Natural variation in autumn FLC levels, rather than epigenetic silencing, aligns vernalization to different climates

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Impact Statement

Alleles of the major floral repressor vary in their initial expression to underpin the ability of Arabidopsis to survive year-on-year climatic fluctuations.

Abstract

Plants monitor temperatures over long timescales to assess seasons and time developmental transitions. In Arabidopsis thaliana, winter is registered during vernalization through the temperature-dependent repression and epigenetic silencing of floral repressor FLOWERING LOCUS C (FLC). Natural Arabidopsis accessions show considerable variation in vernalization, however which aspect of the FLC repression mechanism is most important for adaptation to different climates is not clear. By analyzing FLC silencing in natural variants throughout winter in three field sites, we find that FLC starting levels and early phases of silencing are the major variables underlying vernalization response, rather than establishment of epigenetic silencing. This results in an intricate interplay between promotion and delay of flowering to balance survival, and through a post-vernalization effect of FLC, reproductive effort via branch production. These data reveal how non-coding FLC variation aligns vernalization response to different climatic conditions and year-on-year fluctuations in natural temperature profiles.
Main Text

Introduction

Developmental transitions in plants are aligned with specific seasons to synchronize with pollinators and optimal climatic conditions (Andrés and Coupland, 2012). A major seasonal cue used to time the transition to flowering is temperature. How temperature affects flowering has been studied genetically in controlled laboratory conditions. However, recent work has shown the importance of analysing this process in natural field conditions (Fig. 1A) (Wilczek et al., 2009; Duncan et al., 2015; Kudoh, 2016; Antoniou-Kourounioti et al., 2018; Hepworth et al., 2018; Rubin et al., 2018; Song et al., 2018; Nagano et al., 2019; Taylor et al., 2019).

In Arabidopsis thaliana, expression of FLOWERING LOCUS C (FLC), a MADS-box transcription factor that represses flowering, plays a major role in determining the timing of the transition to flowering. FRIGIDA (FRI) up-regulates FLC, establishing an active transcription state at FLC chromatin. FLC expression is then progressively epigenetically silenced by winter cold, in a process called vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999).

Vernalization represses FLC expression through a Polycomb Repressive Complex 2 (PRC2) mechanism that establishes and holds epigenetic memory at FLC. This mechanism also involves a temperature-integrating accessory protein, VERNALIZATION INSENSITIVE3 (VIN3) (Sung and Amasino, 2004; De Lucia et al., 2008). Cold-induction of VIN3 leads to formation of a PHD-PRC2 complex, which nucleates Polycomb silencing at an intragenic site within FLC (De Lucia et al., 2008). The resulting epigenetic switch and subsequent epigenetic memory is a cis-based mechanism that depends on the local chromatin environment (Angel et al., 2011; Berry et al., 2015), which is influenced by non-coding cis polymorphism at the locus (Lempe et al., 2005; Shindo et al., 2006; Li et al., 2015; Bloomer and Dean, 2017; Sasaki et al., 2018; Qüesta et al., 2020). This non-coding variation defines a small number of major FLC haplotypes within the worldwide Arabidopsis thaliana population, which confer different vernalization responses and appear to have been maintained in the A. thaliana population due to their contributions to life history diversity (Shindo et al., 2006; Li et al., 2014).

Previous field work in Sweden and Norwich, UK, with the vernalization reference genotype Col FRÎ², and mutants in VIN3 (vin3-4) had shown that initial FLC down-regulation requires cold temperature, whereas the epigenetic silencing requires the absence of daily temperatures above 15 °C (Hepworth et al., 2018). In different climates the autumn conditions greatly influenced when epigenetic silencing initiated (Hepworth et al., 2018). These data were used to develop a mathematical model.
of vernalization that can predict FLC silencing in natural field conditions (Antoniou-Kourounioti et al., 2018; Hepworth et al., 2018). However, the importance of cis FLC polymorphism in delivering the different phases of FLC silencing in different climates was unknown. Here, we exploit our field studies in three climatically very distinct locations, over multiple years with a high degree of climate variation, to investigate genotype versus environment interaction of FLC cis polymorphism in vernalization response (Fig. 1).

Our results demonstrate how the major FLC haplotypes, differing only through non-coding variation, have different starting FLC levels and rates of response to autumn cold, but show remarkably similar epigenetic silencing rates in winter. This generates a uniform vernalisation response in most years across haplotypes and climates in the field. However, in capturing an unusual year, our experiments also reveal effects of the haplotypes on reproductive success through branching and higher silique number. By studying gene expression across years and climates, we have been able to dissect how non-coding cis variation effects are important in the adaptation of vernalization response to natural fluctuating environments.

Results

Field experiments

Li et al. (2014) identified 20 haplotypes representing the major allelic variation at the FLC locus across a worldwide panel of more than a thousand Arabidopsis accessions. Although the variation characterising these haplotypes is entirely due to non-coding or synonymous single-nucleotide polymorphism, these haplotypes conferred different responses to vernalization in laboratory conditions (Li et al., 2014). To investigate their function in field conditions, we selected accessions to represent each of the five most populous haplotypes, in total representing more than 60% of tested accessions, as well as a further accession, Löv-1, for which there is evidence of local adaptation to the climate in the region of our North Sweden field site (Duncan et al., 2015; Qüesta et al., 2020). To compare these alleles in a common genetic background, we exploited extant and developed new Near Isogenic Lines (NILs) in which the FLC allele from each accession had been repeatedly backcrossed to Col FRI(2) (“Col FRI”; Duncan et al., 2015; Li et al., 2015). These genotypes were tested across two years and three field sites, with the exception of three NIL lines which were synthesised during the experiments (Fig. 1A, 2B-J). The three field sites in Norwich, UK, in Ullstorp, Sweden (“South Sweden”) and in Ramsta, Sweden (“North Sweden”) were chosen to represent different climates, and are close to the source sites of several of the tested accessions; Vår2-6 and Ull2-5 in...
Skåne, near or at Ullstorp; Löv-1 near Ramsta (Fig. 1A). In the second year of experimentation, we also included the vin3-1 mutant in the Col FRI background. The experiments ran from August 2014 until spring 2015 and again from August 2016 to the spring of 2017. In the first year, two plantings were performed in North Sweden, two weeks apart. The temperatures that the plants experienced are shown in Fig. 1A. We measured the levels of spliced and unspliced FLC, and mRNA levels of the key cold-responsive input to FLC, VIN3, to follow the progress of vernalization in the field. The transition to flowering (bolting) was assessed both in the field and by transfers to warm inductive conditions.

Figure 1. Field experimental setup

(A) Map showing locations of field sites (dots) and the origins of five of the accessions (flowers) used in this study. These accessions, with the addition of Col-0, represent the five major and one intermediate (Löv-1) FLC haplotypes identified by Li et al. (2014). The lab genotype Col FRI was also used in this study as a vernalization-requiring reference. (B-D) Temperature profiles experienced by plants at the three field sites, North Sweden – Ramsta (B), South Sweden – Ullstorp (C) and Norwich, UK (D) (Source Data 1). Flowers above temperature profile indicate the time of the first bolt of each of the natural accessions and of Col FRI (legend at bottom left corner). Black dots below temperature profile indicate the timepoints when plant material was collected for expression analysis. Black arrows below temperature profiles indicate time of transfer to greenhouse with long-day, warm conditions to assess degree of vernalization based on bolting time.
Natural variation in different phases of FLC silencing in the field

Across all the genotypes we tested and all seven field experiments, as expected, FLC expression reduced over weeks in response to autumn and winter temperatures, whereas VIN3 was upregulated. Previously, we had noted that in 2014-5 in Norwich, substantial VIN3 upregulation did not occur until ~65 days after sowing, although temperature conditions were suitable for VIN3-independent FLC downregulation for most of this time (Hepworth et al., 2018). In the following field season, this pattern occurred again, with VIN3 upregulation delayed until 48 days after sowing (Antoniou-Kourounioti et al., 2018). For the Col FRI reference, we had found that we could fit two separate exponential decay curves to FLC; the first for the initial, slow, VIN3-independent phase and the second for the faster, VIN3-dependent phase of the downregulation (Fig. 2A; Hepworth et al., 2018). Thus three features of the FLC profile contribute to the level of FLC at any time: firstly the ‘starting level’ of FLC before vernalization, secondly the rate of downregulation in the initial VIN3-independent phase, and thirdly the rate of VIN3-dependent downregulation (Fig. 2A). This pattern was consistent across the accessions and NILs, allowing us to investigate the effect of natural variation on different aspects of FLC regulation. The time of upregulation of VIN3, and thus the time
of switching from the VIN3-independent to the VIN3-dependent shutdown, also affects FLC levels, but this was very similar between genotypes at the same site.

Figure 2. Downregulation in 2014/15 in Norwich, North Sweden (2 plantings) and South Sweden for all NILS and accessions.

(A) Experimental data for Col FRI in Norwich 2014-5, showing the temperature profile (top), FLC (middle) and VIN3 (bottom) expression. Different shades indicate the separation of the VIN3-dependent (grey) and -independent (black) phases of FLC silencing (Hepworth et al., 2018) and equivalent times in VIN3 and temperature profiles. Expression data was normalised to the control sample for 2014-5 (see Methods). N=6 except where samples were lost to death or degradation (see Methods and Source Data 2). The initial measurement in the field (Starting Levels), the rate of downregulation before induction of VIN3 expression (VIN3-independent, estimated from the slope of the fitted line) and the rate of downregulation after VIN3 induction (VIN3-dependent) are the three features that were analysed and compared for each genotype and treatment. Error bars show standard error of the mean (s.e.m). (B-J) FLC downregulation analysed as level at first time point (Starting levels), and rate of downregulation (Slope) for North (B-E) and South Sweden (F-G), or rate of downregulation before (Slope, dark bars) and after (Slope, translucent bars) VIN3 induction for Norwich (H-J). Features of genotypes that are significantly different to the reference line Col FRI are indicated by *. p-values for all comparisons are given in Supplementary file 1. Rates of downregulation are given in units of “a.u. per day”, where the arbitrary units (a.u.) correspond to the normalised concentration of FLC mRNA. VIN3 induction started at ~58 days in Norwich (Fig. S2-2, S2-3). Expression data was normalised to the control sample for 2014-5 (see Methods). Error bars show s.e.
In Norwich 2014-15, the ‘Rapid Vernalizing’ (RV) FLC accession Edi-0 (haplotype group RV1) behaved very similarly to Col \textit{FRI} (RV2). In comparison, the ‘Slow Vernalizing’ (SV) haplotypes (Löv-1, Ull2-5, Bro1-6 and Var2-6) show higher levels of \textit{FLC} throughout the winter (Fig. 2, S2-1). Ull2-5 started with similar levels of \textit{FLC} as Col \textit{FRI}, but slower rates of downregulation in both the VIN3-independent and VIN3-dependent phases generated higher levels of \textit{FLC}. For both Var2-6 and its NIL, an apparently slower VIN3-independent rate of downregulation contributed to its raised \textit{FLC}, indicating that the cis variation at \textit{FLC} was responsible for this difference. The VIN3-dependent phase was also slower in Var2-6 and Löv-1 (Fig. 2), but not in their NILs, suggesting that this effect is not governed by the \textit{FLC} locus.

In Sweden 2014-5, at both sites the VIN3-independent and -dependent phases occurred concurrently (Fig. 2, S2-1; Antoniou-Kourounioti et al., 2018). There was little variation observed in the overall rate of \textit{FLC} downregulation between genotypes, though VIN3 induction was more variable (Fig. S2-2). Instead, most of the natural variation in \textit{FLC} levels throughout winter in Sweden was generated by differences in the early expression level. In North Sweden again both RV accessions had similar starting levels whereas the SV Swedish accessions were higher to different degrees. However, in South Sweden Löv-1 starts similarly to Col \textit{FRI}.

For the 2016-7 experiment, we sowed plants in North Sweden and Norwich two weeks earlier and South Sweden three weeks earlier than for the 2014-5 season (Fig. 1A, 3, S3-1, S3-2). In North Sweden this was followed by a warm autumn and produced a delay in VIN3 induction similar to that seen in both years in Norwich. However, the first stage of shutdown was still more rapid in North Sweden than Norwich, despite higher average temperatures in North Sweden (Fig. 1A), so that both VIN3-independent and VIN3-dependent phases had similar rates, resulting in the appearance of a single decline. As in the previous experiment in Sweden, these rates of downregulation were generally similar between different genotypes, with higher \textit{FLC} levels in SV accessions and the Var NIL due to higher starting levels (Fig. 3, S3-1).

In Norwich the patterns seemed to be largely repeated, though our statistical power was lower in the 2016-7 experiment due to fewer timepoints. Bro1-6, Löv-1, Var 2-6 and their NILs appeared to have higher starting \textit{FLC} levels, though of these only Bro1-6 was significant in our analysis. Ull2-5, Var2-6, Edi-0 and Löv NIL2 showed slower VIN3-independent downregulation and Ull2-5 and Var2-6 also again showed slower VIN3-dependent downregulation (Fig. 3). Other than this slower VIN3-independent rate in Norwich in Edi-0, the RV alleles behaved similarly to each other again in 2016-7.
The slower rate of the later phase in Var2-6 was consistent across years in Norwich (Fig. 2, 3). Unlike the other accessions, this change in the VIN3-dependent phase in Var2-6 was consistently mirrored in lower levels of VIN3 (Fig. S2-2, S3-1). The circadian clock is an important regulator of VIN3 (Antoniou-Kourounioti et al., 2018; Hepworth et al., 2018). When sampled over 48 hours in the Norwich field experiment (Fig. S3-3), VIN3 expression in Var2-6 is much lower compared to our previous results from Col FRI (Fig. S3-3B, D; Antoniou-Kourounioti et al., 2018), as is the expression of circadian clock component CIRCADIAN CLOCK ASSOCIATED1, the protein of which binds to the VIN3 promoter (CCA1; Fig. S3-3; Nagel et al., 2015). Therefore, variation in circadian regulation may underlie some of the difference in FLC regulation in Var2-6.

Figure 3. Downregulation in 2016 in Norwich and North Sweden for NILS and accessions show similar patterns of response to the first year.

(A-D), FLC downregulation in Col FRI, Var2-6 and the Var NIL, as measured for Norwich and North Sweden in the winters of 2014-5 and 2016-7. (E-J), FLC downregulation as starting level and VIN3-independent and dependent rates. Features of genotypes that are significantly different to the reference line Col FRI are indicated by *. p-values for all comparisons are given in Supplementary file 1. Expression data was normalised to the corresponding control sample (2016-7, see Methods). N=6 except where samples were lost to death or degradation (see Methods and Source Data 3). Rates of downregulation are given in units of “a.u. per day”, where the arbitrary units (a.u.) correspond to the normalised concentration of FLC mRNA. Error bars of bar plots show s.e., of line graphs show s.e.m.
Chromatin modifiers control FLC regulation in the field

In Norwich, our contained site allowed us to investigate mutants in genes known to affect FLC levels before cold or in response to cold to look at what trans factors may provide temperature information to the different phases of shutdown (Fig. 4).
As previously reported (Hepworth et al., 2018), mutants in VIN3 (vin3-4, vin3-1) did not show the increase in downregulation rate that marks the later epigenetic phase of silencing (Fig. 4C, F). This effect was also seen in other epigenetic memory mutants; VERNALIZATION1 (vrn1-4); the PRC2 component VRN2 (vrn2-1); the VIN3-related protein VRN5 (vrn5-8); and LIKE HETEROCHROMATIN1 (lhp1-3), in accordance with this phase representing epigenetic silencing. Loss of the H3K36 methyltransferase SET DOMAIN GROUP8 (sdg8) generated the same effect, correlated with its delayed upregulation of VIN3 (Kim et al., 2010; Finnegan et al., 2011).

In the initial, VIN3-independent silencing phase, both Col fri and sdg8 FRI were hyperresponsive, consistent with SDG8’s role with FRI in the establishment of high FLC transcription (Hyun et al., 2017). Mutants in the ‘autonomous flowering’ pathway, which upregulate FLC expression in the absence of FRI, had no significant effect on VIN3-independent silencing, but behaved mostly like Col FRI, although fve-3 reduced the rate of downregulation non-significantly in both years. The mutant with the most dramatically reduced VIN3-independent response was the B3-binding transcription factor VAL1 (VP1/ABI3-LIKE 1 ), required for PRC2 action at FLC (Fig. 4E; Qüesta et al., 2016; Yuan et al., 2016), followed closely by the vrn mutants, vrn1 and vrn5. Conversely, the lhp1-3 and vin3 mutants clearly show no impairment in the early phase of downregulation. Overall, it seems that epigenetic silencing components are required for both phases of downregulation in the field.

**Figure 4.** Starting levels and rates of downregulation of FLC in mutants and transgenics in field conditions in Norwich, UK. (A-F), FLC downregulation analysed as level at first time point (Starting levels, A, D), rate of downregulation before induction of VIN3 expression (Slope, dark bars, B, E) and rate of downregulation after VIN3 induction (Slope, translucent bars, C, F). Features of genotypes that are significantly different to the reference line Col FRI are indicated by *. p-values for all comparisons are given in Supplementary file 1. Rates of downregulation are given in units of “a.u. per day”, where the arbitrary units (a.u.) correspond to the normalised concentration of FLC mRNA. VIN3 induction started at: Norwich 2014, ~58 days, see Fig. S2-2; Norwich 2016, ~48 days, South Sweden 2016, ~35 days, North Sweden, ~46 days, see Fig. S3-1). All mutants are in the Col FRI background unless otherwise stated. Expression data was normalised to the corresponding control sample (for 2014-5 or 2016-7, see Methods). N=6 except where samples were lost to death or degradation (see Methods and Source Data 2 and 3). Error bars show s.e.
Initial levels of FLC are the major variables in vernalization response

To identify the major variable aligning vernalization response to different climates, we estimated the coefficient of variation for the rates of shutdown and starting levels for all the natural accessions, NILs, and vin3-1 where available (from Fig. 2, 3, S2-1, S3-1, Supplementary Table 3). In Sweden the starting levels are significantly more variable than the slopes (p-value=2.8 \times 10^{-8} for the first year, Fig. 5A) but in Norwich the VIN3-independent shutdown rate is more variable than the starting levels and VIN3-dependent rate (p-value=2.1 \times 10^{-5} for 2014, p-value=5.0 \times 10^{-4} for 2016, Fig. 5B).

Combining Norwich and Sweden data, the early shutdown rate is again most variable (p-value=1.9 \times 10^{-10} for 2014, p-value=2.0 \times 10^{-5} for 2016, Fig. 5C). On the other hand, there was no significant difference in the variability of the starting levels (Fig. 5D) between the different field sites and years, and similarly for the shutdown rates (Fig. 5E-G). What we describe as the starting level was measured after some days in the field, so it is not equivalent to a non-vernalized control. Some FLC shutdown, most likely VIN3-independent, will have occurred at that time. Therefore, it is likely that the combination of these two determinants of FLC levels early in the field (starting levels, VIN3-independent shutdown) provides most of the potentially adaptive variation.
Figure 5. Causes of natural variation in FLC levels across sites and years

(A) The coefficient of variation for the rates of shutdown and for the starting levels in all Sweden experiments in the first year. (B) Similarly in Norwich 2014 (top) and 2016 (bottom) but separately for the VIN3-independent (V-indep) and VIN3-dependent (V-dep) shutdown rates. (C) Comparison of the variability of the starting levels and the shutdown rates, separating V-dep and V-indep where appropriate, combining data from all sites in 2014 (top) and 2016 (bottom). “Shutdown” refers to the combined V-dep/V-indep shutdown rate that was fitted in Sweden 2014, and so is not present in the 2016 results. (D) The coefficients of variation of the starting levels for each site/year. (E) The coefficients of variation of the single shutdown rates for the different plantings and sites in Sweden in 2014. (F-G) Similarly, for Sweden 2016 and Norwich in both years, separating the V-indep rates (F) and V-dep (G). Data from Source Data 2 and 3.
Vernalization is saturated before midwinter in the field

Having characterised the variation in FLC response to field conditions, we investigated the effect of this variation on the floral transition in the field.

In the 2014-15 season, accessions and NILs bolted quite synchronously in Norwich and Sweden, with only the Bor1-6 accession in Norwich showing much relative delay in the floral transition (Figure S6-1 and S6-2). These results suggested that the vernalization effect on FLC is normally saturated across accessions and climates in the field. To test this hypothesis, in 2016-17 we removed plants from field conditions to heated, long-day conditions to induce flowering and scored the length of time until floral buds were visible at the shoot apex (bolting). There was wide variation in the delay to bolting between and within the genotypes for plants transferred to the warm greenhouses early in autumn, at all sites, indicating that vernalization requirement had not been saturated at this point (Fig. 6A, C, E). However, as winter progressed, time to bolt reduced and became more uniform for plants within and between each genotype (Fig. 6B, D, F), although vernalization saturated at different rates in different genotypes (Fig. S6-3, S6-4). All accessions and NILs bolted broadly synchronously after removal on 21\textsuperscript{st} December in Norwich, in South Sweden by 17\textsuperscript{th} December, and in North Sweden accessions were almost synchronous by the date of the final transfer, 24\textsuperscript{th} November (Fig. 6, S6-3, S6-4), in line with previous findings for North Sweden (Duncan et al., 2015). Therefore, in current climates almost all Arabidopsis plants have probably saturated their requirement for vernalization well before midwinter.

Figure 6: Vernalization requirement for FLC downregulation is saturated in natural winters.

(A-F) Bolting time for accessions and NILs after transfer to floral-induction conditions from ‘natural’ winter 2016-17, in (A) Norwich 21/10/16 (B) Norwich 21/12/16 (C) South Sweden 01/10/2016 (D) South Sweden 17/12/16 (E) North Sweden 06/09/2016 (F) North Sweden 24/11/2016. Plants that did not flower within 70 days (C, D) or 205 days (E, F) not shown. (G-H) North Sweden 2016 transfers for accessions and NILs, (G) mean time to bolting after transfer to floral-inductive conditions plotted against mean FLC expression per genotype at transfer, Norwich 2016-17, $R^2=0.68$, $p<0.001$. (H) mean time to bolting after transfer to floral-inductive conditions plotted against mean FLC expression per genotype at transfer, North Sweden 2016-17, genotypes that did not bolt within 205 days not shown, $R^2=0.85$, $p<0.001$. N=12 plants except where plants died or (E, H) did not bolt within 205 days (Source Data 5). Error bars show s.e.m.
The same expression levels from different FLC alleles predict different flowering transition responses in transfer experiments.

Across genotypes, in Norwich and North Sweden 2016-7, the time to the floral transition correlated closely with the FLC expression at the time of transfer to floral inductive conditions, as expected (North Sweden, $R^2 = 0.85$, $p<0.001$, Norwich, $R^2 = 0.68$, $p<0.001$, linear regression Fig. 6G, H).

Accessions and NILs with high starting levels and slower downregulation rates generally bolted later and took longer to saturate their vernalization requirement.

However, each accession differed in the relationship between FLC levels at transfer and subsequent bolting time (Fig. S6-5, S6-6). This is partly due to variation at the many other genes that regulate the
floral transition, but it was also observed among the NILs, suggesting that this relationship is also
controlled by cis variation at FLC. For example, for Löv-1 and the Löv NIL1, the time to bolt for a
given level of FLC at transfer is longer than for Col FRI, but for Edi-0, it is shorter (Fig. S6-5, S6-6).
This analysis allowed us to extract a further feature of FLC regulation, the relationship of FLC levels
at the time of transfer to the floral transition time. We named this feature the FLC-post-vern value
(m in Table 1). An allele with a higher m value suggests that at a given level of FLC, this allele results
in later flowering than an allele with a lower m value would at the same expression value of FLC at
the time of transfer. We calculated this value from both the Norwich and the North Sweden
transfers. There was substantial variation between the different glasshouses in the estimates for the
accessions (Table 1). However, for the lines in the common Col FRI background the estimates
generally correlated well, with the exception of the Ull NIL. Therefore, within the Col FRI
background, we can quantify our previous observations and use the FLC levels to roughly predict the
expected bolting time.

Table 1. Linear regression relationship between bolting time and FLC mRNA expression,
as shown in Fig. S6-5 and S6-6, where days to bolting = m[FLC mRNA] + c, and m is the 'FLC-
post-vern' value and c is a fitted constant relating to non-FLC-mediated bolting delay. NA
– estimate only based on two data points, so no standard error is calculable. n.d – no data.

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High FLC reduces precocious flowering in a warm autumn

At all sites in the 2014-15 season, and in Norwich in 2016-17, none of the natural accessions or NILs flowered in the field until after mid-winter (Fig. S6-1, S6-2). Therefore, in at most sites in most years the variation in FLC levels in autumn that we had observed had no phenotypic consequence.

However in North Sweden winter 2016-17 many of the plants with the Col background (NILs, Col FRI, and vin3-1 FRI) transitioned to flowering early, by the 18th November (65 days after sowing), before winter and snowfall (Fig. 7A). Precocious bolting was rare in the SV accessions and was much reduced even in the RV accession Edi-0. Over all the genotypes, the percentage of plants transitioning to flowering before winter negatively correlated with genotype FLC expression on 5th October, one day after the first recorded bolting for plants in the field (p<0.001, GLM for binomial data, Fig. 7B). Within the Col FRI\(^{F2}\) background, the SV Var and Löv FLC alleles, the NILs with the highest FLC levels during autumn (Fig. S3-1), substantially reduced precocious bolting (p=0.005, binomial proportions test, Fig. 7A).

Figure 7: High FLC genotypes reduce precocious bolting in North Sweden in warm years.

(A) Percentage of plants bolting before winter in the North Sweden 2016 experiment by genotype. Plants in the field are less likely to flower precociously before winter (18th November 2016) if they are accessions from more northerly latitudes or, to a lesser degree, if they are FLC introgression lines from these SV haplotypes. (B) The percentage of plants transitioning to flowering before winter per genotype negatively correlated with FLC expression (normalised to control) on 5th October (R\(^2=0.59\), p = 0.0058). (C) Total number of siliques produced per genotype, showing contribution from plants that bolted before winter and plants that bolted after. Within the Col FRI genetic background. no overall penalty in average silique number for surviving plants bolting before versus after winter (92 and 77 per plant respectively, not significant in Mann-Whitney U test). (D) Mean silique production in plants surviving to set seed positively correlated to their mean rosette branch production for Col FRI genetic background genotypes (NILs and vin3-4) (R\(^2 = 0.56\), p-value = 0.002). (E) Rosette branch production of Col FRI genotypes surviving to set seed is strongly negatively correlated with the FLC post-vern value for that genotype as from Table 1 (R\(^2 = 0.86\), p-value < 0.002). (F) Total number of siliques produced by Col FRI background genotypes plotted against FLC post-vern, linear regression for post-vern effect alone, R\(^2 = 0.35\), p-value = 0.1. (G) Total number of siliques produced by Col FRI background genotypes plotted against percentage survival of that genotype to point of seed set, linear regression for survival effect alone, R\(^2 = 0.64\), p-value = 0.019. N=36 plants sown (A-C), n for surviving plants (D-G) varies per genotype, see Source Data 6.
Across all genotypes, plants that bolted precociously were not observed to set seed before winter and were less likely to survive the winter (42% of bolting plants survived, whereas 67% of non-bolting plants survived to spring, p<0.001, binomial proportions test, Fig. S7-1A). Therefore, high FLC levels in October correlated with higher survival to seed set in the field (p<0.003, GLM for binomial data, Fig. S7-1B).

**Variation at FLC affects fitness in the field through branching and silique number**

Although survival is critical for fitness, subsequently the number and viability of the seeds set by the plant determines reproductive success. We confirmed the promotive effect of vernalization on branching in *A. thaliana* in our core accessions and NILs in controlled conditions (Fig. S7-2, S7-3) and noted that NILs bearing different FLC alleles showed different rates of branch production in response to differing lengths of vernalization (Fig. S7-3C). Moreover, in the 2016 Norwich transfer experiments, plants that were transferred before the vernalization requirement was saturated produced lower and more variable amounts of seed (Fig. S7-4).

To investigate the role of FLC specifically on silique numbers, we looked at the behaviour of the genotypes within the Col FRI genetic background. In these plants, there was no overall penalty in average silique number for plants that bolted before winter compared to those that transitioned afterwards, provided that they survived (Fig. 7C, S7-1D).

Silique production occurred only after winter, after saturation of vernalisation requirement (Fig. 6F) and yet there was large variation in branching and silique production within the Col FRI background plants. We asked whether the likelihood of FLC reactivation after winter might relate to fitness. To estimate this, we tested the relationship between FLC and bolting time in the warm, the ‘FLC-post-vern value’ as derived from Fig. S6-S and Fig S6-6 (Table 1), however, this did not seem to affect survival to seed set or date of bolting of the survivors (Fig. S7-1E, F). Instead, within the Col FRI background, the average silique set amongst survivors correlated with rosette branching (Fig. 7D, Fig. S7-1G) and branch number was strongly negatively correlated with FLC-post-vern value (Fig. 7E).

Between them, survival to seed set and post-vern value explained a large part of the variation in total silique set per Col FRI genotype (linear model adjusted R²= 0.94, p-value = 0.001 for model, post-vern p-value = 0.006, percentage survival to silique set p-value = 0.002, Fig. 7F, G).

**Discussion**

Investigation of the dynamics of key floral integrators in the field has recently led to important insights into their molecular response to natural environments (Antoniou-Kourounioti et al., 2018; Song et al., 2018). Here we integrate experiments on the expression of FLC in field conditions with...
investigation on how natural genetic variation and induced mutation interact in distinct climates to affect phenotype and fitness. In our previous work, we found that, as expected from laboratory studies, FLC was repressed and VIN3 was induced by winter cold (Antoniou-Kourounioti et al., 2018; Hepworth et al., 2018). However, in 2014-5, winter conditions in Norwich generated a subtly different response to those in Sweden, activating the VIN3-independent transcriptional shutdown before the VIN3-dependent epigenetic pathway. In the following season, this pattern was triggered again, not only in Norwich but also in North Sweden, implying that this is a common occurrence that plants must adjust to across climates, although with different frequencies at different locations.

We find that FLC level variation in both accessions and NILs is largely due to differences in the starting FLC level and the VIN3-independent rate of shutdown, which vary widely between accessions but not between climate sites or years (Fig. 5). This variation in response, as well as absolute levels of FLC, partly explains why FLC levels measured at any one time may not correlate with the final flowering time phenotype in different accessions (Sasaki et al., 2018).

In Norwich, we investigated the mechanisms of the responses. Contrary to our expectations, mutants of the autonomous pathway, despite upregulating FLC in fri plants in laboratory conditions (Ausín et al., 2004; Liu et al., 2010; Wu et al., 2016) generally behaved in a remarkably similar manner to Col FRI in terms of vernalization response (Fig. 2, 3). However, the reduced response of the fve mutants in the VIN3-independent phase, though not significant in each year, may be worth further investigation, as FVE has been implicated in intermittent cold-sensing through histone deacetylation at FLC, independently of vernalization (Kim et al., 2004; Jung et al., 2013).

All three factors that set FLC levels in the field (starting levels, VIN3-independent and VIN3-dependent phases) require chromatin modifiers for their correct function. Whether this is a direct effect on FLC chromatin, or due to epigenetic control of unidentified trans factors that govern the VIN3-independent response, is unclear. The VIN3-independent phase does not generate a strong epigenetic memory of cold, supporting a trans effect (Hepworth et al., 2018). However, the VAL1 transcription factor, which binds directly to FLC and is required for PRC2 action there (Qüesta et al., 2016; Yuan et al., 2016), has a strong effect on the VIN3-independent pathway (Fig. 3), suggesting it acts directly, whether via PRC2 or another mechanism.

These epigenetic factors are rarely found in genome-wide-association studies for flowering time, likely because their modification would have pleiotropic effects. In the natural accessions we find that the VIN3-dependent phase is indeed the least variable (Fig. 5). This may explain why so much...
variation in vernalization maps to FLC itself (Lempe et al., 2005; Sánchez-Bermejo et al., 2012; Dittmar et al., 2014; Sasaki et al., 2015; Bloomer and Dean, 2017; Sasaki et al., 2018).

The importance of FLC variation in adaptation for natural populations is well established (Méndez-Vigo et al., 2011; Sánchez-Bermejo et al., 2012; Dittmar et al., 2014; Li et al., 2014; Duncan et al., 2015; Ågren et al., 2016; Bloomer and Dean, 2017). Nevertheless, we found that even in a challengingly warm year, across three climates vernalization requirement saturated well before midwinter (Fig. 6). This response had been seen previously in North Sweden in the locally adapted Löv-1 accession as a reaction to the extreme winters (Duncan et al., 2015). However, we find that this is a general response in all our tested accessions. The consequences of incomplete vernalization are severe — in our 2016-17 transfer experiments this led to delayed flowering and reduced fecundity (Fig. 6, S7-4). Hence, there is strong selection pressure on survival for RV FLC alleles in warm conditions, as demonstrated in field experiments in Italy (Ågren et al., 2013; Grillo et al., 2013; Dittmar et al., 2014; Ågren et al., 2016).

However, in reciprocal transplant experiments, Ågren and coworkers found that the Italian RV FLC allele generally had a neutral effect on survival in Sweden, as expected for survival alleles when outside their adapted locality (Fournier-Level et al., 2011; Ågren et al., 2013; Ågren et al., 2016).

Likewise, in 2014-5, the low mortality and synchronicity of flowering did not indicate any strong effect of the FLC allele after winter across our field sites (Fig. S6-2), suggesting that the autumn saturation of vernalization had removed its influence. Nevertheless, we observed that the Swedish, cold-winter-adapted accessions and SV alleles expressed the highest FLC levels during autumn (Fig. 2, 3, S2-1, S3-1). In North Sweden in 2016-7, an unusually warm growing season revealed an adaptive role for these high FLC levels. In plants with an SV allele, including the locally-adapted Swedish accessions, the higher level of FLC expression protected against precocious flowering and its consequent reduced survival (Fig. 7, S7-1). These rare, but highly selective occurrences, may be a driving force for local adaptation. That the effects of these alleles are only revealed occasionally is a logical consequence of the fact that the flowering time genes can have strong or weak effects depending on the environment (Wilczek et al., 2009; Burghardt et al., 2016; Fournier-Level et al., 2016; Taylor et al., 2019).

FLC also controls fecundity as well as survival (Ågren et al., 2013). Li et al. (2014) found that SV FLC alleles produced lower seed weight compared to RV alleles in non-saturating vernalization conditions. In Arabidopsis, saturation of vernalization requirement is known to increase flowering branch production, particularly rosette branch production, and this effect is linked to FLC (Huang et al., 2013; Jong et al., 2019). In the Arabidopsis relative Arabis alpina, this effect has been linked to
the FLC homologue PEP1, and has a subsequent effect on fitness by influencing silique production (Lazaro et al., 2018). We confirmed the effect of the FLC allele in A. thaliana on branching response to vernalization in laboratory conditions (Fig. S7-2). In the field, silique production, and hence fitness, in surviving plants was closely linked to branch production (Fig. 7). As expected, FLC levels in autumn did not correlate with branch production in spring (Fig. S7-1H), and in Norwich 2014-15, there was little variation in branch production. However, we found that under the conditions in the field in North Sweden, branch production was negatively related to a factor we named ‘FLC post-vern’, which encoded the relationship between FLC levels and subsequent flowering in warm controlled conditions (Fig. 7). Variation in this factor probably derives from regulatory differences at FLC post-cold, such as the reactivation phenotype of the Löv-1 FLC allele (Shindo et al., 2006; Coustham et al., 2012; Qüesta et al., 2020), allowing FLC repression of flowering to saturate at the shoot apical and axillary meristems at different rates, a phenomenon that occurs in the perennial relative Arabis alpina (Wang et al., 2009; Lazaro et al., 2018). Why the field conditions in Sweden, but not Norwich, revealed these conditions is not yet clear. This property of FLC appears to be regulated separately to that of FLC vernalization response, as a slow VIN3-independent shutdown does not necessarily cause slow flowering in the warm. As such, it is an example of how one gene can directly regulate independent phenotypes in response to different environmental cues, mitigating evolutionary constraints in which selection for one phenotype may be traded-off against concomitant changes to another (Auge et al., 2019). Given that in many cases there is stronger selection for high branch production than flowering time in the field (Taylor et al., 2019), this is likely to be an important evolutionary constraint for FLC alleles.

In summary, our detailed analysis of the different phases of FLC silencing through winters in distinct climates, over multiple years, has given a clear picture of the mechanistic basis of adaptation in vernalization response. FLC starting levels and early phases of FLC silencing are the major determinants for variation in vernalization. Non-coding FLC SNP variation aligns vernalization response to different climatic conditions and year-on-year fluctuations in natural temperature profiles. In a changing climate, understanding the complex genotype by environment interactions that govern timing mechanisms will become ever more important.
Materials and Methods

Plant materials

Sources of previously described mutant lines and transgenics are presented in Supplementary Table 1.

NILs

All near-isogenic lines were produced by six rounds of backcrossing to the Col FRI parent, selecting for the introgressed FLC in each generation, before one round of selfing and selection of homozygous families.

Supplementary Table 1. Sources of previously published mutants and transgenics.

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Near Isogenic Lines

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Mutants


Experimental conditions

Field experiments

Field experiments have been described previously (Antoniou-Kourounioti et al., 2018 (2016-7 winter); Hepworth et al., 2018 (2014-5 winter)). Briefly, seeds were stratified at 4°C for three days.

For gene expression measurements, for all field sites and sowing dates and timepoints within them, six replicate tray-cells were sown using a block-randomised design within 5.7cm² cell trays (Pöppelman, Lohne, Germany), and where each replicate included material from at least three plants. For flowering time, plants were thinned to a single plant per cell in 3.9cm² cell trays (Pöppelman). Trays were watered when necessary.

In Norwich, trays were placed on benches in an unheated, unlit glasshouse, and bedded in vermiculite. For expression, plants were randomised within 6 single-replicate sample-sets, which were then randomised using Research Randomiser (Urbaniak and Plous, 2015) in a 3 complete block design lengthwise along the greenhouse, adjusting to ensure each of the two replicates per block were on different benches. For flowering time, plants were block-randomised per tray and per block.
In Sweden, trays were germinated and grown outside under plastic covers for two weeks at Mid Sweden University, Sundsvall (North Sweden) or Lund University (South Sweden). Trays were then moved to the experiment sites dug into the soil. The experiment site in the North was at Ramsta (62° 50.988’N, 18° 11.570’E), and in the South at Ullstorp (56° 06.6721’N, 13° 94.4655’E). Expression sample-sets were randomised in three blocks. Flowering time plants were in a completely randomised design across two (2014-15) or three (2016-17) blocks.

Plants were sown and moved to the field site on: Norwich first year, sown into position on 29th September 2014, second year, sown into position on 15th September 2016; South Sweden first year, sown 24th September 2014, moved 8th October 2014, second year sown on 6th September 2016, moved on 21st September 2016; North Sweden first year, first planting ‘A’ sown 26th August 2014, moved 11th September 2014; second planting ‘B’ sown 8th September 2014, moved 24th September 2014; North Sweden second year, sown on 12th August 2016, and moved 24th August 2016.

For the transferred plants, in Norwich 2016-7, for each transfer 6 trays (each holding 2 replicates, total n=12) were moved from the unheated, unlit, ventilated greenhouse to a greenhouse with supplementary lighting (600W HPS lamps) and heating set to 22°C/18°C, 16 light/8 hour dark, and 70% humidity on 21st October 2016, 3rd November 2016, 17th November 2016, 30th November 2016, 21st December 2016 and 26th January 2017. For selected time points, plants were covered with ventilated clear plastic bags to collect seed for weighing. In South Sweden, trays were transferred from the field site to heated, lit greenhouses at Lund University, on 1st October, 22nd October, 19th November and 17th December 2016. In North Sweden, for each transfer 3 trays (each holding 4 replicates, n=12) were moved from the field site to a greenhouse set to 16 hr light, 22°C, at Midsweden University, Sundsvall (as in Duncan et al., 2015), on 6th September, 4th October, 1st November and 24th November 2016.

For all expression analysis except the 48hr sampling (Fig. S3-3), 6 replicates per timepoint per genotype were chosen in order to allow sufficient number of samples for statistical analysis while allowing for losses in the field and to allow duplication within randomization blocks. Where resulting samples are smaller, this is due to experimental or processing loss (e.g., death of plants in the field, degradation due to poor sample quality or processing, see RNA extraction and QPCR). For Fig. S3-3, 3 replicates per timepoint per genotype were chosen due to space constraints. Each expression sample (single replicate) was of at least 3 plants pooled. For flowering time, 12 plants per genotype per transfer condition were chosen to provide replication across blocks and trays while remaining within size constraints. For field flowering in North Sweden 2016-17, 36 plants per genotype were sown to allow for losses, although in the event these were more substantial than anticipated.

Temperature was recorded at plant level at each site with TinyTag Plus 2 dataloggers (Gemini Data Loggers (UK) Ltd, Chichester, UK). Bolting was scored when flower buds were visible at the shoot apical meristem. For the North Sweden 2016-7 field experiment, plants were scored for survival and flowering in the field from planting to December 2016, and then from March to May 2017. Plants were harvested and scored for branching and silique production after the end of flowering, in July 2017.

**Branching analysis**

Fig. S7-2, S7-3; Seeds were sown on soil, stratified after sowing for three days at ~4-5°C, and transferred to a Norwich long-day glasshouse set to 18°C/15°C, 16/8 hour light/dark conditions for 7
days before being returned to vernalization conditions (a 4°C growth chamber under short day, low light conditions; 8/16 hour light/dark) for 12, 8, 4, and 0 weeks. Sowing was staggered so that after vernalization all plants were transferred to glasshouse conditions simultaneously. Plants were scored for their flowering time, total branch number, cauline branch number and rosette branch number. In all cases, plants were randomized into blocks and at least three replicate plants for each accession/cultivar per treatment were scored for their flowering and branching phenotypes. Primary rosette and cauline branch number were scored at senescence.

**RNA extraction and QPCR**

RNA extraction and QPCR for field experiments were performed as described in Hepworth *et al.* (2018) and Antoniou-Kourounioti *et al.* (2018). Field data, was unified across sites and timepoints within the yearly datasets and normalised to a synthetic control sample, as described in Hepworth *et al.* (2018). QPCR results were analysed using LinReg (Ruijter *et al.*, 2009), and normalised to the geometric means of At5g25760 (‘PP2A’) and At1g13320 (‘UBC’) control genes (Czechowski *et al.*, 2005; Yang *et al.*, 2014).

QPCR samples that showed high Cp values (UBC Cp>28 for LinReg analysis) of the control genes, indicating possible degradation, were excluded if test amplicons (FLC, VIN3) results were also anomalous (criteria: completely absent, or varying from non-flagged samples by an order of magnitude). Any measurements where amplicon Ct values varied by more than 0.6 were also excluded if insufficient sample was available for a repeat.

Primers used are described in Supplementary Table 2.

**Supplementary Table 2. Primers used for PCR.**

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Statistics

For the analysis presented in Supplementary file 1 and Figures 2-4, the Starting level comparisons and the Slope comparisons were done separately, using the following methods. For the starting levels, ANOVA was performed in the R (R Core Team, 2018) statistical language using the lm function, followed by a Dunnett post hoc test comparing all genotypes against the control Col FRI.

For the slopes, the lmer function from the lmerTest package in R was used to perform the same comparison on the genotype-timepoint interaction, controlling for blocks as random factors.

We used the R package cvequality (Version 0.1.3; Marwick and Krishnamoorthy, 2019) with the asymptotic test (Feltz and Miller, 1996) to assess differences between the coefficients of variation of different groups as described in the text and Supplementary Table 3. The significance limit was adjusted to control the false discovery rate using the Benjamini-Hochberg procedure with a false discovery rate of 0.05 (Supplementary Table 3, Supplementary file “coef_var_comparison_analyses.xlsx”). This analysis was performed including all the natural accession and NILs combined (Fig. 4), but also for all the accessions separately and for all the NILs separately (including Col FRI in both cases). The different analyses do not change our overall conclusions and all three are reported in Supplementary Table 3.

For multiple regression on field data, R was used to obtain minimal adequate models using linear regression (lm function), except when n>10 for count data, for which general linear models (GLM, glm function) using Poisson error distributions were used (total numbers of siliques), or for proportion data for which GLMs with binomial errors were used (survival, bolting before winter).

Supplementary Table 3. List of all comparisons of coefficients of variation.

Differences between the coefficients of variation of different groups (sites, features, years; 24 comparisons).

<table>
<thead>
<tr>
<th>#</th>
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<th>Groups</th>
<th>Signif. Acc</th>
<th>Signif. NILs</th>
<th>Signif. combined</th>
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<td>Features (2014 sites combined)</td>
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<td>Features in Norwich 2014-5</td>
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<td>Starting levels</td>
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For genetic materials, we are indebted to Prof. Johanna Schmitt (University of California Davis) for the kind gift of *vin3-1 FRI*, and Huamei Wang for creating the Ull and Bro NIL lines and preparing seed stocks. For the Swedish field sites, many thanks to family Öhman and Nils Jönsson. Thanks to Prof. James Brown (JIC) for advice on the statistics, and to Ian Baldwin (Senior Editor at eLIFE) for helpful suggestions on the initial submission of the manuscript. Finally the authors would like to...
reiterate their appreciation of all those from the Dean, Howard, Holm, Säll and Irwin groups who helped in cold, heat, wind, rain and laboratory with the field studies.

Competing interests
The authors declare that they have no competing interests.

Data Sources
Supplementary File 1 – Statistics for Fig. 2, 3 and 4.
Source Data 1 – Field temperatures
Source Data 2 – RNA Expression for all field experiments 2014-15
Source Data 3 – RNA Expression for all field experiments 2016-17
Source Data 4 – Flowering time and phenotypes for all field experiments 2014-15
Source Data 5 – Flowering time and phenotypes for all transfer experiments 2016-17
Source Data 6 – Flowering time, survival and phenotypes for North Sweden field experiments 2016-17
Source Data 7 – Flowering time and branching for accessions and NILs in constant-condition vernalisation treatments.

References
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Yang H, Howard M, Dean C (2014) Antagonistic Roles for H3K36me3 and H3K27me3 in the Cold-Induced Epigenetic Switch at Arabidopsis FLC. Curr Biol 24: 1793–1797

Supplementary Information

Figure S2-1. FLC downregulation in accessions and NILs in Norwich, North Sweden and
South Sweden 2014-5.

Expression normalised to control sample for 2014-5 (see Methods). (A-D) Norwich, (E-H) South
Sweden, (I-L) North Sweden first planting, (M-P) North Sweden second planting. N=6 except where
samples were lost to death or degradation (see Methods and Source Data 2). Error bars show s.e.m.
Figure S2-2. VIN3 upregulation in accessions in Norwich, North Sweden and South Sweden 2014-5.

VIN3 expression normalised to control sample for 2014-5 (see Methods). N=6 except where samples were lost to death or degradation (see Methods and Source Data 2). Error bars show standard error of the mean (s.e.m).
Figure S2-3. Expression of VIN3 in NILs with the Col-0 VIN3 allele in the field in 2014-2015.

(A) Norwich, (B) North Sweden first planting, (C) South Sweden, (D) North Sweden second planting.

N=6 except where samples were lost to death or degradation (see Methods and Source Data 2).

Error bars show s.e.m.
Figure S3-1. FLC downregulation and VIN3 upregulation in accessions in Norwich and North Sweden in autumn/winter 2016

Expression normalised to control sample for 2016-7 (see Methods). (A-E) Norwich FLC mRNA, (F-J) Norwich VIN3 mRNA, (K-O) North Sweden FLC mRNA, (P-T) North Sweden VIN3 mRNA. N=6 except where samples were lost to death or degradation (see Methods and Source Data 3). Error bars show s.e.m.
Figure S3-2. Downregulation of *FLC* and upregulation of *VIN3* in South Sweden in 2016.

(A) Hourly temperature readings from plant-level in South Sweden 2016. (B) *FLC* mRNA levels in the Col *FRI* and *vin3-1 FRI* accessions over autumn, with *vin3-1* showing less repression, especially later in the season. (C) unspliced *FLC* levels. (D) *VIN3* mRNA levels, with *vin3-1 FRI* showing no induction.

Expression normalised to control sample for 2016-7 (see Methods). N=6 except where samples were lost to death or degradation (see Methods and Source Data 3). Error bars show s.e.m.
Figure S3-3. Low VIN3 upregulation in Var2-6 is correlated with perturbation of the circadian clock.

VIN3 and CCA1 expression measured over 48 hours in the field glasshouse in Norwich in 2016 in Col FRI and the Var2-6 accession. CCA1 shows a circadian pattern throughout autumn in Col FRI, as does VIN3 when it is upregulated later in the year. In Var2-6, CCA1 expression is low, as is VIN3 expression later in the year. (A) Expression in Col FRI in October. (B) Expression in Col FRI in November. (C) Expression in Var2-6 in October. (D) Expression in Var2-6 in November. N=3, Source Data 3. Error bars show s.e.m.
Figure S4-1. Expression of FLC and VIN3 in all mutants in the field in Norwich 2014-2015.

Expression normalised to control sample for 2014-5 (see Methods). (A-D) FLC mRNA, (E-H) unspliced FLC transcript, (I-L) VIN3 mRNA. N=6 except where samples were lost to death or degradation (see Methods and Source Data 2). Error bars show s.e.m.
Figure S4-2. Expression of FLC and VIN3 in all mutants in the field in Norwich 2016-2017.

Expression normalised to control sample for 2016-7 (see Methods). (A-D) FLC mRNA, (E-H) unspliced FLC transcript, (I-L) VIN3 mRNA. N=6 except where samples were lost to death or degradation (see Methods and Source Data 2). Error bars show s.e.m.
Figure S6-1. Flowering after winter in Norwich 2014-5 in the field was largely synchronous. 

(A) Time to bolting for each genotype in the ‘field’ glasshouse in Norwich 2014-5 experiment. (B) Number of rosette branches for plants shown in A. (C) Number of rosette and cauline branches for plants shown in A. Box-and-whiskers plot for time to bolting for each genotype, showing mean (grey circle), interquartile range (box), range (whiskers) and outliers (red crosses, values more than 1.5 times the interquartile range outside of the interquartile range). N=12, except where plants died,

Source Data 4. Survival data for Norwich 2014-2015 not shown as only 2 plants died.
Figure S6-2. The transition to flowering after natural winters in South and North Sweden 2014-5 in the field was largely synchronous, while later bolting had a negative effect on survival only in South Sweden.

(A, D, F) Box-and-whiskers plot for time to bolting for each genotype, showing mean (grey circle), interquartile range (box), range (whiskers) and outliers (red crosses, values more than 1.5 times the interquartile range outside of the interquartile range). (B, E, G) Percentage of germinated plants of each genotype surviving to date of bolting, plotted against the mean date of bolting for that genotype. (B) South Sweden, Generalised Linear Models (GLM) for binomial distribution, survival versus date of bolting, \( p=0.0416 \). (C) South Sweden, percentage survival versus mean \( FLC \) mRNA per genotype (normalised to control sample for 2014-5) on 30\(^{th}\) March, GLM for binomial distribution, ns. (E, G) GLM for binomial distribution, ns. N=12, except where plants died, Source Data 4. Error bars on scatter plots show s.e.m.
Figure S6-3. Bolting after transfer to warm, long-day conditions from winter in the field 2016-7 saturates at different rates in different genotypes in Sweden.

Figure S6-4. Bolting after transfer to warm, long-day conditions from winter in the field 2016-7 saturates at different rates in Norwich.

Bolting from sequential transfers to long day warm conditions from the field, for each genotype and transfer, n=12. (A) 21/10/2016, (B) 03/11/2016, (C) 17/11/2016, (D) 30/11/2016, (E) 21/12/2016, (F) 26/01/2017. N=12, except where plants died, Source Data 5.
Figure S6-5. The relationship between time to floral transition and FLC expression at the end of cold (Norwich winter 2016-7) varies among accessions, both due to trans effects and due to the FLC alleles themselves. (A-F) Mean time to bolting of plants moved to a greenhouse lit for 16 hours, and maintained at 22°C/18°C light/dark, plotted against the mean FLC mRNA expression from plants sampled in the Norwich field condition greenhouse on the day of transfer, with linear regression lines plotted. For all accessions and NILs over 3-6 transfers at different times during the winter, $R^2 = 0.68$ for linear regression, $p<0.001$. N=6 for expression data, N=12 for bolting data, except where plants died, Source Data 3 and 5. Error bars show s.e.m.
Figure S6-6. The relationship between time to floral transition and FLC expression at the end of cold in North Sweden winter 2016-7
Mean time to bolting of plants moved to a greenhouse lit for 16 hours, and maintained at 22°C, plotted against the mean FLC mRNA expression from plants sampled in the North Sweden field on or adjacent to the day of transfer, with linear regression lines plotted. (A-F) For all accessions and NILs over 3-6 transfers at different times during the winter, $R^2= 0.68$ for linear regression, $p<0.001$. For D, there is no regression for Löv-1 as no Löv-1 plants from the first two transfers flowered within the 120 days of the experiment. N=12 for bolting data, except where plants died, Source Data 3 and 5. Error bars show s.e.m.
Figure S7-1. FLC controls fitness in North Sweden through bolting time and branching.
Survival, branching and silique set in North Sweden are all correlated to aspects of FLC regulation. 

(A) Survival over winter of plants that bolted before winter in different genotypes versus survival of plants that did not bolt before winter. 
(B) Survival to seed set plotted against FLC levels (normalised to control sample for 2016-7) in the field in North Sweden 2016 (p<0.003, GLM for binomial data). 
(C) Percentage mortality before setting seed was high for all genotypes. 
(D) Mean number of siliques for plants surviving to set seed that bolted before or after winter. 
(E) Survival in the field does not correlate with FLC post-vern for the Col FRI background (GLM with binomial distribution, p-value > 0.1). 
(F) Date of bolting in the field does not correlate with FLC post-vern for the Col FRI background (linear regression, p-value > 0.1). 
(G) Silique production by surviving Col FRI background plants correlates with number of rosette branches, though more weakly at the individual level than at the genotype average level (linear regression, R² = 0.23, p-value 0.004). N=36 plants sown (A, B, C, E) subsequent data based on survivors to seed set (D, G) and plants that survive to bolting (F), see Source Data 6.
Figure S7-2. Increased vernalization increases the amount of rosette branch production and reduces the variability of bolting time.

(A) Time to bolting for accessions in a heated, lit greenhouse without vernalization (NV) or after weeks of vernalization at constant 5°C ($n$WV), experiment 1. (B) Number of rosette branches for plants shown in A, experiment 1. (C) Time to bolting for selected accessions and NILs in a heated, lit greenhouse without vernalization (NV) or after weeks of vernalization at constant 5°C ($n$WV), experiment 2. (D) Number of rosette branches for plants shown in C, experiment 2. Median (central line), mean (cross), interquartile range (box), range (whiskers) and outliers (circles, values more than 1.5 times the interquartile range outside of the interquartile range). Plants that did not flower within 120 days of transfer not shown.
Figure S7-3. Increasing vernalization correlated with greater branch production with subtly different effects depending on FLC haplotype in the Col FRI background.
Means per genotype and vernalization length treatment of rosette branch data presented in S7-2, plotted against days to bolting. (A) Data from S7-2A-B, experiment 1, accessions only. (B) Data from S7-2A-B, experiment 1, NILs only. (C) Data from S7-2C-D, experiment 2. Error bars show s.e.m.
Figure S7-4: Increased vernalization increases the amount and reduces the variability of seed set.

(A) Flowering time for Col FRI, NILs and the vin3-4 FRI mutant after transfer to floral-induction conditions from ‘natural’ winter in Norwich 2016-7 on 21/10/16 and 17/11/16 (as in Fig. S6-A, B).

(B) Total seed mass produced by plants in A. Box-and-whiskers plot for time to bolting for each genotype, showing median (central line), mean (cross), interquartile range (box), range (whiskers) and outliers (circles, values more than 1.5 times the interquartile range outside of the interquartile range). The time to bolt per plant negatively correlated with seed mass produced, $p<0.001$, Kenward-Roger’s t-test on REML Linear mixed model with date of transfer as a random factor.