a critical life-history event, particularly in sessile organisms such as plants. Relative to plants that reproduce early in the season, those that delay reproduction until later will accumulate more resources and potentially increase the quantity and quality of resulting offspring. Additionally, the timing of reproduction can influence how parents and offspring interact with the biotic environment (e.g. available mates, pollinators, predators, herbivores, etc.) and abiotic environment (e.g. photoperiod, temperature, water availability, etc.). In angiosperms, much effort has been directed towards identifying the genes and intragenic variants that underlie phenotypic variation in flowering phenology (Michaels & Amasino, 2000; Simpson & Dean, 2002; Corbesier & Coupland, 2005) and thus can be favoured by selection. The use of forward and reverse genetics has resulted in the characterization of many flowering-time genes. Because the action or the magnitude of effect of individual loci can vary across environmental settings (Tonsor *et al.*, 2005), recent attempts to annotate flowering-time genes have employed more realistic field conditions (Stinchcombe *et al.*, 2004; Korves *et al.*, 2007). One overlooked issue, however, is that the genes regulating the timing of biological events, like reproduction, could also indirectly influence the (co)variation of traits developing later in the life history by regulating environmental conditions encountered by parents (or offspring). Such indirect effects could influence both the opportunity for and response to selection. Here we utilize a common-garden field experiment and subsequent growth-chamber experiments to test if a gene of major effect in the flowering time pathway affects (co)variation of floral traits in *Arabidopsis thaliana* by determining the environment of developing flowers.

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Vernalization, the extended exposure of plants to cold temperatures, can release floral repression, yielding plants that are more sensitive to pathways that promote flowering (e.g. the photoperiod pathway, shade-avoidance pathway, and autonomous pathway). Napp-Zinn (1955, 1957) utilized natural variation in *A. thaliana* accessions to identify one of the major genes involved in the vernalization pathway, *FRIGIDA* (*FRI*). Expression of functional *FRI* promotes the flowering repressor, *FLOWERING LOCUS C* (*FLC*), which in turn is repressed by the mitotically stable action of vernalization (Michaels & Amasino, 2000). In natural populations of *A. thaliana*, variation at *FRI* is due primarily to the emergence of multiple, independent loss-of-function alleles (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Shindo *et al.*, 2005). Despite the occurrence of hundreds of flowering-time genes, allelic variation at *FRI* can account for a substantial proportion of variation in flowering time in natural populations of *A. thaliana* (between 12.6% and 70% depending on experimental conditions) (Shindo *et al.*, 2005; Scarcelli *et al.*, 2007). In addition, homologues in the vernalization pathway have been shown to regulate flowering time responses in related Brassicaceous species, suggesting that the genetic characterization of flowering time in *A. thaliana* may well apply to other species (Schranz *et al.*, 2002; Kuittinen *et al.*, 2008).

Molecular evolutionary (Le Corre *et al.*, 2002; Le Corre, 2005) and selection analyses (Korves *et al.*, 2007; Scarcelli *et al.*, 2007) suggest that variation at *FRI* may be partially maintained by geographically variable selection. For example, in geographic regions with long cold winters, plants with functional *FRI* and *FLC* alleles will overwinter as vegetative rosettes and flower in the subsequent spring (i.e. plants express a winter annual life history). Plants homozygous for nonfunctional *FRI* alleles lack floral repression due to *FLC* and are sensitive to additional genetic mechanisms that promote flowering. These nonfunctional *FRI* alleles are hypothesized to lead to a rapid-cycling phenotype (i.e. plants express a spring or fall annual life-history), whereby plants germinate, reproduce, and senesce within a single season. Rapid-cycling phenotypes, which can also arise from nonfunctional or weak *FLC* alleles (Gazzani *et al.*, 2003; Michaels *et al.*, 2003), are hypothesized to be selectively advantageous under milder climates (Korves *et al.*, 2007). Thus, natural variation at *FRI* results in plants that can flower under exceedingly different seasonal conditions, with winter/spring annuals germinating in the fall and spring, respectively, but both flowering under warm long-day conditions of spring, whereas fall annual plants germinate and reproduce under cooler shorter photoperiod conditions of autumn. In fact, plants from southern latitudes with putatively functional *FRI* alleles are more sensitive to vernalization conditions and flower significantly earlier than those from more northern latitudes in field environments (Stinchcombe *et al.*, 2004, 2005).

If floral traits (e.g. petal, stamen, and pistil length) are dependent on either internal resource status or on external abiotic conditions that vary with the timing of reproduction (e.g. photoperiod, temperature, water availability), the direct role of *FRIGIDA* in regulating the timing of flowering may also *indirectly* influence floral morphology. Moreover, if some floral traits are more sensitive to environmental conditions than others, trait (co)variation will likely vary across environments. Floral traits are commonly less plastic to variation in the environment than vegetative traits (Berg, 1960; Andersson, 1994; Armbruster *et al.*, 1999; Brock & Weinig, 2007); however, correlative and experimental studies have found that floral traits vary across abiotic gradients. As water availability declines corolla area also declines (Galen, 1999, 2000; Elle & Hare, 2002; Lambrecht & Dawson, 2007), which may be a plastic response to reduce costly floral transpiration or an indirect effect of reduced carbon assimilation in dry sites. Corolla length is also positively correlated with ambient temperature prior to flowering in the morning glory, *Ipomoea trichocarpa* (Murcia, 1990). Finally, shifts in day length should influence total daily plant carbon assimilation, which may in turn influence floral morphology. Independent of total irradiance, previous research also indicates that floral meristem identity genes (*APETALA1* and *APETALA2*) in the 'A' whorl of the ABC floral model influence floral/inflorescence traits differentially across photoperiods (Okamuro *et al.*, 1996, 1997).

Here, we test how reproductive timing, partially controlled by the major flowering time locus, *FRIGIDA*, influences (co)variation in the size of floral organs by examining flowers collected from the field experiment originally described in Stinchcombe *et al.* (2004). We then explore whether shifts in the abiotic environment (photoperiod, temperature, and rainfall patterns) that coincide with variation in bolting day can account for possible clinal variation in floral morphology. Finally, we directly test the influence of photoperiod and water availability on floral morphology in separate growth-chamber and greenhouse experiments to explore whether variation in these abiotic factors directly influence floral morphology.

Materials and methods

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) is a small weedy annual plant native to Europe and central Asia. Eurasian populations of *A. thaliana* grow and reproduce across a wide range of abiotic conditions associated with the species' expansive latitudinal distribution that ranges from ~68°N to ~0°N (Koornneef *et al.*, 2004). Plants of *A. thaliana* germinate predominantly in the fall, overwinter as vegetative rosettes, and reproduce during the spring and early summer months (i.e. most populations exhibit a winter annual life history) (Baskin & Baskin, 1983; Nordborg & Bergelson, 1999), although populations with

spring or fall rapid-cycling strategies exist (i.e. spring or fall annual life histories) (Griffith *et al.*, 2004). At bolting, the apical meristem produces an indeterminate inflorescence bearing largely autonomously self-fertilizing flowers (Abbott & Gomes, 1989), which are composed of sepals (4), petals (4), stamens (4 long + 2 short), and a compound pistil (2 carpels).

Rhode Island common-garden experiment

On October 2, 2001, seeds of 89 *A. thaliana* accessions were planted in common garden plots near the greenhouses at Brown University in Providence, RI, USA (41°49.417′N, 71°25.333′W) and allowed to germinate, overwinter, and flower under natural abiotic conditions (see Supporting Information Table S1 for accessions). The common-garden experimental design, methods for determining *FRI* genotype, and discussion of the interaction between *FRI* functionality and latitude of accession origin on flowering time are detailed in Stinchcombe *et al.* (2004). Briefly, one replicate of each accession was randomly assigned to a peat pot in each of four spatial blocks nested in a raised soil bed. There were three beds for a maximum of 12 replicate plants for each accession. We planted 3–5 seeds of a given accession in a 5.08 cm × 5.08 cm pot filled with Metromix 360 (Scotts-Sierra Horticultural Products, Marysville, OH, USA). Pots were sunk into the beds to a level flush with the soil surface and separated from adjacent pots by 2.54 cm. Fine wire mesh was placed over the beds to limit potential seed movement by rainfall. Following germination, the wire mesh was removed, and seedlings were thinned to one plant per pot (for further details and monthly abiotic conditions see Stinchcombe *et al.*, 2004).

Plants were censused daily for days to bolting (i.e. time between planting and differentiation of the primary inflorescence from the basal rosette) and rosette diameter at bolting. Bolting time is a fundamental determinant of plant fitness (Mitchell-Olds & Schmitt, 2006; Korves *et al.*, 2007), because it affects the likelihood that fruits mature prior to the end of the growing season and, as hypothesized here, because it potentially determines the abiotic and biotic conditions that maturing buds and flowers experience. Once plants began to flower, a total of three newly opened (first-day) flowers were collected from each replicate plant and stored in 70% ethanol. Flowers were collected from inflorescence nodes 4–12, which are developmentally equivalent floral positions (Diggle, 1997). Flowers were dissected and photographed under a Nikon SMZ800 Stereoscope fitted with a digital camera. Using ImageJ (ImageJ ver 1.31; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), we measured the following floral traits: petal length of one haphazardly selected petal, filament length (as a proxy for stamen length) of one haphazardly selected long stamen, and pistil length (from receptacle to stigma). Additionally, we estimated herkogamy distance, or the separation between stigma and anther, as the difference between pistil and filament length. Floral metrics from the three flowers were averaged for each plant.

Data analysis

We estimated least-squares means (lsmeans) of each floral trait (petal length, stamen length, pistil length, and herkogamy) for each accession using a mixed model analysis of variance (ANOVA; Proc GLM, SAS, vs 8.02; SAS Institute Inc., Cary, NC, USA) with the following random terms: planting bed, block nested in bed, accession, and the accession by bed interaction. We used ANCOVA to test for an effect of latitude of origin, *FRI*, or the latitude by *FRI* interaction on lsmeans of petal length, stamen length, pistil length, and herkogamy. Results of general linear models (tests of significance and resulting lsmeans) are presented for ease of comparison with results presented in a previous manuscript (Stinchcombe *et al.*, 2004); no qualitative differences between significance tests were found using this approach vs. restricted maximum likelihood estimates. We further explored significant latitude by *FRI* interactions by testing for an effect of latitude of origin on floral morphology separately for each *FRIGIDA* functional class. For putatively functional *FRI* alleles, which showed significant latitudinal variation in floral morphology, we ran two additional regression models. First, we tested if the effect of latitude on floral morphology could be explained by average days to bolting, with the expectation that bolting time determines floral exposure to abiotic conditions that affect floral morphology. We also evaluated whether variation in average rosette diameter could explain variation in the size of floral organs, under the hypothesis that larger more vigorous genotypes might also have larger flowers. In addition, we examined if abiotic conditions occurring on bolting day influenced floral morphology. Using climate data obtained from the National Climate Data Center (Providence, RI, USA; T.F. Green Airport, 12.3 km S of Brown University), we tested if latitude of origin, length of the day (photoperiod) at bolting, average temperature at bolting (average temperature on the day of bolting and one day prior), or average rainfall (total daily rainfall averaged for the bolting day and four days prior) explained variation in floral morphology.

To explore the relationship between floral genetic architecture and flowering phenology, we tested for differences between genetic variance-covariance matrices (**G**-matrices) of putatively functional and nonfunctional *FRIGIDA* allele classes. **G**-matrices traditionally refer to the genotypic (co)variance among traits within a population of randomly mating individuals and can be used to predict the response of a trait (or vector of traits) to multivariate selection within a population (Lande, 1979; Lande & Arnold, 1983). Here, *A. thaliana* accessions are from distinct populations sampled over a broad

geographic range (see Supporting Information Table S1); comparisons of **G**-matrix structure are used to explore developmental and genetic mechanisms underlying floral morphology. Floral **G**-matrices for each allele class were estimated from the variances and covariances of genotypic lsmeans of petal length, stamen length, pistil length, and herkogamy (Proc CORR, SAS, vs 8.02). **G**-matrices were then compared using common principal components analysis (CPC), which distills each matrix into a hierarchical range of relatedness and tests for significant differences between allelic class (Phillips & Arnold, 1999). First, the CPC model tests if the matrices share eigenvectors by testing for differences within each principal component level of relatedness, that is do **G**-matrices differ at principal component 1 (*CPC1*), *CPC2*, …, to *CPC* (p-2), where p equals the number of traits in the **G**-matrix. Next, the model test for full *CPC* indicating matrices share all principal components but have at least one significantly different eigenvalue (i.e. the amount of variance explained by each principal component). Matrices are then compared for *Proportionality*, where eigenvalues of each shared principal component are proportional by a single constant. Finally, matrices are *Equal* and thus share eigenvectors and eigenvalues. We tested an additional set of **G**-matrices (petal, stamen, and pistil length), because herkogamy is a mathematical function of pistil and stamen length. We used the 'jump-up' approach when evaluating CPC results which compares each level of relatedness to the null hypothesis of unrelated structure (Phillips & Arnold, 1999). Because each level of the hierarchy includes the lower levels of relatedness, testing stops once a significant difference is obtained. CPC analysis can be sensitive to normality; as a consequence, we used lsmean trait values in CPCrand, a programme that determines test significance via a randomization procedure (Phillips & Arnold, 1999). Tests were based on 10 000 permutations, and matrices did not require 'bending' to ensure a positive definite matrix.

The above **G**-matrix comparisons explore how multivariate relationships among floral traits change with an allelic substitution at a major flowering time locus; however, CPC analyses do not identify the specific matrix elements that underlie potential significant differences. To examine bivariate floral–floral elements that may contribute to **G**-matrix differences across seasonal settings, we estimated pairwise correlations between floral traits within *FRI* backgrounds using the same traits as above (Proc CORR, SAS, vs 8.02) and then tested for significant differences between *FRI* backgrounds using Fisher's Z-test.

Growth-chamber and greenhouse experiments

Because many factors vary simultaneously in field settings, we carried out growth-chamber and greenhouse experiments to directly test the effects of two abiotic factors (photoperiod and water availability) that were correlated with shifts in floral morphology in the field experiment. We manipulated the abiotic variables of photoperiod in a growth-chamber experiment and tested the effects of water availability in a greenhouse drought experiment. In the photoperiod experiment, we grew a subset of accessions used in the field experiment (N = 58, see Supporting Information Table S1) in two long-day chambers (16L : 8D) and two short-day chambers (8L : 16D). For each of the four chambers, the 58 accessions were randomly assigned to three cells within four Araflat trays (Betatech bvba; Gent, Belgium). We planted 3–5 seeds per cell containing Metromix 200 (Sun Gro Horticulture; Bellevue, WA, USA) and then stratified seeds for 4 days in the dark at 4 °C to promote synchronous germination. Each of the four sets of trays was placed in a Conviron E7/2 compartment (Conviron Controlled Environments Limited, Winnipeg, MB, Canada) and seeds were germinated under 12L : 12D light cycles (~350 μmol m^{-2} s^{-1}) at 22 °C. Six days later, we initiated photoperiod treatments (16L : 8D or 8L : 16D) and thinned seedlings to 1 seedling/cell. Plants were censused for flowering date, and three newly opened flowers were collected and placed into 70% ethanol for storage. Flowers were harvested from developmentally equivalent nodes (positions 5–12; Diggle, 1997) 2 h after subjective dawn (i.e. 2 h after the lights turned on in the chamber), which approximated the time of flower collection in the field. Flowers were then dissected and imaged as described above.

To test the influence of water availability on floral morphology, we grew nine accessions of *A. thaliana* (see Supporting Information Table S1), including the wild-type Landsberg *erecta* (L*er*) and Columbia (Col) accessions in two moisture-level treatments. We also added two near isogenic lines (NILs) in which functional *FRI* alleles from the San Feliu-2 (sf-2) accession had been introgressed into wild-type L*er* and Col backgrounds, which carry different null *FRI* alleles (Lee *et al.*, 1994; Lee & Amasino, 1995). Comparison of wild-type vs. introgressed genotypes enables us to test for possible direct effects of *FRI* on floral morphology. Each of the 11 total genotypes was grown under watered (control) and drought treatments in a greenhouse at the University of Wyoming (Laramie, WY, USA). We sowed 3–5 seeds of each genotype in 6 cm square × 5 cm tall pots containing a mixture of Sunshine LP5 planting mix and sand (2 : 1, respectively) and 60 mg of Osmocote slow-release fertilizer (18-6-12; Scotts, Marysville, OH, USA). In a fully-randomized design, we planted six replicates per treatment of the NILs and four replicates per treatment of the remaining *A. thaliana* accessions. Seeds were stratified in the dark for 5 days at 4 °C and were then placed in growth chambers (20 °C; 10L : 14D) for 3 days to ensure metabolic activity (Michaels & Amasino, 1999; Michaels *et al.*, 2003; Stinchcombe *et al.*, 2005). Plants were then vernalized in a cold room under simulated short photoperiods (4 °C; 10L : 14D) for 20 days and finally moved to the green-

house with supplemental lighting (12L : 12D). Seedlings were thinned to 1 plant per pot and allowed to grow for 2 weeks with ample water, after which time replicates were assigned to treatments and positions within a fully-randomized design.

Plants in the control treatment received 20 ml of water daily while those in the drought treatment received 10 ml once soil water content (SWC) fell below −4.8% (EC-5 ECH_2O system; Decagon Devices, Pullman, WA, USA)—a previously estimated wilting point for *A. thaliana* inflorescences (Brock & Weinig, unpublished data). Treatments were imposed 8 days prior to bolting, which was predicted by daily monitoring of two additional replicates of each genotype that had been planted 8 days prior to the experimental plants and taken through the exact vernalization process described above. Experimental plants were censused for date of first flower production, at which time two newly opened flowers were collected, preserved, and dissected as described previously. Following flower collection, we also recorded soil water content and collected the third youngest leaf to determine the effectiveness of drought treatments on plant water status. Leaves were immediately weighed to the nearest milligram (fresh mass) and scanned to estimate leaf area. Leaves were then floated on distilled water (5 °C) for 24 h prior to reweighing (saturated mass) and then dried at 65 °C for 48 h to obtain leaf dry weight. For each leaf we estimated specific leaf area (SLA; surface area/dry mass) and leaf relative water content [RWC; (fresh mass-dry mass)/(saturated mass-dry mass)], which have been shown to covary with water availability (Cunningham *et al.*, 1999; Lawlor & Cornic, 2002; Reich *et al.*, 2003).

Data analysis

Variation in floral morphology (petal length, stamen length, pistil length, and herkogamy) in response to experimental photoperiod was examined using ANOVA (Proc MIXED, SAS, vs 8.02), including photoperiod treatment as a fixed factor and the following random terms: chamber nested within treatment, accession, and the accession by treatment interaction. Because we were specifically interested in the direct effects of abiotic factors on floral morphology, we did not include the *FRI* locus as a factor in our analyses of this experiment. Moreover, our sample of natural accessions was not large enough ($N = 58$) to attain sufficient sample sizes in each allelic category to test for potentially variable responses of *FRI* genotypes to photoperiod. Significance of random terms in these models (and in Proc MIXED hereafter) was calculated by the log likelihood method (Littell *et al.*, 1996).

We tested for effects of drought treatment on floral morphology (petal length, stamen length, pistil length, and herkogamy) using ANOVA (Proc MIXED, SAS, vs 8.02) with treatment designated as a fixed factor and accession and the accession by treatment interaction as random factors. This ANOVA model was used for all nine wild-type accessions (i.e. including L*er* and Col wild-type backgrounds). We also examined the effect of drought treatment on SWC, RWC, and SLA of plants of the nine wild-type accessions using the same ANOVA model. To test for a direct effect of allelic variation at *FRI* on floral morphology, we partitioned variation in petal length, stamen length and pistil length among *FRI*, the drought treatment, and the *FRI* by treatment interaction in separate analyses for each genetic background (i.e. L*er* and Col). Plant mortality during the experiment reduced sample sizes; however, all genotypes had at least two replicates in each treatment (average replicates per treatment 4.8 for wild-type and *FRI* NILs and 3.6 for remaining accessions).

Results

Rhode Island common-garden experiment

In our common-garden experiment, *A. thaliana* plants reproduced across a wide range of seasonal conditions (early January to late March). Collectively, accessions with putatively functional *FRI* alleles bolted earlier (due to increased vernalization sensitivity of southern accessions, see Stinchcombe *et al.*, 2005, 2004) and exhibited a broader reproductive phenology than those with non-functional *FRI* alleles (mean days to bolting = 155.6 and 164.5, respectively; variance = 340.8 and 41.3, respectively). ANCOVA demonstrates that the length of individual floral organs (petal, stamen, and pistil) varies significantly with the latitude of origin by *FRI* interaction (Table 1, Fig. 1). Herkogamy distance (i.e. stigma–anther separation) was significantly greater among accessions from lower latitudes but was not influenced by *FRI* class or the latitude by *FRI* interaction (Table 1, Fig. 1). To further explore the origin of the *FRI* by latitude interaction, we tested for a latitudinal cline after splitting the dataset by *FRI* functional class; floral morphology (petal length, stamen length, and pistil length) varies significantly with latitude in the functional *FRI* background ($F_{1,\ 27} = 10.48$, $P = 0.0032$; $F_{1,\ 27} = 22.35$, $P < 0.0001$; $F_{1,\ 27} = 4.51$, $P = 0.0429$, respectively), but not in the nonfunctional *FRI* background ($F_{1,58} = 1.88$, $P = 0.18$; $F_{1,58} = 0.14$, $P = 0.71$; $F_{1,58} = 2.56$, $P = 0.12$, respectively). This latitudinal cline in floral morphology of functional *FRI* plants became nonsignificant after including average days to bolting in the model, suggesting that abiotic factors experienced by the plants from germination to bolting strongly influence floral morphology (Table 2a). Rosette diameter (a positive measure of plant vigour) did not significantly contribute to variation in floral morphology (Table 2a). The final analysis explored if exposure to three abiotic factors at bolting (photoperiod, temperature, and precipitation) accounted for variation in floral morphology. ANCOVA

Table 1 Analysis of covariance of petal length, stamen length, pistil length, and herkogamy for 89 accessions of *Arabidopsis thaliana* grown in a common garden in Rhode Island. ANCOVA partitioned variation in floral traits among the latitude at the site of origin of each accession, the allelic functionality at the *FRIGIDA* locus (putatively functional or nonfunctional), and their interaction. Significant effects are highlighted in bold.

	Petal length			Stamen length		Pistil length		Herkogamy	
Source of variation	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Latitude	1, 85	1.88	0.17	11.39	**0.0011**	0.03	0.87	13.54	**0.0004**
FRI	1, 85	11.47	**0.0011**	15.23	**0.0002**	6.60	**0.0119**	1.03	0.32
Latitude × FRI	1, 85	11.07	**0.0013**	15.16	**0.0002**	6.66	**0.0116**	0.98	0.33

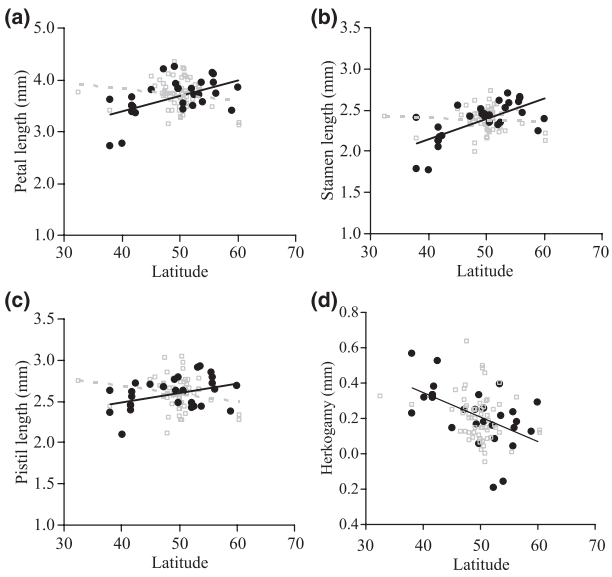

Fig. 1 Graphs illustrating *FRI* × Latitude interactions for (a) petal length, (b) stamen length, and (c) pistil length and a latitudinal cline in (d) herkogamy of 89 *Arabidopsis thaliana* accessions from a common-garden experiment in Rhode Island. Filled circles represent accessions with putatively functional *FRI* alleles that exhibit significant clinal variation (a–c) indicated by the thick solid line. Open gray squares signify nonfunctional *FRI* accessions, which lacked clinal variation in floral traits (a–c; nonsignificant relationship indicated by gray dashed line for comparison). In (d), *FRI* genotype symbols are maintained to illustrate that the cline in herkogamy (thin solid line) was not significantly influenced by *FRI*.

indicates that increases in photoperiod at the time of bolting significantly increased the length of all floral traits measured (Table 2b). Petal length and pistil length also increased significantly with increasing amounts of precipitation during the time of bolting (Table 2b).

G-matrices composed of petal length, stamen length, pistil length, and herkogamy differed significantly across *FRI* backgrounds (CPC1; $P = 0.0187$), indicating that the first principal component differed significantly across *FRI* **G**-matrices. We detected strong correlations between petal, stamen, and pistil length in both putatively functional and nonfunctional *FRI* backgrounds (Table 3; see Supporting Information Table S2 for *P*-values). Interestingly, correlations with herkogamy shifted in both significance (stamen-herkogamy and pistil-herkogamy correlations) and direction (petal-herkogamy) across the *FRI* backgrounds (Table 3; Fig. 2). Fisher's *Z*-tests between pairs of bivariate correlations in petal-herkogamy, stamen-herkogamy, and pistil-herkogamy were all significantly different across *FRI* backgrounds ($Z = 3.10$, $P = 0.002$; $Z = 2.78$, $P = 0.005$; $Z = 2.91$, $P = 0.004$, respectively); however, the remaining bivariate correlations between petal, stamen, and pistil length were not significantly different (Table 3; *Z*-tests, all $P > 0.37$).

Growth-chamber and greenhouse experiments

On average across photoperiod treatments, accessions differed significantly in the size of all floral organs (Accession; Table 4). Although the main effect of photoperiod treatment was nonsignificant (Treatment; Table 4), accessions exhibited significant variation in responsiveness to photoperiod (Accession × Trt; Table 4; Fig. 3). Many of the accessions that responded to shifts in photoperiod produced longer petals, stamens, and pistils under long days, which is the same direction of the observed floral trait-photoperiod correlation in plants grown under common-garden conditions (Table 2b). Although, on average, plants flowered significantly faster under long photoperiods (data not shown), days to flower did not significantly account for variation in floral morphology when included as a covariate in these previous ANOVA models (data not shown), suggesting that observed differences across photoperiod treatments resulted from the direct action of photoperiod on flowers rather than from an indirect effect on developmental rate.

On average across moisture treatments, accessions again differed significantly in the expression of all floral traits (all Accession *P*-values ≤ 0.0007; Supporting Information Table S3). Relative to plants in the control moisture treatment, droughted plants exhibited a significant reduction in the size of all measured floral traits (Fig. 4; see Supporting Information Table S3 for ANOVA). The accession by treatment interaction was nonsignificant for all floral traits (all Accession × Trt *P*-values ≥ 0.27; Supporting Information Table S3).

Table 2 Results of multiple linear regression analyses testing the effects of (a) latitude of origin, rosette diameter, and bolting day and (b) latitude of origin and three abiotic factors during reproduction on petal, stamen, and pistil length for accessions of *Arabidopsis thaliana* with putatively functional *FRI* alleles. Significant effects are highlighted in bold.

	Petal length					Stamen length				Pistil Length			
Source of variation	d.f.	Estimate	T	P	PVE (%)	Estimate	T	P	PVE (%)	Estimate	T	P	PVE (%)
(a)													
Latitude	1,25	0.01	0.77	0.45	1.9	0.01	1.68	0.1	6.8	0	0.3	0.77	0.3
Rosette Diameter	1,25	0.06	0.52	0.61	0.9	0.05	1.05	0.3	2.7	0.08	1.27	0.22	5.5
Days to bolting	1,25	0.01	2.24	**0.0341**	16.3	0.01	3.53	**0.0016**	30.1	0	1.69	0.1	9.8
(b)													
Latitude	1,24	0	0.24	0.81	0.2	0	0.84	0.41	1.6	0	−0.28	0.78	0.2
Photoperiod	1,24	0.28	2.77	**0.0106**	20.6	0.23	4.36	**0.0002**	42.9	0.13	2.15	**0.0416**	13.6
Temperature	1,24	−0.01	−0.85	0.4	1.9	0	−0.63	0.54	0.9	0	−0.05	0.96	0.0
Precipitation	1,24	0.27	2.21	**0.037**	13.1	0.03	0.49	0.63	0.5	0.16	2.28	**0.0318**	15.3

Table 3 Bivariate correlations between four floral traits in accessions of *Arabidopsis thaliana* with (a) putatively functional *FRI* alleles and (b) nonfunctional *FRI* alleles grown in a common-garden experiment in Rhode Island, USA. Superscripts indicate significance of individual correlations and bold formatting indicates that Fisher's *Z*-test comparisons of correlations across *FRI* backgrounds were significantly different (all significant *Z*-test results $P < 0.01$).

	Petal length	Stamen length	Pistil length	Herkogamy
(a) Putatively function FRI				
Petal length	–	0.78***	0.69***	**−0.33**†
Stamen length	–		0.71***	**−0.61****
Pistil length			–	**0.11**
Herkogamy				–
(b) Non Functional FRI				
Petal length	–	0.70***	0.79***	**0.36****
Stamen length	–		0.71***	**−0.06**
Pistil length			–	**0.66*****
Herkogamy				–

† = 0.07, **< 0.01, ***< 0.001

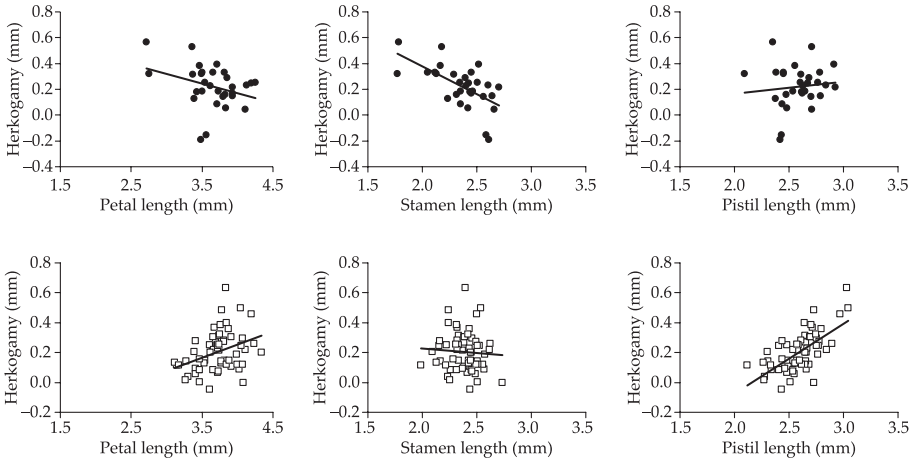

Fig. 2 Bivariate correlations between herkogamy (i.e. stigma–anther separation) and the length of three floral traits (petal, stamen, and pistil) measured from 89 *Arabidopsis thaliana* accessions raised under common-garden conditions in Rhode Island. Filled circles represent accessions with putatively functional *FRI* alleles while open squares indicate accessions with nonfunctional *FRI* alleles.

Our experimental drought treatment successfully reduced water-availability at flowering. Average soil water content was reduced by 75.4% for plants in the drought treatment ($F_{1,21} = 63.52$, $P < 0.0001$) and average leaf relative water content declined by 8.5% under drought [86.7 ± 1.3% (mean ± SE) and 79.3 ± 1.6% RWC for control and drought, respectively; $F_{1,76} = 13.78$, $P < 0.0004$]. Accession and the accession

Table 4 Mixed model ANOVA results partitioning variation in floral traits among the fixed photoperiod treatment and random effects of chamber, accession, and accession by treatment interaction. Significant effects are highlighted in bold.

	Petal length			Stamen length			Pistil length			Herkogamy		
Sources of variation	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Photoperiod treatment	1, 2	0.89	0.44	1, 2	8.46	0.09	1, 2	5.58	0.13	1, 2	0.09	0.78
Random factors	d.f.	Chi	*P*	d.f.	Chi	*P*	d.f.	Chi	*P*	d.f.	Chi	*P*
Chamber (Trt)	1	5.7	**0.0085**	1	8	**0.0023**	1	12	**0.0003**	1	0.3	0.29
Accession	1	318	**< 0.0001**	1	286	**< 0.0001**	1	274.1	**< 0.0001**	1	294.4	**< 0.0001**
Accession × Trt	1	11.9	**0.0003**	1	9.4	**0.0011**	1	14.3	**< 0.0001**	1	3.9	**0.0241**

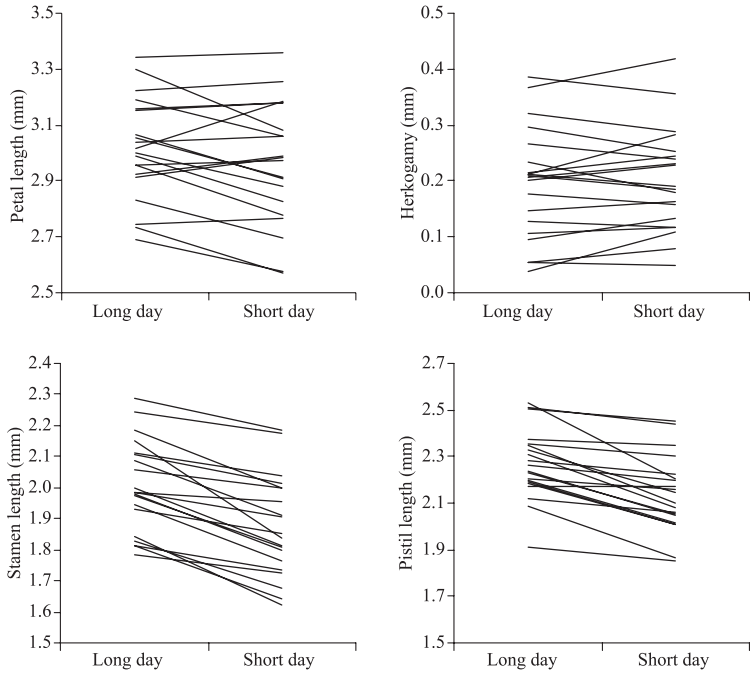

Fig. 3 Reaction norms of floral morphology from *Arabidopsis thaliana* plants grown under long-day (16 h) and short-day (8 h) photoperiods. For illustrative purposes, we present best linear unbiased predictors of 20 accessions that are the most plastic (10 accessions with the greatest increase in each long and short-day treatment).

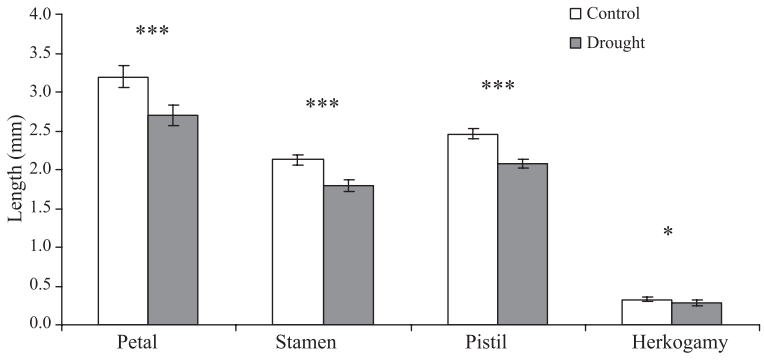

Fig. 4 Average floral trait expression (± SE) of nine accessions of *Arabidopsis thaliana* accessions raised in the greenhouse under control and drought treatments. Significance levels of drought treatment indicated by asterisks for each floral trait (*$P \leq 0.05$; *** $P < 0.001$).

by treatment interaction did not explain a significant amount of the variation in SWC or RWC ($P \geq 0.16$ for all tests). Specific leaf area was 16.3% lower among plants in the drought treatment relative to those in the control treatment [0.48 ± 0.029 cm^2 mg^{-1} (mean ± SE) and 0.57 ± 0.027 cm^2 mg^{-1}, respectively; $F_{1,\ 68.7} = 24.61$, $P < 0.0001$]. Accessions expressed significant genetic variation for SLA ($\chi^2 = 36.5$, d.f. = 1, $P < 0.0001$), but

responded similarly to treatments (Accession × Trt, $\chi^2 = 0$, d.f. = 1, $P = 1.0$).

Allelic differences at the *FRI* locus (e.g. the introgressed Sf-2 allele vs. the wild-type Col allele) did not affect petal, stamen, or pistil length (all *FRI* *P*-values ≥ 0.11, Supporting Information Table S4). As was observed in the accessions, drought treatment again explained a significant amount of variation in petal, stamen, and pistil length in the *FRI* near-isogenic lines (all Treatment *P*-values ≤ 0.0126, Supporting Information Table S4). The *FRI* by treatment interaction was nonsignificant (all *FRI* × Trt *P*-values ≥ 0.18, Supporting Information Table S4).

Discussion

Ecological evaluation of FRI effect

In a common-garden field experiment along the Northeast coast of North America (RI, USA), we utilized the natural range in flowering phenology of *A. thaliana* accessions to explore the relationship between reproductive timing and floral morphology. We observed that allelic variation at the major flowering time locus, *FRI*, was associated with significant shifts in the (co)variation of floral traits. This association most likely arises from the effects of flowering time on exposure of developing flowers to seasonally variable abiotic factors, and highlights the potential effects of major genes on genetic architecture and, thus, evolutionary dynamics.

Floral morphology of 89 *A. thaliana* accessions varied significantly with the *FRIGIDA* by latitude interaction. This interaction resulted from the significant clinal variation in floral traits (petal, stamen, and pistil length) in plants homozygous for putatively functional *FRI* alleles, which was not detected in plants with nonfunctional *FRI* alleles (Fig. 1). The cline in floral morphology is similar in direction and pattern to the *FRI* association with flowering time (Stinchcombe *et al.*, 2004; Korves *et al.*, 2007). It is worth noting that population structure can produce spurious results in association mapping studies (Pritchard *et al.*, 2000; Cardon & Palmer, 2003); however, the *FRI*-bolting association was robust following a statistical correction for population structure (Korves *et al.*, 2007) and was later validated in a segregating population (Scarcelli *et al.*, 2007).

The possibility still exists that population structure causes a spurious association between *FRI* and the expression of floral traits. This, however, seems unlikely, because not only functional but also nonfunctional *FRI* genotypes exhibited floral plasticity to photoperiod in our growth-chamber experiment (data not shown), suggesting *FRI* is not in linkage disequilibrium with a gene that directly affects the expression of quantitative variation in floral organ size. Furthermore, near isogenic lines, harbouring either functional or nonfunctional *FRI* alleles in an otherwise similar genetic background, were likewise responsive to moisture levels in our greenhouse study. Thus, although floral (co)variation observed in the common-garden experiment could be due to population structure, to us, a more parsimonious explanation is that abiotic factors coincident with reproductive timing underlie the observed variation in floral morphology. Several independent pieces of evidence support this interpretation: there is strong genetic evidence for the effects of *FRI* on flowering time in controlled crosses and transformations (Napp-Zinn, 1955, 1957; Johanson *et al.*, 2000), clinal trends in flowering time in accessions are mediated by *FRI* as mentioned above (Stinchcombe *et al.*, 2004; Lempe *et al.*, 2005; Korves *et al.*, 2007), variation in flowering time exposes accessions to different environmental cues (this common garden study), and experimental manipulation of those environmental cues affects the phenotypes of interest (these chamber and greenhouse experiments). Further support for this interpretation could come from studies utilizing large segregating progenies (i.e. F2's, RIL's, backcrosses), in which recombination can break up associations between *FRI* and other genes.

We examined the influence of seasonal conditions on floral morphology by testing if three abiotic factors accounted for the clinal variation observed in putatively functional *FRI* accessions. Variation in photoperiod and precipitation at the time of bolting were both positively associated with the length of floral organs (Table 2b). Our results indicate that putatively functional *FRI* accessions exhibited a broad flowering phenology (January to March) and suggest that exposure to seasonal variation in photoperiod and precipitation directly affected the average expression of floral morphology. Similar patterns between corolla size and water availability have been shown in other systems (Galen, 2000; Carroll *et al.*, 2001; Lambrecht & Dawson, 2007), and although the influence of photoperiod needs further examination in wild species, horticultural studies demonstrate that *Fragaria* × *ananassa* produces longer stamens under longer daylengths (Voyiatzis & Paraskevopoulou-Paroussi, 2002). Average floral trait expression did not vary with temperature at bolting in the field. Temperature could still influence floral morphology via a significant genotype by environment interaction; however, we cannot test for floral plasticity in this common-garden experiment, because accessions are exposed to only one set of seasonal conditions. Not only do our results support a direct role of abiotic factors in shaping floral morphology, but they further suggest the possibility of 'environmentally mediated epistasis,' whereby *FRI* influences the timing of reproduction and thus the phenotypic expression of other loci that directly influence floral morphology (e.g. the phenotypic effect of floral-organ size loci may be sensitive to seasonal abiotic factors). The observed clinal variation in floral morphology could also arise from the direct effects of an epistatic interaction between *FRI* and a gene (or genes) distributed in a clinal pattern, as suggested by the *FRI* × latitude interaction.

The genetic architecture of floral traits, as estimated by floral **G**-matrices differed significantly across putatively functional and nonfunctional *FRI* backgrounds. CPC analysis revealed that these floral **G**-matrices did not share their first eigenvector (i.e. the principal component axis that explains the most variation), suggesting fundamental differences in floral genetic architecture across *FRI* allelic groups. After splitting this dataset into early and late flowering cohorts to explicitly test whether flowering time mediates observed the differences in genetic architecture across *FRI* alleles, we found parallel differences in the first principal component across phenology cohorts (data not shown). These results strongly suggest that allelic variation at a major flowering-time gene impacts floral genetic architecture, which presumably results via floral exposure to different seasonal conditions.

Environmentally induced shifts in (co)variation of floral organs may impact plant reproductive success and evolutionary responses to selection. For predominantly selfing species, like *A. thaliana*, selection favours reduced spatial separation between stigma and anther (herkogamy) (Kalisz *et al.*, 2004; Moeller & Geber, 2005; Brock & Weinig, 2007). Likewise, traits such as overall floral size or corolla size (Alexandersson & Johnson, 2002; Bloch & Erhardt, 2008) and stamen and pistil length (or their exsertion from the corolla) (Cresswell, 2000; Morgan & Conner, 2001) can alter the rate and efficiency of pollen import and export in outcrossing species. Given these patterns of selection, the observed differences in **G**-matrices could ultimately result in divergent evolutionary trajectories of floral morphological traits between early and late flowering plants. For example, examination of floral correlation matrices demonstrated striking differences in the direction and significance of bivariate correlations between floral organ lengths and herkogamy across *FRI* backgrounds (Table 3, Fig. 2). Because variation in herkogamy influences proportional fruit set in *A. thaliana* (Brock & Weinig, 2007), selection for reduced stigma–anther separation should result in larger petaled flowers in early-flowering cohorts (negative herkogamy-petal length correlation; Table 3) while producing smaller petaled flowers in later cohorts (positive herkogamy-petal length correlation; Table 3). Taken together, the observed shifts in floral trait expression and genetic architecture demonstrate that annotation of this and other genes that regulate phenological traits should be evaluated in natural settings for potential effects on traits expressed later in ontogeny.

Causal tests for effects of abiotic factors on floral morphology

We experimentally manipulated photoperiod in a growth-chamber experiment and water availability in a greenhouse experiment to test for a direct relationship between these abiotic factors and floral morphology. *A. thaliana* is a facultatively long-day plant, and as a consequence, the vegetative phase of the life history was shorter on average under long photoperiods (data not presented). Nevertheless, many accessions produced larger petals, stamens, and pistils under long days (similar to the positive relationship observed between floral organ size and photoperiod in the common-garden field conditions). Floral plasticity to the photoperiod treatment is not therefore mediated through variation in flowering time, and may arise from plant responsiveness to variation in cumulative photosynthetic photon flux density across long and short days or to the photoperiod cue itself. Manipulations in the day-length cue can alter regulation of floral meristem identity in mutants of *APETALA2* (*AP2*), a floral meristem/organ identity gene integral to the *Arabidopsis* ABC model of floral development (Okamuro *et al.*, 1993, 1997). Under short-day cues, mutants in *AP2* have enhanced inflorescence-like characteristics and produce secondary flowers. Interestingly, these short-day dependent phenotypes can be suppressed by ectopic addition of gibberellins (GA) (Okamuro *et al.*, 1997), which are themselves regulated by photoperiod (Metzger & Zeevaart, 1980, 1982).

In the greenhouse, water availability also strongly influenced floral size. In comparison with control plants, those in the drought treatment exhibited an average reduction of 15.2% (range: 13.9–15.8%) for measured floral traits (Fig. 4). Several proxies for plant water status (SWC and RWC) also declined significantly in drought treatments, as did specific leaf area, which varies negatively with plant water use efficiency (Cunningham *et al.*, 1999; Reich *et al.*, 2003). Together these plant and soil measures indicate the efficacy of our drought treatments and support a direct effect of water limitation on floral morphology. Floral size has been shown to decline under limited water availability in other study systems in both greenhouse and field settings (Galen, 2000; Carroll *et al.*, 2001; Lambrecht & Dawson, 2007). Reductions in plant water status can severely impact plant physiological processes. For example, high leaf to air vapour pressure deficits and attendant increases in transpiration lead to stomatal closure in water-limited plants, which in turn can limit access to ambient CO_2 and reduce rates of carbon assimilation. Floral structures have a limited ability to regulate the loss of water through transpiration (Nobel, 1977; Galen *et al.*, 1993) and as a consequence, selection may favour reduced floral size to limit costs associated with plant water stress. Our greenhouse results, in concert with those of other studies, support a causal role between precipitation at bolting and floral variation in our common-garden experiment (Table 2b).

Conclusions

The timing of reproduction is a crucial component of fitness, because it can influence the expression of traits in the maternal plant (e.g. plant size, number of flowers,

likelihood of fruit maturation) as well as traits expressed in the progeny generation. For instance, maternal flowering phenology can influence progeny life-history strategy (e.g. winter annual, spring annual, autumn flowering), by regulating the seasonal conditions to which offspring are exposed (Galloway, 2002; Donohue, 2005; Donohue et al., 2005a,b). Here, we find evidence that variation in *FRI* mediates an indirect effect on floral morphology by regulating flowering time, which exposes bolting plants to systematic shifts in seasonal conditions that in turn directly influence floral morphology. Our growth-chamber experiment manipulating photoperiod and greenhouse experiment on plant water relations support the role of these seasonal abiotic factors in shaping floral morphology. In addition, floral morphological responses similar to those reported here in *A. thaliana* have been observed in seasonal cohorts of the outcrossing relative *Brassica rapa* (Brassicaceae) (Weinig & Brock, unpublished data), suggesting that floral plasticity to abiotic factors may be prevalent across species and breeding systems. Interestingly, our common-garden experiment suggests that allelic differences in a single gene can also be associated with substantial differences in the genetic (co)variation of complex phenotypes, which influences these traits' evolutionary potential. Finally, these results stress the need for gene characterization in natural settings, because novel functions can be exposed.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 A complete list of *Arabidopsis thaliana* accessions, associated latitude of origin, and functionality of the *FRIGIDA* locus (*F* = putatively functional *FRI* and *N* = nonfunctional *FRI*) used in three experiments detailed in this manuscript.

Table S2 Bivariate correlations and associated *P*-values. Accessions with putatively functional FRI alleles (*N* = 30) are above the diagonal while those with nonfunctional alleles are below the diagonal (*N* = 60).

Table S3 Mixed model ANOVA results of drought treatment on four floral traits in nine accessions of *Arabidopsis thaliana*. Significant effects are highlighted in bold.

Table S4 ANOVA for variation in petal, stamen, and pistil length across *FRIGIDA* alleles and drought treatment in the (a) Columbia and (b) Landsberg *erecta* backgrounds of *Arabidopsis thaliana*. Significant effects are highlighted in bold.

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