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RESEARCH COMMUNICATION

Variation in the epigenetic silencing of *FLC* contributes to natural variation in *Arabidopsis* vernalization response

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Vernalization, the cold-induced acceleration of flowering, involves the epigenetic silencing of the floral repressor gene *FLOWERING LOCUS C* (*FLC*). We investigated the molecular basis for variation in vernalization in *Arabidopsis* natural accessions adapted to different climates. A major variable was the degree to which different periods of cold caused stable *FLC* silencing. In accessions requiring long vernalization, *FLC* expression was reactivated following nonsaturating vernalization, but this reactivation was progressively attenuated with increasing cold exposure. This response was correlated with the rate of accumulation of *FLC* histone H3 Lys 27 trimethylation (H3K27me3). Thus, variation in epigenetic silencing of *FLC* appears to have contributed to *Arabidopsis* adaptation.

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Arabidopsis thaliana (*Arabidopsis*) grows in a wide range of environments, so it provides an excellent system to dissect the molecular variation underpinning adaptive variation, a major goal in ecological and evolutionary genetics. Flowering time is a key trait in adaptation, as it is vital for reproductive success. Molecular and genetic analyses in *Arabidopsis* have begun to elucidate the network of pathways that regulate flowering (Simpson and Dean 2002; Henderson and Dean 2004; Sung and Amasino 2005). An important component in this network is the floral repressor FLOWERING LOCUS C (FLC), a MADS-box protein that represses the expression of genes needed to switch the meristem to a reproductive fate (Michaels and Amasino 1999; Sheldon et al. 1999). Vernalization—the acceleration of flowering by exposure to prolonged cold—represses *FLC* expression, thus promoting flowering, whereas FRIGIDA (FRI) activates *FLC* expression and represses flowering (Michaels and Amasino 1999; Sheldon et al. 1999). In the natural context, therefore, the activities of *FRI* and *FLC* actively delay flowering until winter has passed. Plants that have such a vernalization requirement are classified as winter annuals. There is considerable variability in flowering time and vernalization response between accessions, and indications of latitudinal clines in these traits support the idea that this variation is important for adaptation (Caicedo et al. 2004; Lempe et al. 2005; Shindo et al. 2005; Stinchcombe et al. 2005; Werner et al. 2005).

Our understanding of the molecular basis of vernalization in *Arabidopsis* has improved over recent years. Prolonged cold reduces *FLC* expression, and this repression is maintained by an epigenetic mechanism as plants continue to grow in spring and summer. Mutant screens identifying seedlings unable to suppress and epigenetically maintain *FLC* repression have identified proteins that mediate vernalization: VERNALIZATION 2 (VRN2), a homolog of the *Drosophila* Polycomb group gene (PcG) Su(Z)12 (Gendall et al. 2001); VERNALIZATION 1 (VRN1), a plant-specific protein with DNA-binding (B3) domains (Levy et al. 2002); and VERNALIZATION INSENSITIVE 3 (VIN3), a PLANT HOMEODOMAIN (PHD)-finger-containing protein (Sung and Amasino 2004). Prolonged cold induces expression of VIN3 but other factors must also be cold-induced, as constitutive expression of VIN3 does not result in cold-independent early flowering (Sung and Amasino 2004). The activities of VIN3, VRN2, and VRN1 result in histone modifications characteristic of PcG-induced chromatin silencing—namely, reduced histone acetylation and increased histone K27 and K9 methylation (Bastow et al. 2004; Sung and Amasino 2004). These modifications probably recruit LIKE HP1 (LHP1), the *Arabidopsis* HETEROCHROMATIN PROTEIN (HP1) homolog that is also involved in the stabilization of *FLC* repression (Mylne et al. 2006; Sung et al. 2006).

This understanding of vernalization has opened up the opportunity to analyze how this process has changed during the adaptation of *Arabidopsis* accessions to very different growth conditions. Here, we describe the analysis of vernalization response in *Arabidopsis* natural accessions chosen from a range of habitats differing in winter conditions. The expression of the molecular target of vernalization, *FLC*, and an early component of the response, *VIN3*, were analyzed in order to understand the molecular basis of the observed phenotypic variation. Interestingly, no correlation was found between their expression and vernalization response. Instead, variation in the epigenetic maintenance of silencing of *FLC* was found to be the parameter correlated with vernalization response, and Quantitative Trait Locus (QTL) analysis suggests that at least part of this variation maps to *FLC* itself. Thus, variation in epigenetic silencing of *FLC* appears to have been a significant factor in the adaptation of *Arabidopsis* to different winter conditions.

Results and Discussion

Arabidopsis *accessions show a wide range of vernalization responses*

A previous study of the acceleration of flowering by 8 wk vernalization in 192 *Arabidopsis* accessions had shown a very wide range of responses (Shindo et al. 2005). In order to further analyze this variation we focused our effort on those collected in Sweden, as they represented a wide

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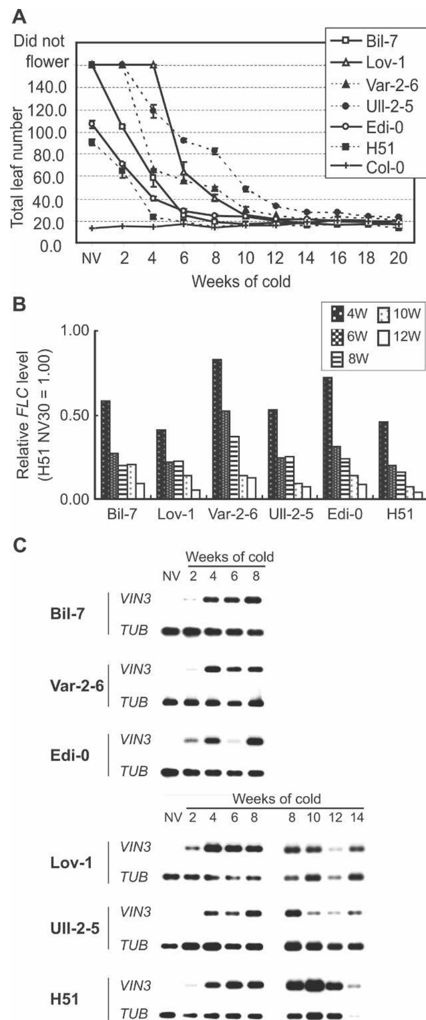

Figure 1. Variation in vernalization response and *FLC* expression of the selected accessions. (*A*) Flowering time was assayed as final LN. Data is mean of at least three experiments with standard error. Nonvernalized plants (NV) of four Scandinavian accessions did not flower when the experiments were terminated in 5 mo since germination, whereas Edi-0 and H51 completed flowering but were still very late. (*B*) *FLC* expression after different lengths of cold. (W) Weeks of cold period. (*C*) *VIN3* expression in the accessions assayed after different periods of cold. Semiquantitative PCR analysis was used to analyze *VIN3* RNA levels (Supplemental Material). Twenty-day-old nonvernalized (NV) whole seedlings were compared with 7-d-old seedlings exposed to cold for 2, 4, 6, and 8 wk and up to 14 wk for Lov-1, Ull-2-5, and H51 and then harvested in the cold.

range of latitudes and their collection sites are very well-documented (Supplementary Fig. 1). Two accessions, Bil-7 and Lov-1, were chosen from northern Sweden and two, Var-2-6 and Ull-2-5, from southern Sweden. In addition, we analyzed Edi-0, a late-flowering accession from Edinburgh, and a control genotype, H51 (Napp-Zinn 1957). The plants were given up to 20 wk in the cold and then grown in a controlled environment room, and the flowering time was assayed by counting total leaf number (LN) (Supplemental Material). The accessions showed different vernalization characteristics (Fig. 1A). The flowering of Bil-7, Edi-0, and H51, but not Var-2-6, Lov-1, and Ull-2-5, was accelerated by 2 wk vernalization. Flowering time variation between accessions was maximal after 4 wk vernalization; the acceleration of flowering of H51 was almost complete (LN = 20), whereas the flowering of Lov-1 was still unchanged. All Ull-2-5 plants did flower after 4 wk vernalization but considerably later than H51. After 6 wk vernalization, Lov-1 flowered earlier than Ull-2-5, suggesting that Lov-1 might be insensitive to short periods of cold but then respond quickly after that. Despite responding to 4 wk of cold, Var-2-6 and Ull-2-5 still flowered late after >6 wk of cold; i.e., the vernalization response was relatively slow. This was especially true for Ull-2-5, which flowered extremely late after 8 wk vernalization and required at least 14 wk of cold to fully saturate the vernalization requirement.

Our data suggest that the microclimate of each location, rather than general climatic factors varying with latitude, is likely to be a significant factor in selecting the most productive reproductive strategy. The extreme conditions found at the Bil-7 collection site—long, cold winters on a rocky mountain side—may lead to very limited growth through most of the winter months, necessitating a rapid vernalization response once winter has passed. In climates found in southern Sweden, where winter temperatures are somewhat variable, a "slow" vernalization response may be favored to ensure flowering is not initiated during an occasional warm period.

Regulators of vernalization show similar expression profiles during the prolonged cold

FLC and *VIN3* are central regulators of the vernalization response in *Arabidopsis* (Sung and Amasino 2005). In

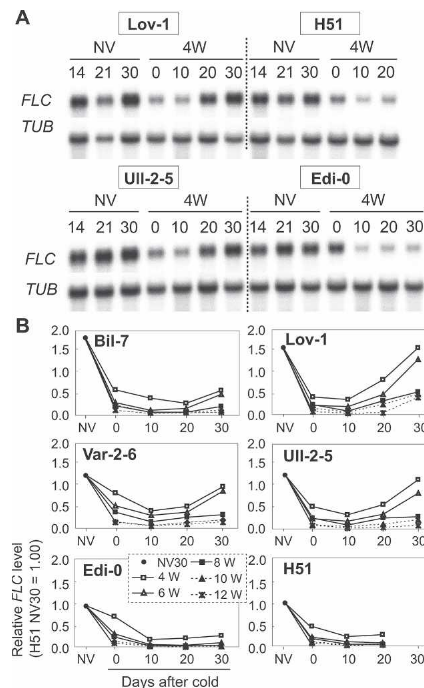

Figure 2. Variation in the maintenance of repression of *FLC*. *FLC* expression was assayed by Northern hybridization of RNA extracted from whole seedlings. (*A*) Nonvernalized (NV) plants grown for different days were compared with seedlings grown for 7 d, vernalized for 4 wk (4W), and then transferred to 23°C for 0, 10, 20, or 30 d. (*B*) Repression of *FLC* following different periods of cold. Thirty-day-old nonvernalized plants (at a developmental stage similar to the 30-d-post-vernalization plants) were compared with seedlings grown at 23°C in long-day photoperiods for 0, 10, 20, and 30 d following each cold treatment (4W, 6W, 8W, 10W, 12W). The 30-d value is missing for H51 as the plants had already flowered.

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order to determine if changes in their expression contributed to adaptive changes in vernalization response, we assayed *FLC* and *VIN3* RNA levels in nonvernalized seedlings or seedlings that had been vernalized for different lengths of time (4, 6, 8, 10, or 12 wk), with the tissue harvested immediately at the end of the cold period. Although a wide variation in *FLC* levels was found in the different accessions, there was no correlation between the *FLC* level in nonvernalized tissue (Supplementary Fig. 2) and the observed vernalization response, in agreement with previous results (Shindo et al. 2005; Werner et al. 2005). *FLC* levels did not change significantly in nonvernalized seedlings as they developed (Supplementary Fig. 2), but decreased as the plants were given increasing weeks of cold, with no apparent plateau (Fig. 1B). Despite the varying initial levels of *FLC* in the accessions the relative decrease of *FLC* expression differed only slightly between them and there was no correlation between *FLC* decrease (Fig. 1B) and flowering time. This is most clearly shown in the comparison of Bil-7 and Lov-1—accessions that responded to cold rapidly and slowly, respectively, yet both showing very similar rates of *FLC* decrease over the time period studied (Fig. 1B). Interestingly, while *FLC* decreased in Lov-1 plants over a 4-wk cold period, flowering time was not accelerated (Fig. 1A,B). *VIN3* expression that was undetectable in the nonvernalized tissue of all of the accessions analyzed, was first detected after 2 wk of cold (Fig. 1C) and then increased, with the maximum expression being reached after ~4 wk of cold. Where we extended this analysis to 14 wk (Lov-1, Ull-2-5, H51) *VIN3* expression did not appear to increase further (Fig. 1C).

Stability of the repression of FLC *varies in the accessions*

To understand why some accessions required 14 wk of cold for the full acceleration of flowering despite *FLC* expression being reduced significantly in all accessions by 8 wk of cold, we analyzed the stability of *FLC* repression. In the accessions where flowering time is rapidly accelerated by vernalization (Edi-0 and the control genotype H51), the level of *FLC* RNA decreased during the cold and then was relatively stable following 4 wk vernalization (Fig. 2A). The slight reduction in *FLC* levels immediately after transfer of plants to the 23°C controlled-environment room (0 d points in Fig. 2A)—perhaps due to either further reduction in *FLC* transcription or increased turnover of the mRNA as plant growth accelerated—was not correlated to variation in vernalization response. In the accessions that responded more slowly to vernalization, Lov-1 and Ull-2-5, *FLC* levels decreased during the cold but then increased significantly after the plants were returned to 23°C (Fig. 2A).

We extended this analysis to all six accessions, and in an independent experiment assayed *FLC* expression over a 30-d period as plants grew at 23°C following cold periods of different lengths (4–12 wk). After 4 wk vernalization, *FLC* levels had increased in Lov-1 and Ull-2-5 (by 30 d after transfer to 23°C) to the same level as nonvernalized plants, while the repression of *FLC* expression in H51 and Edi-0 was maintained (Fig. 2B). Bil-7 showed an intermediate effect, with *FLC* returning to a moderately high level after 4 wk vernalization (Fig. 2B).

After 6 wk of cold, *FLC* levels were lower in all of the accessions (as compared with 4 wk) and were then maintained at that level in Edi-0 and H51. However, they increased slightly in Bil-7 and considerably in Lov-1, Var-2-6, and Ull-2-5 (Fig. 2B). After 8 wk vernalization, *FLC* levels were stable during subsequent development in all of the accessions except for Lov-1. Indeed, even after 12 wk vernalization *FLC* levels increased in Lov-1 after 30 d growth post-cold (Fig. 2B). Analysis of these northerly accessions thus revealed that increasing weeks of cold quantitatively enhances the stability of *FLC* repression, and the variable that shows the most correlation with vernalization response is the ability to epigenetically suppress *FLC* expression in post-cold growth.

QTL analysis of four populations suggests that allelic variation at four to five genes accounts for the range of phenotypic variability in vernalization response

To determine the genetic basis of the variation in vernalization response we performed QTL analysis on F2 populations derived from crossing four accessions—Lov-1, Var-2-6, Ull-2-5, and Edi-0—to Columbia (Supplemental Material). F2 plants were scored for flowering time after either 4 wk (for Lov-1 and Edi-0) or 8 wk (Var-2-6 and Ull-2-5) vernalization. These treatments were chosen to maximize the phenotypic differences shown by the parental accessions. Analysis of the data revealed significant QTL on chromosomes 1, 4, and 5 (Fig. 3). A common QTL in all four populations maps on chromosome 4 at *FRIGIDA.* This was anticipated given that Columbia carries a loss-of-function *FRI* allele and the four accessions carry active *FRI* alleles (Shindo et al.

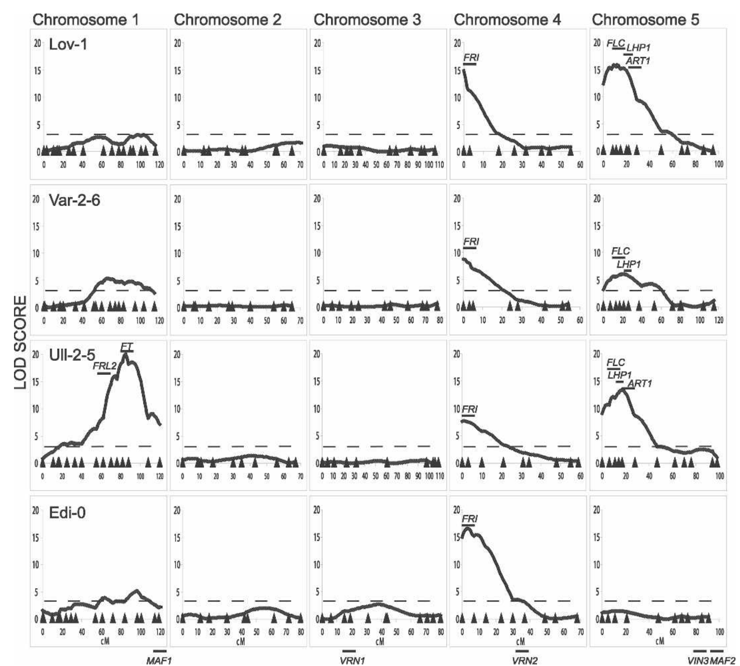

Figure 3. QTL analysis to map genes contributing to the variation in vernalization response. Each chromosome is shown separately with the markers used indicated as triangles. LOD (Logarithm of the odds) score is calculated by the MapMaker Version 3.0b with LOD 3 considered significant. The map positions of candidate genes are indicated.

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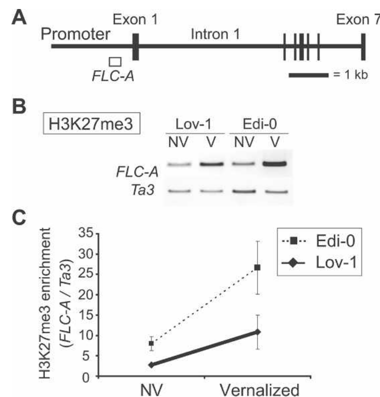

Figure 4. ChIP analysis (*A*) Genomic structure of *Arabidopsis FLC* and the region examined—*FLC-A* (as indicated). (*B*) PCR analysis of DNA immunoprecipitated with anti-trimethyl-histone H3 Lys 27 (H2K27me3). (*C*) Quantification of the H3K27me3 enrichment on *FLC* locus relative to the heterochromatic locus *Ta3* (Johnson et al. 2002). The graph comprises the quantification of two ChIP experiments (error bars are standard deviation of six replicates). Chromatin was prepared from nonvernalized seedlings (20 d old) and seedlings that had been vernalized 5 wk following 7 d pregrowth at 23°C and then grown for 15 d after vernalization at 23°C.

2005). There appear to be two QTL mapping to the top of chromosome 5 in Lov-1, Var-2-6, and Ull-2-5. A major one mapping very close to *FLC* shows an epistatic interaction with the QTL on chromosome 4, supporting the idea that it is *FLC* (A. Strange and C. Dean, unpubl.). This is also consistent with the observation that in accessions requiring >12 wk of cold to saturate the vernalization requirement, *FLC* expression is reactivated if the plants are returned to warm temperatures prematurely. The different vernalization phenotypes of the accessions (Fig. 1A); the lateness after 8 wk vernalization of F2 individuals carrying that region from Lov-1, Var-2-6, and Ull-2-5; and the lack of a QTL on chromosome 5 in the Edi-0 × Col-0 population all suggest allelic variation at *FLC* in the Scandinavian accessions. The second putative QTL on chromosome 5 maps in the region of *LHP1* and *ART1,* the latter previously implicated in determining natural variation in flowering time (Poduska et al. 2003). One or more QTL also map on chromosome 1 and, given their position, possible candidates are *FT* (Kardailsky et al. 1999) and *FRL2* (Michaels et al. 2004) but not *MAF1/FLM* (Ratcliffe et al. 2001; Scortecci et al. 2001).

A clear conclusion that emerges from this analysis is that the natural variation in vernalization response does not map to three of the cloned *trans*-factors regulating vernalization—VIN3, VRN1, or VRN2—that are thought to act directly on *FLC* and result in a stably silenced state (Bastow et al. 2004; Sung and Amasino 2004; Sung et al. 2006). A possible scenario, given the QTL detected, is that polymorphism at *cis* elements in *FLC*, required for both vernalization and up-regulation by FRI, contributes to the variation in vernalization response, together with unknown factors mapping to chromosome 1. *LHP1* has recently been reported to act in *FLC* silencing (Mylne et al. 2006) and so remains another candidate to be analyzed.

FLC *chromatin modifications accumulate differentially in the accessions*

The finding that cold induced a quantitative accumulation in the epigenetic silencing of *FLC* suggested that vernalization-induced chromatin modifications may accumulate differently in the different accessions. To explore this further, chromatin immunoprecipitation (ChIP) experiments were undertaken on two accessions: Edi-0, a representative of an accession with a relatively rapid vernalization response, and Lov-1, a representative of a slow-responding accession. Levels of histone H3 Lys 27 trimethylation (H3K27me3), a histone modification characteristic of PcG silenced genes in *Arabidopsis* and other species (Cao and Zhang 2004; Schonrock et al. 2006), were analyzed in seedlings that had not been vernalized or had been vernalized for 5 wk and then grown for 15 d in a controlled-environment cabinet. Vernalization increased H3K27me3 levels in the *FLC* promoter region (Fig. 4A) in both accessions. However, due to a combination of a different starting level and a different rate in the acquisition of silencing marks, the accumulated levels of H3K27me3 were 2.5 times higher in Edi-0 compared with Lov-1 after 5 wk vernalization (Fig. 4B,C).

Variation in the efficiency of FLC *chromatin silencing accounts for natural variation in vernalization response*

Our data show that quantitative differences in the epigenetic silencing of *FLC*, rather than the rate of *FLC* suppression or *VIN3* induction, contribute to the natural variation in vernalization response. QTL mapping suggests *FLC* itself may contribute to this variation, so polymorphism in key *cis* elements in *FLC* may alter interaction with *trans*-acting proteins and change the efficiency of chromatin silencing. These *cis* elements could be in the 5′ region or intron 1 as both have been shown to be important in the maintenance of *FLC* repression (Sheldon et al. 2002; Sung et al. 2006). Lempe et al. (2005) have documented substantial sequence variation in *FLC*, in particular several large insertions in intron 1. Whatever the nature of the variation, the outcome is a differential accumulation of H3K27me3, a histone modification that increases significantly at *FLC* after vernalization. Combining these data with the flowering time and *FLC* expression analysis suggests a possible model shown schematically in Figure 5. Three phases of vernalization are proposed: an insensitive phase, a phase where silencing occurs (and histone modifications accumulate), and a

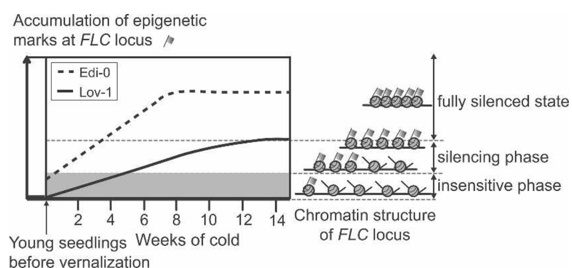

Figure 5. Model proposing that quantitative accumulation of epigenetic modifications in *FLC* contributes to natural variation of vernalization response. Three phases of vernalization are envisaged: an insensitive phase, a phase where silencing occurs (and histone modifications accumulate), and a phase where *FLC* is fully silenced. The dashed and solid lines, estimated from the ChIP analysis shown in Figure 4C, represent the accumulation of epigenetic marks in Edi-0 and Lov-1, respectively. Due to a high initial level and higher accumulation rate, full silencing would be reached after 4 wk of cold in Edi-0 and 10–12 wk of cold in Lov-1.

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phase where *FLC* is fully silenced. Only when sufficient histone modifications have accumulated would a fully silenced state be reached. Full silencing could be attained by 4 wk of cold in Edi-0 (and perhaps other accessions that respond rapidly to vernalization) due to a high initial level and higher accumulation rate. In contrast, the lower initial levels in Lov-1 (and perhaps other accessions that respond slowly to vernalization) necessitate a 10- to 12-wk cold period to reach the same degree of H3K27 methylation.

We are continuing the genetic analysis on these accessions to definitively identify the genes and causative variation conferring this natural variation in vernalization response. Generation of lines differing only at these loci, either through introgression or transformation, will also provide material optimal for determination of fitness consequences of that phenotypic variation. This, together with analysis of the distribution of different alleles in the *Arabidopsis* populations, should be very revealing with respect to the evolution of this adaptive trait.

Materials and methods

Quantification of FLC *expression by Northern analysis*

Vernalized plants were harvested at 0, 10, 20, and 30 d after the return to a 23°C controlled-environment room. All above-ground material was harvested and used for extraction of total RNA as described in Etheridge et al. (1999). *FLC* expression levels were assayed by Northern hybridization as described previously (Shindo et al. 2005). The analysis was repeated with RNA extracted from independently grown plants. Quantification of expression levels was calculated relative to *TUBULIN* expression and shown as values relative to that of nonvernalized H51 plants (i.e., H51 NV30 = 1.00).

ChIP and real-time quantitative PCR analysis

ChIP assays were performed as previously described (Bastow et al. 2004) with minor modifications. Chromatin preparations from *Arabidopsis* seedlings were immunoprecipitated with anti-trimethyl-histone H3 Ly 27 (Thomas Jenuwein, IMP Research, Vienna, Austria; rabbit 6523, 5 bleed) and magnetic Dynabeads Protein A (Dynal Biotech). ChIP DNA was analyzed by real-time quantitative PCR using an OPTICON2 instrument from MJ Research and SYBR Green JumpStart Taq ReadyMix from Sigma. Specific oligonucleotides used are described in the Supplemental Material. Thermal cycling consisted of 2 min at 94°C, followed by 40 cycles of 10 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C. After that, a melting curve was generated to check the specificity of the amplified fragment.

Acknowledgments

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