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Research

Fluctuating, warm temperatures decrease the effect of a key floral repressor on flowering time in *Arabidopsis thaliana*

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Summary

- The genetic basis of growth and development is often studied in constant laboratory environments; however, the environmental conditions that organisms experience in nature are often much more dynamic.
- We examined how daily temperature fluctuations, average temperature, day length and vernalization influence the flowering time of 59 genotypes of *Arabidopsis thaliana* with allelic perturbations known to affect flowering time. For a subset of genotypes, we also assessed treatment effects on morphology and growth.
- We identified 17 genotypes, many of which have high levels of the floral repressor *FLOWERING LOCUS C* (*FLC*), that bolted dramatically earlier in fluctuating – as opposed to constant – warm temperatures (mean = 22°C). This acceleration was not caused by transient *VERNALIZATION INSENSITIVE 3*-mediated vernalization, differential growth rates or exposure to high temperatures, and was not apparent when the average temperature was cool (mean = 12°C). Further, in constant temperatures, contrary to physiological expectations, these genotypes flowered more rapidly in cool than in warm environments. Fluctuating temperatures often reversed these responses, restoring faster bolting in warm conditions. Independently of bolting time, warm fluctuating temperature profiles also caused morphological changes associated with shade avoidance or 'high-temperature' phenotypes.
- Our results suggest that previous studies have overestimated the effect of the floral repressor *FLC* on flowering time by using constant temperature laboratory conditions.

Introduction

In natural environments, temperatures fluctuate diurnally, with the lowest temperatures often at dawn and the warmest in the afternoon. The magnitude of the difference between temperature minima and maxima varies by location and season. Fluctuating day/night temperatures have been shown to influence growth rates and phenology in many insect species (Hagstrum & Milliken, 1991; Brakefield & Mazzotta, 1995; Radmacher & Strohm, 2011; Malek *et al.*, 2015; Spanoudis *et al.*, 2015; Vangansbeke *et al.*, 2015) and a few plants (Thingnaes *et al.*, 2003; Pyl *et al.*, 2012; Liu *et al.*, 2013). Yet, much of what we know about the pathways regulating growth or phenology in response to temperature has come from experiments in which temperature conditions were held constant. It remains an open question how these genetic pathways contribute to development in more complex environments, and whether we have missed important regulatory pathway behaviors by considering only the effects of contrasting constant temperatures. Taking such a perspective is vital for the accurate prediction of growth and development in natural contexts as climates change. Here, we assess the effect of fluctuating temperatures on flowering time in *Arabidopsis thaliana*. We do so across a wide range of mutants in the 'flowering time pathway' to ascertain whether known genes characterized in constant conditions have similar effects in fluctuating temperatures. Along the way, we also consider whether changes in growth and morphology can account for the flowering patterns we observe.

In *A. thaliana*, the genetic pathways and environmental factors that influence the timing of reproduction (bolting – often referred to as flowering) have been particularly well studied. This pathway combines information from internal and external cues (reviewed in Jarillo & Pineiro, 2011; Srikanth & Schmid, 2011; Andrés & Coupland, 2012). Genetic signals indicating season (temperature, day length, cold exposure) and biotic environment (light quality) converge on key integrator genes, including *SUPPRESSOR OF OVEREXPRESSOR OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*). When the

expression of these integrators is high, the meristem irreversibly switches from a vegetative state to the reproductive state.

Temperature influences reproduction in multiple ways. First, increasing ambient temperature tends to accelerate flowering (Halliday *et al.*, 2003; Salome & McClung, 2004; Salome *et al.*, 2010; Capovilla *et al.*, 2015). Recent work has suggested that this pattern is partially driven by temperature-dependent nucleosome occupancy, preventing the expression of floral promoting genes (Kumar & Wigge, 2010) and/or increasing floral repression as a result of alternative splicing in cool temperatures (Lee *et al.*, 2013; Pose *et al.*, 2013). However, most of this work has been conducted on mutants in standard genetic backgrounds that are early bolting. Accessions vary in the extent to which they accelerate flowering at moderate temperature increases (Lempe *et al.*, 2005), and this variation is frequently linked to the genes *FLOWERING LOCUS C* (*FLC* – a potent bolting repressor) and the closely related repressor *FLOWERING LOCUS M* (*FLM*) (Balasubramanian *et al.*, 2006). Mutations in autonomous pathway genes have also been shown to reduce the difference in bolting time between constant cool and warm treatments (Blazquez *et al.*, 2003).

Second, even though low temperatures delay bolting in an immediate sense, long-term exposure to cold temperatures can ultimately accelerate bolting once favorable conditions, such as warm temperatures and long days, return. This process is called vernalization or winter chilling, and occurs via epigenetic down-regulation of floral repressors (Song *et al.*, 2013; Pyo *et al.*, 2014). Vernalization effects on flowering depend on the expression levels of floral repressors during vegetative development. Accessions with high expression of *FLC*, for example, bolt later when not exposed to an extended period of cold, whereas low floral repression accessions bolt at similar times with and without a prolonged cold cue. Low floral repression ecotypes can occur as a result of variation at the *FLC* locus itself (Michaels *et al.*, 2004; Li *et al.*, 2014) or via loss of function of the *FLC* activator *FRIGIDA* (*FRI*), which has occurred fairly recently and repeatedly (Johanson *et al.*, 2000; Toomajian *et al.*, 2006). Accessions vary in both the length of cold required to repress *FLC* (Shindo *et al.*, 2006) and the upper bound of temperatures that can satisfy this requirement (Wollenberg & Amasino, 2012; Song *et al.*, 2013).

In addition to flowering time, temperature also has a strong effect on *A. thaliana* growth. When grown at low temperatures, wild-type *A. thaliana* rosettes are compact, hypocotyls are short and leaves are horizontal to the soil surface (Patel & Franklin, 2009). However, when plants are grown at warmer temperatures, photosynthetic rates increase (Bunce, 2008), as do leaf addition rates (Granier *et al.*, 2002). A further increase in temperatures causes a suite of morphological changes: hypocotyls are elongated (Gray *et al.*, 1998), petioles are lengthened (van Zanten *et al.*, 2009) and leaves are elevated above the soil surface (Patel & Franklin, 2009). Similar morphological changes also occur in response to light quality changes, indicating the presence of neighbors, and thus often called the 'shade avoidance' response.

Given the importance of temperature for flowering and development, it is perhaps to be expected that diurnal fluctuations in temperature will have important consequences for these traits. Patterns of gene expression shift diurnally, and changes in temperature and co-expression may shape developmental responses (Filichkin *et al.*, 2015). Interestingly, the pathways that determine bolting time and circadian rhythms share a genetic basis: genes originally implicated in the flowering time pathway, in particular *FLC* and some autonomous pathway mutants, have been shown to influence circadian rhythms (Edwards *et al.*, 2006; Salathia *et al.*, 2006), and temperature cycles can entrain the circadian clock (Barak *et al.*, 2000). However, *A. thaliana* may have sophisticated compensatory mechanisms that allow it to develop similarly regardless of temperature fluctuations.

A few previous studies have addressed how alternating constant day/night temperatures influence plant growth, morphology and bolting time. Two experiments have shown that metabolism, photosynthesis and growth primarily depend on daytime temperatures (van Zanten *et al.*, 2009; Pyl *et al.*, 2012). By contrast, other experiments have shown that the transition to flowering is accelerated by warm nights (Thingnaes *et al.*, 2003; Chew *et al.*, 2012; Thines *et al.*, 2014), and that the difference in temperature between night and day can influence hypocotyl and petiole elongation (Thingnaes *et al.*, 2003).

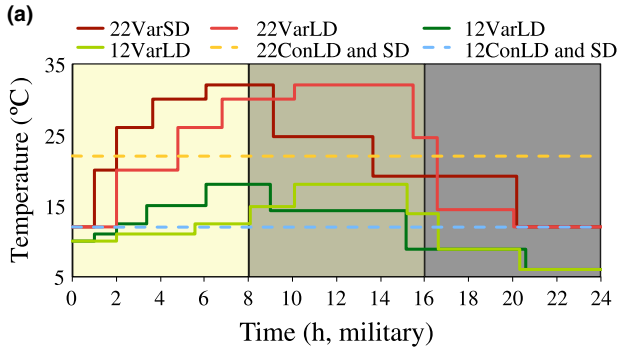
In field experiments conducted across Europe, we observed that genotypes defined as late flowering in the laboratory, including those with a functional *FRI* allele and those with mutations in the autonomous pathway, were far less delayed than in corresponding laboratory experiments (Wilczek *et al.*, 2009). We hypothesized that this effect may be caused by fluctuations in temperature experienced through the day. Here, we test this hypothesis using a panel of *Arabidopsis thaliana* flowering time mutants in the Landsberg *erecta* (L*er*), Columbia (Col) and Col $FRI_{Sf\text{-}2}$ backgrounds. We address the following questions. How do fluctuating temperatures influence flowering time? Do these effects depend on day length, average temperature or exposure to winter chilling? How does mutational perturbation in different genes known to effect flowering time affect flowering responses to thermal fluctuations? Are these responses associated with growth or morphology differences?

Materials and Methods

We performed three experiments to test the phenotypic responses of *Arabidopsis thaliana* (L.) Heynh. to fluctuating temperatures (see Fig. 1 for summary). Experiment 1 focused on a diverse collection of loss-of-function mutants in two genetic backgrounds that were primarily early flowering. Experiment 2 tested whether high temperatures explained the acceleration observed in Experiment 1. Experiment 3 focused on genotypes known to have high floral repression. This experiment also included assessments of the biomass accumulation of two focal genotypes.

Temperature treatments

We tested temperature fluctuations in chambers in which temperature profiles were controlled to closely mimic recorded ground temperatures in Norwich, UK (Wilczek *et al.*, 2009). We chose this location because *A. thaliana* cohorts germinate and establish



(b)

Avg. temperature	Fluctuation	Day length	Vernalization
Cool (12)	Constant (Con)	8 h (SD)	No (NV)
Warm (22)	Variable (Var)	16 h (LD)	Yes (V)

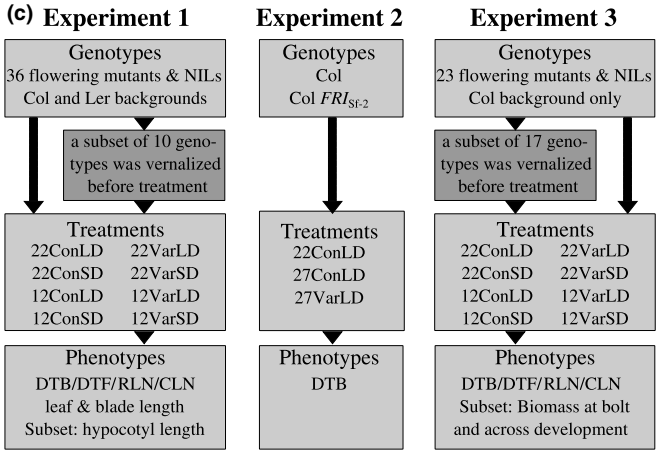


Fig. 1 Temperature profiles and summary of treatment abbreviations used in experiments with *Arabidopsis thaliana*. (a) Variable temperature profiles (solid lines) at high temperature (red) and low temperature (green). Profile shapes differ between long days (lighter colored lines) and short days (darker colored lines) because night lengths differ (8 h for long day vs 16 h for short day). Constant temperature profiles (dashed lines) are the average temperature of the warm (orange) and cool (blue) profiles and are the same across day lengths. (b) Summary of the factor abbreviations used to characterize the environmental treatments throughout this article. (c) Summary diagram of the genotypes, treatments and phenotypes collected in the three experiments reported here. Col and *Ler* refer to the Columbia and Landsberg *erecta* accessions, respectively; DTB and DTF are days to bolt and days to flower, respectively; and RLN and CLN refer to rosette leaf number and cauline leaf number at bolting, respectively. NILs, near-isogenic lines.

in multiple seasons (spring, summer and autumn) in this location (Wilczek *et al.*, 2009, 2010). Arabidopsis rosettes grow extremely close to the soil surface until bolting, so that ground-level temperatures represent more accurately the conditions they experience than do air temperatures. The examination of Norwich temperatures revealed that daily temperature fluctuations in the summer can span 20°C in 1 d – frequently ranging from 12 to 32°C – and temperature profiles in the spring and autumn commonly span 12°C in a day – frequently ranging from 6 to 18°C.

We created variable temperature profiles to mimic temperatures in summer (average 22°C) and spring/autumn (average 12°C) by the identification of criteria from the Norwich daily temperature profiles that defined the profile shape, such as the absolute daily maxima and minima, and the timing of these maxima and minima in relation to the day length. Profiles were optimized using Solver in Excel to match these criteria whilst maintaining the same average profile temperature. The shape of the fluctuating profile in long days and short days differed, so that the timing of the maxima and minima of the profiles would correspond with natural conditions (Fig. 1a).

Control plants were grown in constant conditions reflecting the average temperature of the variable profiles. This is a relevant comparison because plants in the constant treatments accumulate the same number of degree h d^{-1} (a common time unit for plant growth) as plants grown in the variable treatment. These four temperature treatments were crossed with two day lengths (short days (8 h : 16 h, day : night) and long days (16 h : 8 h, day : night)) and two vernalization pretreatments (not vernalized and vernalized) for a total of 16 environmental treatments (Fig. 1b).

Experimental setup

Seeds were bulked in common maternal conditions in a walk-in chamber under both fluorescent and incandescent bulbs with a 14 h 22°C : 10 h 20°C, light : dark/night period. Plants were fertilized and watered as needed (see Fig. 1c and text later for experiment-specific details).

Seeds were stratified for 96 h at 4°C in the dark in 0.125% agar solution. Subsequently, seeds were sown into randomized positions in 50 cell trays (four trays per treatment replicate) into a 4 : 1 promix : perlite medium, and placed into short-day 22°C conditions for 4 d to synchronize germination. Randomized blank positions were left empty for the vernalized seedlings (see later). After 4 d of common germination conditions, the experimental treatments commenced.

Plants in vernalization treatments were started earlier than nonvernalized plants. These seeds were stratified and germinated as outlined and were then placed into a 4°C short-day chamber (see experiment-specific details for lengths). The pots with the vernalized seedlings were moved into the experimental conditions on the day the experimental treatments were begun and, after 3 d of acclimatization, the vernalized seedlings were pricked out into randomized cells in the experimental trays.

Plants grown in long days received twice the daily total photon flux of plants grown in short days. Trays were rotated twice weekly, watered as needed and fertilized sparingly with 50 ppm Scotts 15-5-15 CalMag fertilizer (Marysville, OH, USA). Temperature sensors (HOBO® data loggers, Onset, Cape Cod, MA, USA) confirmed the continuity of treatments.

We recorded days to bolting (DTB), days to first flower opening (DTF), rosette leaf number at flowering (RLN) and cauline leaf number at flowering (CLN). All data have been deposited on Dryad (Burghardt *et al.*, 2015, data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.65d76). The bolting date was determined by macroscopic inspection of

the meristem, and the flowering date was the point at which the first flower unfurled its petals past parallel. RLN and CLN were determined by counting primary leaves and cauline leaves at flowering, respectively. RLN and CLN were combined to obtain the total leaf number (TLN). In warmer temperatures, late-flowering plants produced many secondary leaves, particularly in short days, preventing an accurate estimate of RLN. In these cases, we did not report an RLN. Data collection was blind, except in circumstances that required identification.

Individuals were removed from the experimental trays after flowering to avoid shading other plants. Most experimental treatments were terminated when the last individual flowered. However, in some treatments, after an extended period of time, a few genotypes were left that showed no sign of bolting, were growing extremely slowly and were dying. At this time, treatments were terminated.

Experiment 1 We included 36 loss-of-function flowering time mutants and near-isogenic lines (NILs) in Col and L*er* genetic backgrounds. We used mutants implicated in the photoperiod, autonomous, vernalization, temperature-sensing and light quality-sensing pathways. Both Col and L*er* have weak floral repressor (*FLC*) expression (early-flowering genotypes). Therefore, we also included a high floral repression line (late-flowering genotype) in which a strong *FRI* allele from the San Feliu-2 ecotype was introgressed into the Columbia background (Col $FRI_{Sf\text{-}2}$). A complete list of genotypes can be found in Supporting Information Table S1.

All eight environmental treatments were used. In addition, a subset of genotypes was exposed to a 28-d vernalization treatment. Treatments were replicated in at least two E7/2 growth chambers (Conviron, Winnipeg, MB, USA). There were four replicates of each genotype–vernalization treatment in each chamber for a total of eight genotypic replicates per treatment. As a result of differences in chamber age, light intensities differed among pairs of replicate chambers. In each pair, one chamber produced *c.* 120–130 μmol of photosynthetically active radiation (PAR) and the other produced *c.* 190 μmol. These differences led to a slightly lower red/far red (R/FR) ratio (1 vs 1.3) in the dimmer chamber, but did not cause rank-order reversals in bolting time.

In addition to the previously listed measurements, we also recorded the leaf blade length and total length of the longest leaf at bolting. In several genotypes (Col, L*er* and *phyB-1*), we measured the hypocotyl length 14 d after seed sowing. Experimental treatments were terminated after 162 d.

Experiment 2 To test whether high temperatures could explain the earlier flowering of late bolting lines, we grew Col and the Col $FRI_{Sf\text{-}2}$ NIL in constant and fluctuating 27°C long-day (16 h) treatments. The temperature varied from 22 to 32°C in the fluctuating treatment. These treatments were compared with a 22°C constant long-day treatment. No plants were exposed to vernalization. As a result of space constraints, 12 replicates of each genotype were grown in one chamber replicate. The experiment was terminated after 116 d.

Experiment 3 We used 23 flowering time mutants and NILs exclusively in the Columbia genetic background. Six of the genotypes overlapped with those in Experiment 1 and many were late-flowering genotypes (see Table S1). Experiment 3 was identical to Experiment 1, except for the following: irradiance levels were nearly twice as high (280 PAR for one replicate and 300 PAR for the second replicate); the cool temperature fluctuating treatments were omitted because they did not differ from the constant cool treatments; vernalization treatments lasted 40 d; and data were not collected on leaf/blade/hypocotyl length.

In addition, to assess the influence of the treatments on the growth rates and size at bolting, we collected aboveground biomass data on two genotypes (Col and Col $FRI_{Sf\text{-}2}$). We harvested subsets of plants at bolting and at multiple time points before bolting. To span development, sampling intervals were longer for later flowering genotypes and treatments. At each time point, we harvested eight replicate individuals of each genotype in each treatment by cutting the plant from the root at soil surface level. Plants were dried in an oven at 70°C for 2 wk and each individual was weighed. When plants were very small, we pooled replicates for measurement and divided by the number of plants.

Statistical analysis

Several different measures associated with the reproductive transition can be used. We focused on DTB because bolting is the first macroscopically visible marker of the reproductive transition, calendar time is the most relevant trait scale for ecological processes and leaf number counts became unreliable for late-flowering plants critical to this study. However, for most genotypes, DTB, days to flowering and TLN at flowering were highly correlated (Fig. S1).

We used 16 treatment combinations of four binary factors (abbreviations are summarized in Fig. 1b). Hereafter, the treatments are labeled with these abbreviations (e.g. 22VarLDV refers to 22°C average temperature, fluctuating temperatures, long-day photoperiod and vernalization). Unless otherwise noted, all treatments subsumed within a label are included. For instance, 12SD refers to all treatments that were at 12°C average temperature and in short-day photoperiods (12ConSDNV, 12VarSDNV, 12ConSDV, 12VarSDV). Experiments 1, 2 and 3 use identical notation, except that Experiment 2 had an additional average temperature of 27°C.

We also often employed genotype by fluctuation (genotype × fluctuation) interaction terms in our analysis to test whether specific genotypes responded differently to fluctuating temperatures than the wild-type control. For all analyses, we corrected for multiple tests using sequential Bonferroni (Holm, 1979). We used mixed effect models via the LMER function in the LME4 package (Bates *et al.*, 2015) in R v.3.0.1 (R Core Development Team, 2013). In order to control for the grouping of replicates into two or more environmental chambers, chamber identity was included as a random factor. In cases in which chamber replicates were not available, we used linear models (LM BASE function in R).

DTB measurements

Because variance in DTB measures tended to increase with time to bolting, we log-transformed DTB data before performing statistical analysis.

Experiment 1 – mixed effect models To test whether temperature fluctuations influenced the bolting time of each wild-type genotype in Experiment 1, we subset the data by average temperature, day length and vernalization, and ran the following model:

$$\log\mathrm{DTB}_{ij} = \mu + \mathrm{fluctuation}_i + \mathrm{chamber}_j \quad \text{Eqn 1}$$

We used a likelihood ratio test of a model with and without the fluctuation term to determine whether fluctuations influenced bolting times (Table S2).

We were particularly interested in which kinds of allelic variants altered plant responses to fluctuating temperatures. Therefore, we subset the data into each combination of average temperature, day length and vernalization, and ran the following model:

$$\log\mathrm{DTB}_{ijk} = \mu + \mathrm{genotype}_i + \mathrm{fluctuation}_j + \mathrm{genotype}_i \times \mathrm{fluctuation}_j + \mathrm{chamber}_k \quad \text{Eqn 2}$$

We performed a likelihood ratio test on the interaction term (Table S3).

We also tested whether functionality of the *VERNALIZATION INSENSITIVE 3* (*VIN3*) gene in the Col $FRI_{Sf\text{-}2}$ background altered the response within each treatment using a likelihood ratio test for all combinations of average temperature, day length and fluctuation. We omitted vernalized plants from the analysis (Table S4):

$$\log\mathrm{DTB}_{ij} = \mu + \mathrm{genotype}_i + \mathrm{chamber}_j. \quad \text{Eqn 3}$$

Experiment 2 – regression analysis To test whether extremely high constant or variable treatments changed the bolting response of Col or Col *FRI* when compared with warm conditions, we used the following model and performed a likelihood ratio test contrasting 22ConLD with 27ConLD and 27VarLD (Table S5).

$$\log\mathrm{DTB}_i = \mu + \mathrm{treatment}_i \quad \text{Eqn 4}$$

Experiment 3 – mixed effect models To confirm how certain types of allelic variants altered DTB responses to warm fluctuating temperatures, we ran identical models as those performed in Experiment 1 (Eqn 2), specifically on high floral repression and photoperiod pathway mutants (Table S6).

We also tested the effect of various mutations in the Col $FRI_{Sf\text{-}2}$ genetic background in each environment using the same method as employed for Experiment 1 (see Eqn 3). We omitted autonomous mutants in short days because many never bolted (Table S7).

Morphology and growth measures

Experiment 1 – mixed effect models for blade ratios To test whether petiole elongation changed across treatments, we divided the blade length by the total length of the leaf to create a blade ratio. For all factorial combinations of day length, average temperature and genetic background (Col and L*er*), we analyzed the influence of fluctuations on petiole elongation. We did not test plants that underwent vernalization because of age differences. We controlled for size/age at measurement using bolting time as a covariate and checked that normality assumptions were met. The model used was:

$$\text{blade ratio}_{ijk} = \mu + \mathrm{fluctuation}_i + \text{bolting time}_j + \mathrm{chamber}_k. \quad \text{Eqn 5}$$

For each data subset, we conducted a likelihood ratio test for the fluctuation term (Table S8).

Experiment 1 – linear model on hypocotyl measurements We used a linear model to discern the effect of fluctuation at warm average temperatures on hypocotyl length. We subset the data by day length and genotype (L*er*, Col and Ler *phyB-1*), and included leaf number at measurement as a covariate to control for size differences among treatments. The model used was:

$$\text{hypocotyl length}_{ij} = \mu + \text{leaf number at measurement}_i + \mathrm{fluctuation}_j. \quad \text{Eqn 6}$$

We tested for the influence of the fluctuation term using a likelihood ratio test (Table S9). Leaf number was highly correlated with hypocotyl length for L*er phyB-1* in short days, and so we dropped this test.

Experiment 3 – plant size To test whether fluctuations altered aboveground biomass over time, we subset by genotype (Col and Col $FRI_{Sf\text{-}2}$), day length and plant age, and used the following model:

$$\mathrm{weight}_{ij} = \mu + \mathrm{fluctuation}_i + \mathrm{chamber}_j. \quad \text{Eqn 7}$$

For each time point, we used a likelihood ratio test to determine whether there were weight differences between plants grown at constant and fluctuating warm treatments (Table S10). We used the same model to test whether fluctuations influenced size at bolting, by substituting size at bolting for the dependent variable (Table S11).

Results

Most genotypes showed little response to temperature fluctuation regardless of temperature or day length combination

Genotype-specific bolting times remained largely consistent across both warm and cool conditions and in both long days and

short days (Fig. 2a, many points on one to one line). In particular, fluctuating temperatures had no effect on the bolting times of the two early-flowering accessions L*er* and Col (Table S2; Fig. 2b, c).

However, a set of late-flowering genotypes bolted earlier in warm fluctuating temperatures relative to warm constant temperatures

Most of these genotypes had high *FLC* expression as a result of mutation in the autonomous pathway or introgression of the functional $FRI_{Sf\text{-}2}$ allele into Col (see Figs S2–S4 for all genotypes) (Fig. 2a–c). Relative to the wild-type background (L*er* or Col), the effects of these genetic perturbations on bolting time were much larger in the 22Con treatments relative to 22Var. For example, Col $FRI_{Sf\text{-}2}$ bolted 95 d later than Col in 22ConSD, but only 30 d later in 22VarSD (Table S3). This response to fluctuating temperatures was dependent on the activity of *FLC*, as Col $FRI_{Sf\text{-}2}$ *flc* bolted at the same time in fluctuating and constant temperature conditions (Fig. 2b,c). Further, when late-flowering genotypes were vernalized (a treatment that epigenetically represses *FLC* expression), the effect disappeared (Fig. S4; Table S3).

To confirm these results, we tested additional late-flowering mutants in the Col genetic background in Experiment 3. Many of these mutant genotypes showed a greater difference in bolting time between variable and constant warm temperatures than did Col (genotype × fluctuation interaction). The effects of three in particular – *fca-9*, *fld-3*, *ld-1* – were significant after correction for multiple tests (sequential Bonferroni method, Fig. 3a; Table S6; Figs S5, S6 for all genotypes and vernalization states). We examined the effects of further augmentation of floral repressor expression using lines in which each autonomous pathway mutation was crossed into the Col $FRI_{Sf\text{-}2}$ background. The median bolting day of each doubly modified genotype was later than that of the Col $FRI_{Sf\text{-}2}$ allele by itself in both constant and fluctuating conditions, and all bolted earlier in the 22Var treatments than in the 22Con treatments, and some dramatically so (Fig. 3a; Table S7).

Neither high temperatures alone, nor partial vernalization, explained the earlier bolting time of late-flowering genotypes in warm fluctuating temperatures

To test whether the transient high temperatures of the 22Con treatment (up to 32°C during the day) triggered earlier flowering of Col $FRI_{Sf\text{-}2}$, we measured the bolting times of Col and Col $FRI_{Sf\text{-}2}$ in two treatments with higher average temperatures (constant 27°C and diurnal fluctuations from 22 to 32°C with a mean of 27°C), and compared each with constant 22°C. Neither genotype bolted earlier in either of the high-temperature treatments relative to the constant 22°C treatment (Fig. 3d; Table S5).

Another possible cause of the acceleration could be that vernalization is epigenetically decreasing floral repression during the

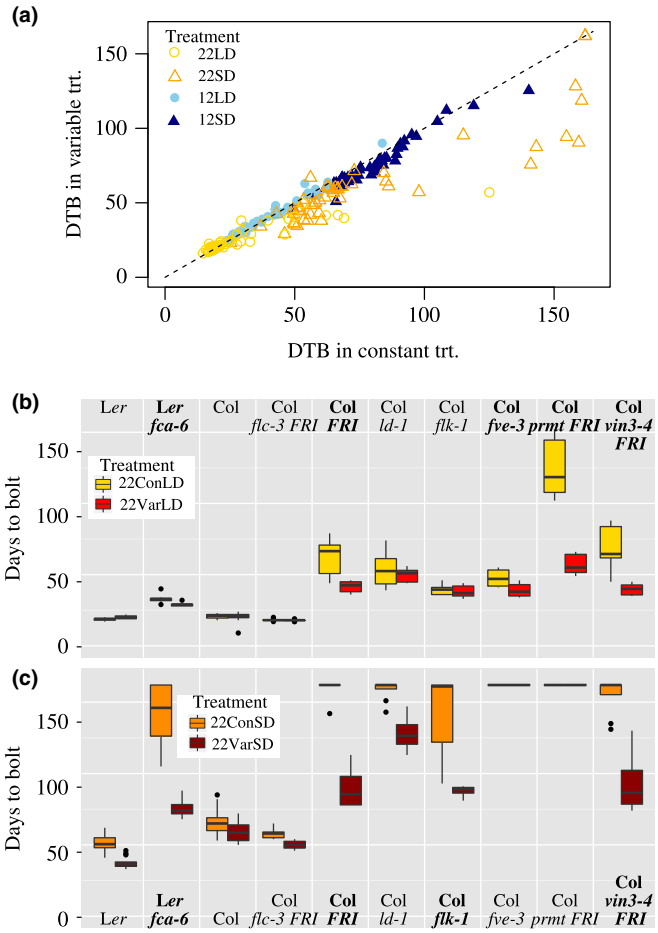


Fig. 2 Days to bolting (DTB) responses of *Arabidopsis thaliana* in constant and variable temperature treatments from Experiment 1. (a) Scatter plot comparing DTB of each genotype in constant (*x*-axis) and variable (*y*-axis) temperature treatments. Blue closed symbols indicate 12°C treatments and orange open symbols indicate 22°C treatments. Circles and lighter colors denote long days (LD) and triangles and darker colors denote short days (SD). Points that fall below the dotted 1 : 1 line indicate an acceleration of bolting in the variable temperature treatments. (b, c) Median bolting responses of selected genotypes in (b) long and (c) short days in Experiment 1. All graphs were drawn with 'ggplot2' package in R. Boxes indicate 25% and 75% quartiles and the heavy black line is the median. Whiskers extend to the highest value that is within 1.5 × the interquartile range. Data outside of this range are outliers and are visualized as points. The experiment was truncated at 162 d and all unbolted plants were assigned this value as their bolting date. A bold genotype label denotes genotypes that behaved significantly differently from the wild-type in the two environmental treatments (significant genotype × fluctuation interaction) after correction for multiple tests. Results for all genotypes and environments can be found in Supporting Information Figs S2–S4. Col and L*er* refer to the Columbia and Landsberg *erecta* accessions, respectively. Con, constant temperatures; Var, variable temperatures.

12°C nights of the fluctuating treatment (12°C causes partial vernalization in this genotype; Wollenberg & Amasino, 2012). However, this explanation is unlikely because the genotype Col $FRI_{Sf\text{-}2}$ *vin3-4*, which carries a mutated *VIN3* gene and is thus insensitive to vernalization, bolted after the same number of days as Col $FRI_{Sf\text{-}2}$ (Fig. 3b; Table S4) in both the 22Con and 22Var

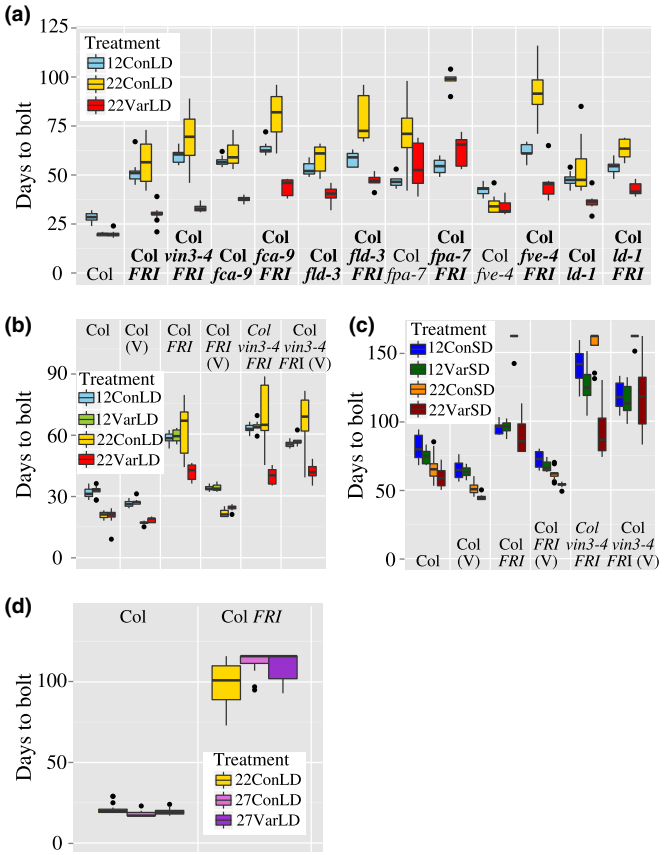


Fig. 3 Median days to bolting (DTB) responses of selected genotypes of *Arabidopsis thaliana*. All graphs were drawn with the 'ggplot2' package in R. Boxes indicate 25% and 75% quartiles and the heavy black line is the median. Whiskers extend to the highest value that is within 1.5 × the interquartile range. (a) Late-flowering, nonvernalized genotypes from long-day treatments of Experiment 3. Bold names denote genotypes that behaved significantly differently from the wild-type in response to temperature fluctuations (significant genotype × fluctuation interaction) after correction for multiple tests. Results for all genotypes and environments can be found in Figs S5 and S6. (b, c) Response of Columbia (Col), Col *FRIGIDA* (*FRI*) and Col *FRI vin3-4* in (b) long days (LD) and (c) short days (SD) in Experiment 1. Genotype names with a (V) were vernalized. (d) Behavior of Col and Col *FRI* in Experiment 2. The experiment was truncated at 116 d. Con, constant temperatures; Var, variable temperatures.

treatments. This result was replicated in Experiment 3 (Fig. S5; Table S7).

By contrast, in cool treatments, regardless of temperature fluctuation treatment, *VIN3* activity is implicated in accelerating the flowering of Col $FRI_{Sf\text{-}2}$. Col $FRI_{Sf\text{-}2}$ *vin3-4* plants flowered slightly later than Col $FRI_{Sf\text{-}2}$ plants in 12°C long days (12Con and 12Var: *c.* 4.5 d later) and substantially later in short days (12Con: *c.* 45.1 d later; 12Var: *c.* 30 d later; Fig. 3c; Table S4). Thus, *VIN3* seems to be involved in accelerating flowering at intermediate temperatures and the effect is most prominent in short days. This result was replicated in Experiment 3 in constant conditions (Fig. S5; Table S7 for statistics). Earlier flowering, primarily in short days, suggests either a photoperiodic gating mechanism or that, in long days, the process is overshadowed by the photoperiodic stimulation of flowering.

In high-FLC plants, warm average temperatures caused later bolting under constant conditions, but earlier bolting in fluctuating thermal environments

When high-*FLC* genotypes were vernalized (i.e. their *FLC* levels were repressed), warmer temperatures led to earlier flowering (Fig. 4, solid lines, except *vin3-4* $FRI_{Sf\text{-}2}$). However, nonvernalized, late-flowering genotypes flowered at the same time or later in warm constant conditions relative to cool conditions (Fig. 4, black dashed lines). The introduction of temperature fluctuations reduced this effect (Fig. 4, gray dashed lines). The strength of this reversal in plasticity was dependent on day length. In long days, fluctuating temperatures often led to faster bolting times in warm conditions vs cool conditions, whereas, in short days, fluctuating temperatures led to similar bolting times in warm and cool conditions.

Flowering acceleration in response to warm temperatures may not occur directly through repression of FLC

Mutants in *GIGANTEA* (*GI*) and *FLAVIN-BINDING KELCH REPEAT 1* (*FKF1*) in a low *FLC* background also displayed earlier bolting in warm variable treatments, but only under long days (Fig. 5a; Table S3). Experiment 3 confirmed this result (Fig. 5c,d; Table S6). Therefore, we looked to see whether any mutants downstream of both *FLC* and the photoperiod pathway caused delayed flowering in variable conditions.

Bolting time was delayed for an *ft* mutant and a limited number of mutations that influence the expression of *FT* (Fig. 5c–f). These effects were dependent on genetic background. *Ft-2* mutants in the L*er* background were delayed in fluctuating conditions, whereas, in the Col background, there was no difference (Tables S4, S6). *PhyB* was also delayed in long days by fluctuating temperatures compared with the wild-type (L*er*), but behaved similarly to the wild-type by being accelerated by variable temperatures in short days. This result was strongly dependent on the measure of flowering time used (Fig. S7).

Interestingly, *co* mutants did not behave like *ft-2*, *fkf1-2* or *gi*. Normally, we would expect no phenotypic effect of photoperiod mutants in short days, and this is what we observed in short-day constant conditions. However, *co-2* mutants actually showed delays in flowering time in the 22VarSD treatment. By contrast, when placed in a Col $FRI_{Sf\text{-}2}$ background, the *co* mutant behaved identically to Col $FRI_{Sf\text{-}2}$ in short days. In long days, bolting was extremely delayed compared with Col $FRI_{Sf\text{-}2}$ in constant conditions (*c.* 76.6 d later) and slightly delayed in fluctuating conditions (*c.* 13 d later; Table S7). In sum, other genes apart from floral repressors could mediate the response to fluctuating temperatures.

A faster growth rate cannot explain the faster bolting in fluctuating temperatures

Aboveground biomass accumulated similarly in the 22Con and 22Var treatments (Fig. 6a). At multiple developmental time points, we found no evidence for differences in plant size for either Col or Col $FRI_{Sf\text{-}2}$ (Table S10). Because growth rates were

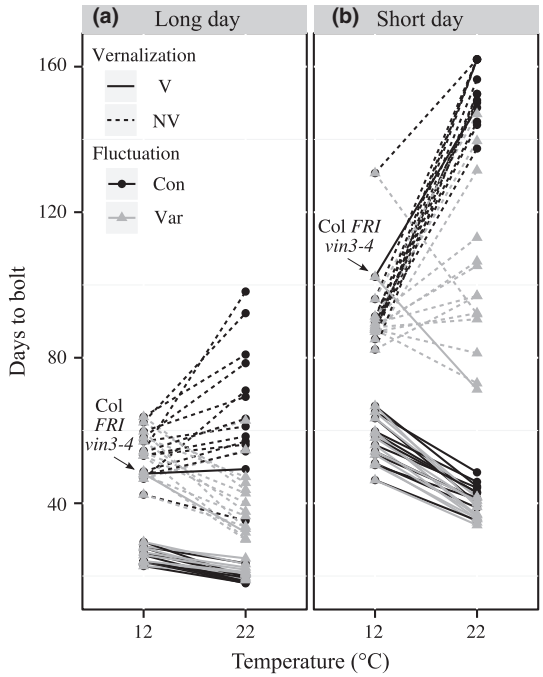


Fig. 4 Reaction norms of late-flowering *Arabidopsis thaliana* genotypes from Experiment 3 to temperature in (a) long days and (b) short days. The behavior of each genotype in 12Con conditions is compared with its behavior in warm constant (black lines/circles) and variable (gray lines/triangles) temperatures. Solid lines indicate reaction norms of the same late-flowering genotypes that were vernalized for 40 d before being placed in their respective treatments. It should be noted that these plants bolt more rapidly in warm temperatures than in cool temperatures. The genotype Columbia *FRIGIDA* (Col *FRI*) *vin3-4* is unresponsive to vernalization and thus is labeled on each graph. V, vernalized; NV, nonvernalized; Con, constant temperatures; Var, variable temperatures.

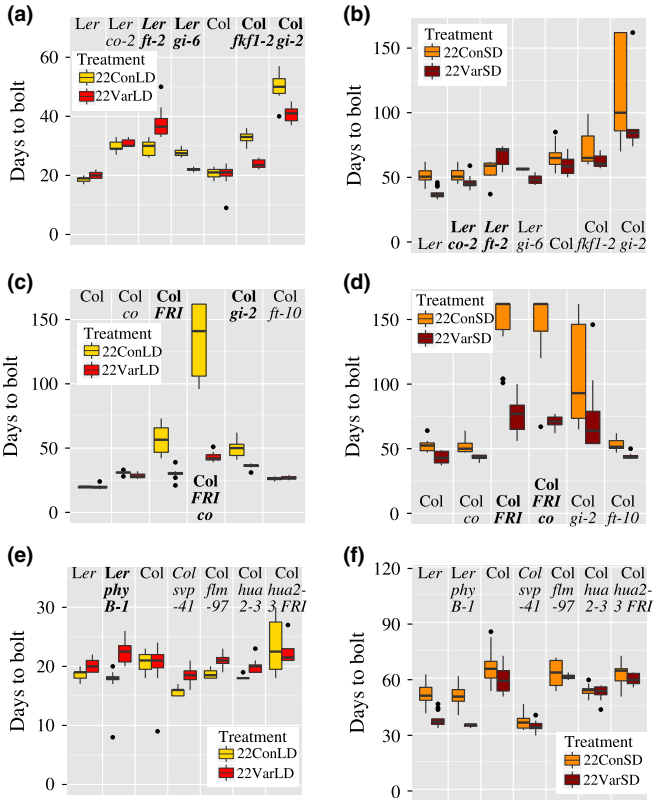


Fig. 5 Median days to bolting (DTB) responses of selected genotypes of *Arabidopsis thaliana* in warm treatments. For all graphs, boxes indicate 25% and 75% quartiles and the heavy black line is the median. Whiskers extend to the highest value that is within 1.5 × the interquartile range. Bold names denote genotypes that behaved significantly differently from the wild-type in response to temperature fluctuations (significant genotype × fluctuation interaction) after correction for multiple tests. (a, b) Photoperiod pathway-associated, nonvernalized genotypes from (a) long-day (LD) and (b) short-day (SD) treatments of Experiment 1. (c, d) Photoperiod pathway-associated, nonvernalized genotypes from (c) long-day and (d) short-day treatments of Experiment 3. (e, f) Additional genotypes from Experiment 1 in (e) long days and (f) short days. Col and Ler refer to the Columbia and Landsberg *erecta* accessions, respectively. Con, constant temperatures; Var, variable temperatures.

similar, but bolting times differed, the relative effect of variable temperatures on size at bolting differed between Col $FRI_{Sf\text{-}2}$ and Col in long days, but not short days. Col $FRI_{Sf\text{-}2}$ plants in the 22VarLD treatments were 88% smaller at bolting than in 22ConLD (0.0379 g vs 0.3121 g), whereas wild-type plants were only 25% smaller (*c.* 0.003 vs 0.004 g). By contrast, in short days, regardless of genotype, variable treatment plants were *c.* 50% smaller than plants grown in constant treatments (Fig. 6b,c; Table S10).

Plants displayed extreme shade avoidance morphology in fluctuating treatments at warm, but not cool, temperatures

In 22Var conditions, we observed a suite of morphological changes associated previously with shade avoidance and exposure to constant high temperature (Fig. 6d). When controlling for days to bolt, petiole lengths were proportionately longer in 22Var treatments compared with 22Con treatments (Table S8; Fig. S8a). This was true in long days for plants with both Col and L*er* backgrounds and in short days for plants with a L*er* background only. Further, in short days, hypocotyls were elongated for both L*er* and Col in the variable warm treatments relative to constant treatments (see Fig. S9; Table S9), and leaf angles in Columbia were more than twice as steep in 22VarSD (*c.* 50°) relative to 22ConSD (*c.* 25°). Interestingly, in our experiment, *phy-B* mutants, which constitutively display a shade avoidance response, showed even more extreme phenotypes in fluctuating warm conditions: each of the three rosette leaves was separated by 1-cm internodes and hypocotyls were further elongated (Fig. S9b; Table S9).

By contrast, there was little morphological difference between variable and constant treatments with an average temperature of 12°C. Rosettes were compact and hypocotyls were short (similar in length to those found in 22ConLD conditions) and blade ratios did not differ (Table S8).

Discussion

We tested 59 genetic perturbations known to effect flowering time to genetically dissect the effect of diurnal fluctuations of temperature on growth, morphology and flowering time. We found that temperature fluctuations, specifically at warm average

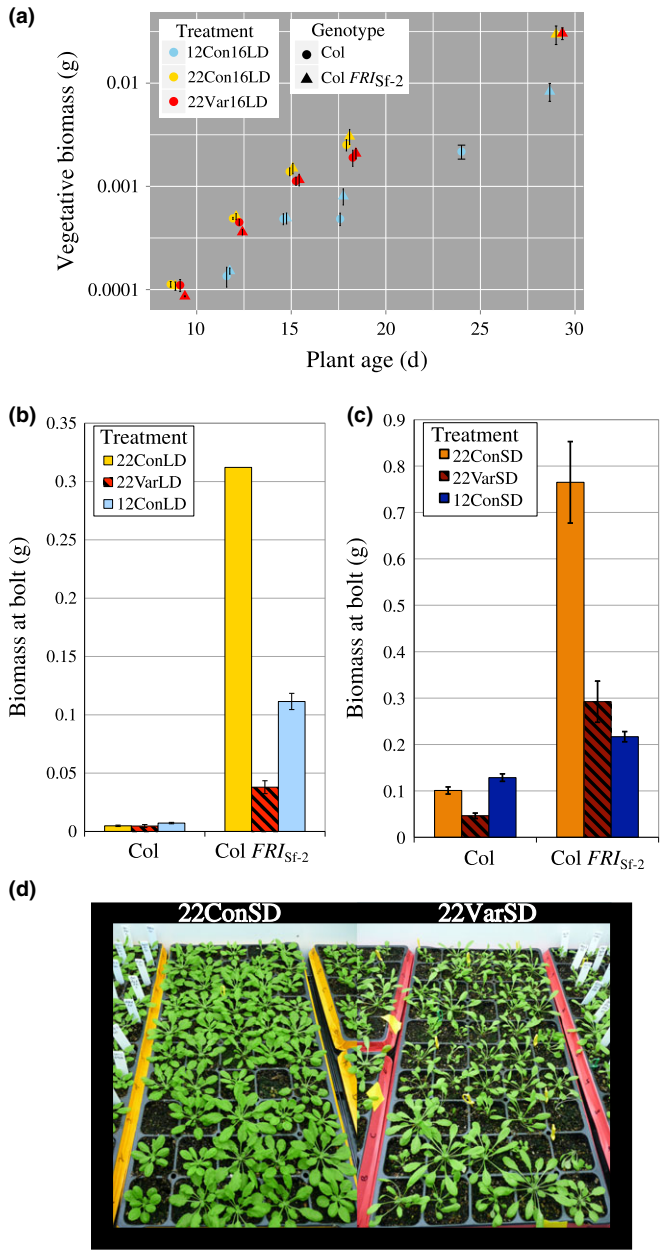


Fig. 6 Aboveground growth and morphology of *Arabidopsis thaliana* in environmental treatments. (a) Biomass accumulation of Columbia (Col, circles) and Col *FRIGIDA* (*FRI*)$_{Sf\text{-}2}$ (triangles) in three long-day (LD) treatments: 12ConLD (blue), 22ConLD (gold) and 22VarLD (red). (b, c) Average plant biomass at bolt in both (b) long and (c) short (SD) days. For (a–c), error bars indicate ± SE of eight replicate plants spread evenly across two replicate chambers of each environment. Note the difference in *y*-axis values between (b, c). (d) Photographs of morphology of plants in the warm constant (left) and warm fluctuating (right) treatments in short days. Plants had been in their treatments for 35 d when the photographs were taken. Some plants pictured had also experienced 28 d of vernalization before experiencing the temperature treatments (see the Materials and Methods section). Con, constant temperatures; Var, variable temperatures.

temperatures, caused a 'shade avoidance' or 'high-temperature response' morphology. Although bolting of many wild-type and mutant genotypes showed little response to temperature variability, a subset of genotypes bolted much more rapidly in warm fluctuating conditions than in constantly warm conditions. Many of these genotypes were late-flowering genotypes (Col *FRI*$_{Sf\text{-}2}$ and autonomous pathway mutants) which are known to have high *FLC* levels. We found that this acceleration was dependent on a functional *FLC* gene and appeared to be dosage dependent, did not occur because plants were being 'vernalized' in a *VIN3*-dependent manner in the fluctuating warm treatment, was not a result of plants growing more rapidly in the variable treatment, and was not caused solely by high temperatures in the variable treatment. In addition, for many of these genotypes, the standard response of faster flowering in warmer temperatures was reversed, so that plants actually bolted more rapidly in cool constant conditions than in warm constant conditions. In total, these results suggest that the state of the *FLC* pathway modulates a multifaceted response to fluctuating temperatures. Therefore, the large flowering delays documented in the laboratory for naturally occurring late-flowering ecotypes may not adequately reflect the behavior of these genotypes in complex natural environments.

We observed a few additional genes not associated with floral repression which, when perturbed, led to different responses to fluctuations than in the wild-type (*GI*, *FKF1*, *PHYTOCHROME-B*, *FT*, *CONSTANS*), hinting that earlier flowering may not be occurring only through the modulation of floral repression. Further, some of these effects were background specific – they were only observed in L*er*. These results are consistent with the idea that the relative importance of each upstream gene pathway can vary by genetic background, as shown recently for germination behavior in these two accessions (Vaistij *et al.*, 2013).

High-FLC lines and autonomous pathway mutations are not temperature insensitive; they reverse plasticity to temperature

Previous research on the thermal sensitivity of flowering time mutants has suggested that autonomous pathway mutants and high-*FLC* lines are 'temperature insensitive' because they flower at similar times in warm and cool conditions (Blazquez *et al.*, 2003; Balasubramanian *et al.*, 2006; Lee *et al.*, 2010). In our study, we replicated these results, but also found that, when floral repression was further increased, these genotypes were delayed at warm temperatures and are thus not 'temperature insensitive'. Because previous work on ambient temperature sensing has been performed in genotypes that bolt more rapidly in warmer conditions, most known ambient temperature mechanisms lead to earlier flowering in warmer conditions: temperature-dependent *FLM* splicing (Pose *et al.*, 2013), changes in repression via *SHORT VEGETATIVE PHASE* (*SVP*) (Lee *et al.*, 2013) or *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*) (Nomoto *et al.*, 2012). It will be worth investigating the mechanisms underlying earlier bolting in cool conditions, because many *A. thaliana* ecotypes have high levels of floral repression and many display no or reversed 'thermal sensitivity' (re-analysis of the data from Lempe *et al.*, 2005; Fig. S9). One possibility is that *VIN3*-dependent vernalization – occurring at higher temperatures than previously suspected (Wollenberg & Amasino, 2012) – may explain the faster bolting in cool conditions. However, this process cannot fully explain our data because cool temperatures

accelerate *vin3-4 FRI* mutants in short days and in fluctuating warm conditions.

Possible mechanisms for the acceleration in warm fluctuating conditions

Later flowering in warm constant temperatures than in fluctuating temperatures was only observed when the network had high levels of *FLC* or had mutations in *GI* or *FKF1*. The fact that vernalization, which reduces the expression of *FLC* and other floral repressors, nullifies the effect supports the notion that floral repression levels are crucial. Because *FLC* levels and autonomous mutations have been shown to lengthen and vernalization shown to shorten the circadian period (Salathia *et al.*, 2006), one possibility is that changes in clock period could delay flowering in warm constant conditions. However, recent work has also suggested another possibility. *FLC* directly represses both *FT* and *SOC1* via protein complexes formed with *SVP*, *FLM*, *MADS AFFECTING FLOWERING 2* (*MAF2*) and *MAF3* (Gu *et al.*, 2013). Because the expression of *FLM*, *MAF2* and *MAF3* cycles diurnally and the splice forms of *FLM* and *MAF3* proteins that are present are temperature dependent, it is possible that the composition of floral repressor complexes may shift over the course of the day (Gu *et al.*, 2013), influencing *FT* expression at critical periods (Krzymuski *et al.*, 2015).

Recently, a double coincidence model has been suggested for the high-temperature-triggered architectural responses, such as hypocotyl elongation and flowering acceleration (Nomoto *et al.*, 2012). This model suggests that *PIF4* expression levels (a promoter of *FT* expression) increase with temperature – with temperatures at dusk in short days being particularly important. In concordance, we observed the largest morphological changes and floral acceleration in short days when high temperatures occur at sunset. However, cool nights, not just hot afternoons, were necessary to observe the floral acceleration.

Fluctuating temperatures could promote flowering in high floral repression genotypes by indirectly overriding repression via a promotive pathway, as occurs with light quality changes (Wollenberg *et al.*, 2008), or actively reducing floral repression in the fluctuating treatment. Preliminary analysis of RNAseq data suggests that the fluctuating treatment decreases *FLC* levels by 30–40% in the late afternoon (D. Runcie, unpublished). In addition, work is needed to identify the particular aspect of the fluctuating profile that promotes flowering: the width of the oscillation, the timing of the fluctuation or the absolute temperatures in the profile.

Contrasting effect sizes in field studies vs controlled chamber environments

Numerous laboratory-based experiments have found that variation at the *FRIGIDA* locus can explain *c.* 23–70% of the variation in flowering time in nonvernalized plants (Lempe *et al.*, 2005; Werner *et al.*, 2005; Shindo *et al.*, 2006). However, experiments conducted in chambers simulating seasonal temperature cycles (Scarcelli *et al.*, 2007; Li *et al.*, 2010) and those conducted in the field (Wilczek *et al.*, 2009) found smaller, although significant, effects of *FRI* in warm natural environments, despite little vernalization. Our results suggest that constant warm temperatures used in laboratory experiments may artificially magnify the effect sizes of floral repression genes.

Our results also hint that higher irradiance levels could play a role in differences between chamber and field studies. We found that high light levels were able to accelerate flowering, particularly in fluctuating treatments and high-*FLC* lines, both within and between experiments (Fig. S10). Higher light levels increase photosynthetic rates, potentially accelerating growth and/or developmental progress (Thornley & Johnson, 1990; Vialet-Chabrand *et al.*, 2013). In sum, the introduction of fluctuating temperature regimes and increasing light levels in chambers may improve the ability to connect genetic effects isolated and studied in the laboratory to behavior in natural environments.

Application of the results to an understanding of plant responses in natural environments

We found that temperature ranges, as well as means, were crucial for the determination of the phenotype in many, but not all, genotypes. Interestingly, although many genotypes met our expectation that the transition to flowering would occur more rapidly in warm than in cool conditions, we discovered a subset of genotypes for which this expectation was only met in fluctuating conditions and not in constant conditions. These results suggest that, once gene networks have been characterized in constant conditions, a necessary next step is to examine the consistency of this response to complex environments. In addition, these results demonstrate genotype-specific responses to fluctuating temperatures – adding complexity to the challenge of predicting how organisms will respond to climate change as variability increases.

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Author contributions

L.T.B., A.M.W., J.L.R., S.M.W. and J.S. designed the experiment, L.T.B., A.M.W. and M.D.C. planned the experiment and collected the data, and L.T.B., D.E.R. and J.S. analyzed the data. L.T.B. wrote the first draft of the manuscript, with all the other authors, particularly D.E.R., contributing to revisions.

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Supporting Information

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

Fig. S1 Scatter plot of the relationship between days from sowing to bolt and days from sowing to flower.

Fig. S2 Summary of results for all nonvernalized Columbia background genotypes from Experiment 1.

Fig. S3 Summary of results for all nonvernalized L*er* background genotypes from Experiment 1.

Fig. S4 Summary of results for all vernalized genotypes from Experiment 1.

Fig. S5 Summary of results for all nonvernalized genotypes from Experiment 3.

Fig. S6 Summary of results for all vernalized genotypes from Experiment 3.

Fig. S7 Number of rosette leaves at bolting for select genotypes.

Fig. S8 Differences in blade morphology and hypocotyl length between environments.

Fig. S9 Re-analysis of the data from Lempe *et al.* (2005) on the flowering time of a diverse panel of ecotypes in multiple constant temperatures.

Fig. S10 Comparison of days to bolt data from high-irradiance and low-irradiance experiments.

Table S1 Description of genotypes, sources and the experiments in which they were used

Table S2 Likelihood ratio tests to determine whether fluctuating temperatures influence bolting times of wild-type genotypes (Col and Ler)

Table S3 Likelihood ratio tests to determine which allelic changes alter plant responses to fluctuating treatments

Table S4 Likelihood ratio tests to determine whether the bolting time of a *VERNALIZATION INSENSITIVE 3* (*VIN3*) mutation in the Col $FRI_{Sf\text{-}2}$ background differs from the Col $FRI_{Sf\text{-}2}$ background

Table S5 Likelihood ratio tests to determine whether high average temperatures influence the bolting time of Col and Col $FRI_{Sf\text{-}2}$ genotypes

Table S6 Likelihood ratio tests querying whether each genotype behaves significantly differently to warm fluctuating conditions when compared with Columbia wild-type

Table S7 Likelihood ratio tests to determine whether mutations in the Col $FRI_{Sf\text{-}2}$ background influence bolting within each treatment

Table S8 Likelihood ratio tests to determine whether fluctuating temperatures influence the blade ratio

Table S9 Likelihood ratio tests for the effect of fluctuating temperatures on hypocotyl lengths for Col, L*er* and L*er phyB-1* in warm treatments

Table S10 Likelihood ratio tests to determine whether fluctuations at warm temperatures alter aboveground biomass at various times throughout development

Table S11 Likelihood ratio tests to determine whether fluctuations at warm temperatures alter aboveground biomass at bolt

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