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**Research Category:** Development and Hormone Action
Title
A single amino acid change in the Enhancer of Zeste ortholog CURLY LEAF results in vernalization-independent, rapid-flowering in Arabidopsis.

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Financial Source:
This work was supported by the University of Wisconsin, the National Institutes of Health (1R01GM079525) and the National Science Foundation (0446440)
Abstract
Many strains of Arabidopsis (*Arabidopsis thaliana*) require exposure to prolonged cold for rapid flowering, a process known as vernalization. Vernalization in Arabidopsis results in the suppression of *FLOWERING LOCUS C (FLC)*, a repressor of flowering. In a screen for mutants that no longer require vernalization for rapid flowering, we identified a dominant allele of the Enhancer of Zeste E(z) ortholog *CURLY LEAF (CLF)*, *clf-59*. *CLF* is a Polycomb Group (PcG) gene, and the *clf-59* mutant protein contains a proline to serine transition in a cysteine-rich region that precedes the SET domain. Mutant plants are early flowering and have reduced *FLC* expression, but, unlike *clf* loss-of-function mutants, *clf-59* mutants do not display additional pleiotropic phenotypes. *clf-59* mutants have elevated levels of tri-methylation on lysine 27 of histone H3 (H3K27me3) at *FLC*. Thus, *clf-59* appears to be a gain-of-function allele, and this allele represses *FLC* without some of the components required for vernalization-mediated repression. In the course of this work, we also identified a marked difference in H3K27me3 levels at *FLC* between plants that contain and those that lack the *FRIGIDA (FRI)* gene. Furthermore, *FRI* appears to affect CLF occupancy at *FLC*, and thus, our work provides insight into the molecular role that *FRI* plays in delaying the onset of flowering.
Introduction

The switch from vegetative to reproductive growth is an important developmental transition in the life history of flowering plants. The proper timing of this switch is critical for reproductive success, especially in temperate climates where flower and seed production often needs to align with favorable weather conditions and/or the presence of pollinators. To ensure that flowering occurs at an optimal time of the year, plants have evolved mechanisms to sense and respond to seasonal environmental cues. One such cue is the prolonged cold of winter. The promotion of flowering by cold occurs through a process known as vernalization (reviewed in (Sung and Amasino, 2005; Dennis and Peacock, 2007)).

Among accessions of Arabidopsis (*Arabidopsis thaliana*), there is variation in the requirement for vernalization. In winter-annual accessions flowering is delayed unless plants undergo vernalization; summer-annual accessions do not require prolonged cold for rapid flowering. Natural variation at two loci in Arabidopsis, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), has a major influence on the requirement for vernalization (Napp-Zinn, 1979; Koornneef et al., 1994; Lee et al., 1994a). Many summer-annual accessions, including Columbia (Col), Wassileskija (Ws), and Landsberg erecta (Ler), flower rapidly without a prolonged cold treatment because they carry mutations in *FRI* (Johanson et al., 2000). *FRI* acts to delay flowering through the up-regulation of *FLC*, a MADS-box transcription factor that represses flowering. Vernalization leads to *FLC* repression in the presence of *FRI* and is thus able to supersede the ability of *FRI* to activate *FLC* (Michaels and Amasino, 1999; Sheldon et al., 1999). Thus, under natural conditions the role of the *FRI/FLC* system is likely to repress flowering in autumn. The prolonged cold of winter then results in vernalization which is manifest in the repression of *FLC* and rapid flowering in the spring.

Arabidopsis also contains a group of genes known collectively as the autonomous pathway which, in contrast to *FRI*, act as repressors of *FLC*. *LUMINDEPENDENS* (*LD*), *FCA*, *FLOWERING LOCUS D* (*FLD*), and *FVE* are among this group of genes (Lee et al., 1994b; MacKnight et al., 1997; He et al., 2003; Ausin et al., 2004). FCA functions in RNA-mediated gene silencing (Baurle et al., 2007). FLD and FVE function in complexes that modify chromatin (He et al., 2003; Ausin et al., 2004). Mutations in autonomous-
pathway genes result in elevated FLC expression and delayed flowering (Michaels and Amasino, 2001). Similar to winter-annual accessions with an active FRI allele, delayed flowering in autonomous-pathway mutants can be suppressed through vernalization.

Chromatin modification appears to play a large role in setting the expression level of FLC. In genetic backgrounds that favor elevated FLC expression, "active" chromatin modifications accumulate at FLC, and several proteins required for the deposition of these modifications have been identified (reviewed in (Schmitz and Amasino, 2007)). During the course of cold exposure, FLC expression is suppressed (Michaels and Amasino, 1999; Sheldon et al., 1999), and several signatures of silenced chromatin accumulate at FLC (Bastow et al., 2004; Sung and Amasino, 2004; Mylne et al., 2006; Schubert et al., 2006; Sung et al., 2006b; Finnegan and Dennis, 2007; Schmitz et al., 2008). One such signature is tri-methylation on lysine 27 of histone H3 (H3K27me3). Changes in the amount and distribution of H3K27me3 at FLC both during and after cold treatment have been described (Schubert et al., 2006; Sung et al., 2006a; Finnegan and Dennis, 2007). H3K27me3 is carried out by Polycomb-Group (PcG) complexes which contain orthologs of Drosophila Polycomb Repressive Complex 2 (PRC2) components and other plant-specific proteins such as VERNALIZATION INSENSITIVE 3 (VIN3) (Wood et al., 2006; De Lucia et al., 2008). PcG proteins have also been shown to repress FLC in accessions that do not require vernalization for rapid flowering (Jiang et al., 2008).

The Arabidopsis protein CURLY LEAF (CLF) is an ortholog of the Drosophila PRC2 component Enhancer of Zeste [E(z)], a methyltransferase with specificity for H3K27 (Goodrich et al., 1997; Czermin et al., 2002; Muller et al., 2002). CLF was initially characterized as a suppressor of floral homeotic genes including AGAMOUS (AG) (Goodrich et al., 1997). In addition, recent work has indicated a role for CLF in the suppression of FLC and the floral promoter FT in a non-vernalized, rapid-flowering accession (Jiang et al., 2008). CLF has also been shown to play a role in the repression of FLC by vernalization (Wood et al., 2006).

Here we describe a gain-of-function allele of CLF that was isolated in a screen for suppressors of delayed flowering in an autonomous-pathway mutant background. This allele leads to elevated H3K27me3 at FLC chromatin, FLC mRNA repression, and rapid
flowering in backgrounds that would otherwise require vernalization. In addition, we also demonstrate that the presence of *FRI* has a substantial effect on the degree of H3K27me3 accumulation and CLF occupancy at *FLC*.

**Results**

**A mutation in *CURLY LEAF* suppresses *FLC*-mediated delayed flowering via reduced *FLC* expression.**

Mutants with lesions in the autonomous-pathway gene *LD* are delayed in flowering due to elevated expression of *FLC* (Michaels and Amasino, 2001). An ethyl methanesulfonate mutagenesis carried out in an *ld-3* mutant background (Ws accession) resulted in the isolation of several mutants with rapid-flowering phenotypes. One mutation suppressed delayed flowering in a semi-dominant manner (Figure 1A; the mutation is designated *clf-59*) and mapped to a region that included *CLF* (Goodrich et al., 1997). Given the dominant nature of this mutation, it was not possible to confirm gene identity by transgenic complementation with the wild-type gene product. Instead, the *CLF* genomic region was cloned from the mutant and introduced into an *ld-3* background. Plants carrying the mutant transgene flowered more rapidly than non-transformed plants (an example of a transgenic versus parental line is shown in Figure 1B). An introduced copy of wild-type *CLF* did not alter flowering time (data not shown). Thus, this semi-dominant mutation is an allele of *CLF* which we designate *clf-59*.

Mutations in autonomous-pathway genes lead to delayed flowering via elevated expression of *FLC* (Michaels and Amasino, 2001). Similarly, plants carrying a functional copy of *FRI* also display *FLC*-dependent late flowering (Koornneef et al., 1994; Lee et al., 1994a; Michaels and Amasino, 1999; Sheldon et al., 1999). To evaluate whether the effect on flowering was specific to *ld, clf-59* was combined genetically with *fca*, another autonomous-pathway mutant (MacKnight et al., 1997) and a Columbia line that contains a functional copy of *FRI* (*FRI*-Col) (Lee et al., 1994a); in both cases, *clf-59* suppressed late flowering in a semi-dominant manner (Figure 1C). Rapid flowering was also observed when *clf-59* was introduced transgenically into the autonomous-pathway mutants *fld* (He et al., 2003) and *fve* (Ausin et al., 2004) (Figure 1D). Because
autonomous-pathway mutants and FRI-containing plants are delayed in flowering due to elevated FLC levels (Michaels and Amasino, 2001), rapid flowering in a clf-59 background could result from FLC repression. Indeed, ld-3, fca-1, and FRI-Col all displayed reduced levels of FLC mRNA when combined with clf-59 (Figure 1E). Thus, rapid flowering in clf-59 results, at least in part, from decreased expression of FLC.

**clf-59 Contains a Single Amino Acid Change in a Cys-Rich Motif**

The CLF protein contains several conserved motifs (Figure 2). The C5 motif functions in protein-protein interactions (Chanvivattana et al., 2004; Ketel et al., 2005), and the SET domain contains the histone methyltransferase catalytic site (Rea et al., 2000). In CLF and other E(z)-like proteins, a conserved cysteine-rich region precedes the SET-domain. clf-59 contains a single amino-acid substitution within this region which converts a conserved proline to a serine (P704S) (Figure 2).

In addition to CLF, Arabidopsis contains two additional E(z)-like genes, SWINGER (SWN) and MEDEA (MEA) (Grossniklaus et al., 1998; Chanvivattana et al., 2004). MEA is essential for endosperm development (Grossniklaus et al., 1998). SWN is partially redundant in function with both CLF and MEA (Chanvivattana et al., 2004; Wang et al., 2006). For example, CLF and SWN act redundantly in the silencing of FLC as a result of vernalization. Both clf and swn single mutants can become vernalized, but down-regulation of both genes simultaneously results in vernalization insensitivity (Wood et al., 2006). Given the pronounced effect of clf-59, it was of interest to determine whether the P to S substitution in SWN and MEA would also cause rapid flowering or possibly other developmental aberrations. Accordingly, this substitution was introduced into genomic clones of both SWN and MEA. The mutant genes were subsequently transformed into Col and FRI-Col backgrounds. All transgenic plants flowered synchronously with the non-transformed parents (data not shown). Thus, the P to S transition in MEA and SWN does not cause enhanced FLC repression. In addition, no visual phenotypic differences were observed in plants containing modified SWN or MEA at any stage of development. Possible reasons for lack of a phenotype in plants with modified SWN or MEA include, but are not limited to, wild-type CLF masking an effect of these modified transgenes or simply the lack of an effect of this substitution on SWN.
or MEA activity. As noted below, the only effect of the P to S transition in clf-59 that we observe is on flowering and *FLC* expression.

**clf-59 mutants do not display phenotypes seen in either clf or agamous loss-of-function mutants.**

Loss-of-function mutations in *clf* cause a number of phenotypes including upward curling of leaves and abnormal flower development (Goodrich et al., 1997). These phenotypes are largely due to ectopic expression of the floral homeotic gene *AG* (Mizukami and Ma, 1992; Goodrich et al., 1997). On the other hand, plants that do not express *AG* fail to produce stamens and carpels (Bowman et al., 1989; Yanofsky et al., 1990). Given that *CLF* is a regulator of *AG*, it was of interest to look for altered *AG* expression in a *clf-59* background. The presence of *clf-59* does not result in hyper-suppression *AG* similar to the effects of this allele on *FLC*, at either the mRNA or phenotypic level. Specifically, in a *clf-59* background, expression of *AG* in either seedling (Figure 3A) or inflorescence tissue (Figure 3B) is indistinguishable from wild type, nor are there any *ag* reduction-of-function phenotypes in *clf-59* mutants (Figure 3C,D). In addition, no phenotypes characteristic of *clf* loss-of-function (and the associated ectopic expression of *AG*) are observed in *clf-59* mutants. Thus, the *clf-59* protein does not appear to affect all targets of wild-type CLF.

The effect of a *clf* loss-of-function allele, *clf-28*, on flowering time and *FLC* expression was also examined. *clf-28* hastened flowering in *FRI*-Col but to a much lesser degree than *clf-59* (Figure 4A) and, unlike *clf-59*, did not have a significant effect on *FLC* expression (Figure 4B). This distinction indicates that *clf-59* is a gain-of-function allele with respect to *FLC* expression as opposed to a dominant negative.

In the absence of *FRI*, *clf-28* also hastened flowering despite slightly elevated *FLC* levels (Figure 4A,B). Such *FLC* de-repression in a loss-of-function *clf* mutant has been reported previously (Jiang et al., 2008). Early flowering in *clf* loss-of-function mutants likely results from de-repression of *FT* (Jiang et al., 2008), a strong promoter of flowering that when over-expressed bypasses the repressive effects of *FLC* (Michaels et al., 2005).
Enrichment of H3K27me3 at FLC chromatin is altered in clf-59 and is dependent on genetic background.

CLF catalyzes the methylation of H3K27. Previous studies have extensively examined the distribution of H3K27me3 across FLC and surrounding chromatin (Bastow et al., 2004; Sung and Amasino, 2004; Schubert et al., 2006; Finnegan and Dennis, 2007). The accumulation H3K27me3 at FLC that occurs as a result of vernalization initiates near the transcriptional start site and then spreads throughout the gene (Finnegan and Dennis, 2007), and thus, we have focused our study on regions of FLC near the transcriptional start site. Levels of H3K27me3 were analyzed in non-vernalized FRI clf-59, Col, and FRI-Col using chromatin immuno-precipitation (ChIP). The level of H3K27me3 in FRI clf-59 was greater than that in FRI-Col indicating that, in a FRI background, clf-59 is capable of elevating H3K27me3 levels in the absence of vernalization (Figure 5A,B).

When assaying H3K27me3 in clf-59 plants, a marked difference in H3K27me3 levels was observed between Col and FRI-Col (Figure 5A,B). To our knowledge, this is the first report of the effect of FRI on H3K27 methylation. Reduced H3K27me3 levels in the presence of FRI indicate that, in a genetic sense, FRI is a negative regulator of H3K27me3 at FLC. Moreover, autonomous-pathway mutants also display reduced levels of H3K27me3 at FLC which are comparable to levels seen in FRI-Col (Figure 5C,D). Thus, in backgrounds that lack FRI, the autonomous pathway is required for the elevated H3K27me3 levels found at FLC.

Given the elevated levels of H3K27me3 in Col relative to FRI-Col in non-vernalized plants, it was of interest to determine whether vernalization simply elevates H3K27me3 levels to those found in Col or if additional H3K27me3 accumulates in Col upon exposure to prolonged cold. H3K27me3 levels are in fact elevated in Col following cold treatment; however, the fold change is not as great as that seen in a FRI-Col background (Figure 5E). In both vernalized and non-vernalized plants, FLC had higher levels of H3K27me3 in Col than in FRI-Col. However, it should be noted that the 30-day cold treatment given in these experiments is not saturating with respect to the acceleration of flowering (Lee and Amasino, 1995). Thus, longer cold treatments may equalize the amount of H3K27me3 at FLC in vernalized Col and vernalized FRI-Col.
**clf-59 causes rapid flowering in the absence of genes required for vernalization**

In addition to *CLF*, several other genes have been shown to play a role in the vernalization-mediated deposition and maintenance of H3K27me3 at *FLC*. These include the PcG gene *VERNALIZATION 2 (VRN2)* (Gendall et al., 2001; Bastow et al., 2004; Sung and Amasino, 2004) as well as *VERNALIZATION 1 (VRN1)*, and the PHD-domain proteins *VERNALIZATION INSENSITIVE 3 (VIN3) and VIN3-LIKE 1 (VIL1, also known as VRN5)* (Levy et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004; Sung et al., 2006a; Wood et al., 2006; Greb et al., 2007; De Lucia et al., 2008). *clf-59* was introduced into lines carrying mutations in these genes in order to evaluate whether any of these genes are required for the rapid-flowering, gain-of-function phenotype in *FRI clf-59*. However, rapid flowering was observed in all tested lines: *FRI vrn1-1*, *FRI vin3-1*, *FRI vil1*, and *fca vrn2-1* (Figure 6A,B) indicating that these genes, which are required for vernalization, are not required for the *clf-59* phenotype.

Although the loss of *vil1* did not affect the ability of *clf-59* to repress *FLC* and cause early flowering (Figure 6C), *FRI vil1 clf-59* plants did display several pleiotropic phenotypes including pale green leaves and downward leaf curling (Figure 6D,E), the opposite of that seen in *clf* loss-of-function mutants. Thus, *VIL1* appears to be required for wild-type leaf morphology in the presence of *clf-59*.

**Genetic background affects wild-type CLF localization to FLC**

As discussed above, *FRI*-Col and autonomous-pathway mutants have relatively low levels of H3K27me3 at *FLC* chromatin when compared to Col (Figure 5). In addition, it has been shown that *clf* loss-of-function mutants have reduced H3K27me3 levels at *FLC* chromatin in a Col background (Jiang et al., 2008). Because the presence of *FRI* or a *clf* mutation has a similar effect on H3K27me3 levels at *FLC*, the effect of *FRI* on CLF localization was investigated. A constitutively expressed, GFP-tagged version of *CLF (GFP:CLF)* (Schubert et al., 2006) was introduced by crossing into a line containing *FRI* (*FRI*-Ws: all lines in the FRI/CLF localization experiments are in a Ws background). ChIP analysis using an anti-GFP antibody revealed that levels of CLF were enriched at *FLC* chromatin in Ws when compared to *FRI*-Ws (Figure 7A). Such a reduction in CLF
occupancy likely contributes to the lower levels of H3K27me3 at FLC seen in FRI-containing backgrounds (Figure 5B).

*GFP:CLF* was also introduced into *fca-9* and *fld-3* backgrounds. In both cases, the autonomous-pathway mutant displayed lower levels of CLF occupancy at FLC chromatin relative to wild type (Figure 7B). Thus, as with *FRI*, the *fca* and *fld* genetic backgrounds create a condition that leads to reduced occupancy of CLF protein at FLC chromatin.

We also examined the effect of prolonged cold on the enrichment of CLF at FLC. After a 35-day cold exposure, a difference in CLF occupancy at FLC could no longer be detected between plants that contain and those that lack *FRI* (Figure 7C). This 35-day cold exposure was sufficient to substantially hasten flowering time in *FRI*-Ws (Figure 7D). Thus, with vernalization the presence of *FRI* no longer affects the amount of CLF that occupies FLC chromatin, and increased CLF occupancy at FLC after prolonged cold correlates with the vernalization-mediated increase in H3K27me3 at FLC.

**Discussion**

We have identified a gain-of-function allele of *CLF*, *clf-59*, that reduces the level of *FLC* expression and thus eliminates the requirement for vernalization in winter-annual types of Arabidopsis. CLF is a PcG protein that functions in a complex, PRC2, which has conserved features in plants and animals. Plant genomes typically contain multiple copies of conserved PRC2 components (for example, Arabidopsis has three E(z) homologs: CLF, SWN and MEA) and these components interact with members of the plant-specific, VIN3-like family of proteins (Wood et al., 2006; De Lucia et al., 2008). Thus, there is potential for much variability in the specific PcG complexes that assemble in plants. The *clf-59* protein may either enhance the activity of a particular PcG complex or allow for the formation of a novel PcG complex that possesses high affinity for FLC chromatin.

*clf-59* harbors a proline to serine amino-acid transition in a cys-rich region. Although the specific biochemical role of this region is not known, it contains two contiguous domains with a unique spacing of cysteines called CXC domains as first described in ENX-1, the human homolog of E(z) (Hobert et al., 1996). Subsequent work has shown this domain to be important for the function of E(z) and E(z)-like proteins.
(Carrington and Jones, 1996; Kuzmichev et al., 2002; Ketel et al., 2005). Several otherwise unrelated proteins from plants and animals have been described that contain CXC domains separated by sequences of varying length, and these domains have been shown to bind zinc in vitro (Andersen et al., 2007). TSO1 is a CXC-containing protein in Arabidopsis that functions in the regulation of cell division, and both the tso1-1 and tso1-2 loss-of-function alleles contain amino-acid substitutions within the CXC domain (Hauser et al., 2000; Song et al., 2000).

Both clf-59 and clf loss-of-function alleles hasten the onset of flowering; however, the two types of alleles do so via different mechanisms. clf-59 causes hyper-repression of FLC expression with no noticeable effect on AG. In contrast, clf loss-of function mutants cause de-repression of several genes including AG, FLC and FT (Goodrich et al., 1997; Jiang et al., 2008), and it is likely that FT de-repression leads to rapid flowering in clf loss-of-function mutants. This occurs despite elevated FLC expression as ectopic FT expression can bypass the repressive effects of FLC (Michaels et al., 2005).

It is interesting to compare the degree of FLC de-repression that we observe in a clf loss-of-function mutant to that reported previously (Jiang et al., 2008). In a Col background, Jiang et al. (2008) report a higher level of FLC de-repression than we observe. In a delayed-flowering background the difference is even greater: Jiang et al. (2008) report extensive de-repression in an fca clf double mutant whereas we do not observe de-repression in FRI clf. Therefore, with respect to FLC de-repression, clf loss-of-function mutants may have different effects in FRI compared to autonomous-pathway mutant backgrounds.

The wild-type function of the autonomous pathway is to repress FLC expression (Michaels and Amasino, 2001). We find reduced levels of both H3K27me3 and CLF at FLC chromatin in several autonomous-pathway mutants. Thus, one aspect of autonomous pathway-mediated FLC repression involves a direct or indirect effect on the occupancy of CLF at FLC chromatin. However, given the large degree of FLC de-repression previously reported in fca clf mutants (Jiang et al., 2008), it is possible that a sufficient amount of CLF still occupies FLC chromatin in autonomous-pathway mutant backgrounds as this would allow for some degree of de-repression when CLF is genetically removed.
In this work we present data that is consistent with FRI playing an antagonistic role with respect to PcG occupancy at *FLC*. We observe that H3K27me3 levels, a mark deposited by PcG protein complexes, were less abundant at *FLC* chromatin in a *FRI*-Col background relative to Col. We provide a possible explanation for the difference in H3K27me3 levels in the presence or absence of FRI by demonstrating that CLF, a PcG protein that catalyzes H3K27me3 deposition, is less abundant at *FLC* in *FRI*-Col relative to Col plants. Because CLF occupancy at *FLC* is reduced in a *FRI* background, one might expect a *clf* loss-of-function mutant to have less of an effect on *FLC* expression in a *FRI*-Col background, and indeed, this is consistent with the lack of *FLC* de-repression we observe in *FRI clf* plants. A molecular signature present in vernalized Arabidopsis plants is the accumulation of H3K27me3 at *FLC* (Schubert et al., 2006; Sung et al., 2006a; Finnegan and Dennis, 2007). Consistent with our results, the antagonism between FRI and CLF is relieved upon vernalization; CLF occupancy and H3K27me3 increases at *FLC* following exposure to prolonged cold. Genes required for vernalization such as *VIN3* may play a role in overcoming the antagonistic effects of *FRI* by helping to recruit CLF to *FLC* chromatin.

Chromatin modification has been shown in many systems to reinforce and/or maintain states of gene expression. Much of the data presented here and in other papers on *FLC* chromatin and flowering show correlations between the transcriptional level of *FLC* and particular chromatin modifications. We show, for example, that levels of H3K27me3, a repressive chromatin mark, and occupancy of CLF, a PcG protein, are decreased at *FLC* in two genetic situations in which *FLC* expression is elevated – the presence of *FRI* and the lack of autonomous-pathway genes. The differential accumulation of marks such as H3K27me3 at *FLC* may very well be an indirect consequence of the effect that *FRI* and autonomous-pathway genes have on *FLC* transcription. Indeed, just as we show that *FRI* leads to a reduction in CLF occupancy at *FLC*, a recent report has shown that *FRI* also correlates with the accumulation of a COMPASS complex component at *FLC* that modifies chromatin to an active state (Jiang et al., 2009). The specific biochemical role of FRI and the autonomous-pathway components in *FLC* regulation remain to be determined.
The unique gain-of-function *clf-59* allele results in an increase in the abundance of H3K27me3 at *FLC* in the presence of *FRI*. Thus, the *clf-59* protein might be immune to the ability of *FRI* or loss of autonomous-pathway components to reduce CLF occupancy at *FLC* chromatin in the absence of vernalization, and thus *clf-59* may be able to accumulate to higher levels than wild-type CLF at *FLC* in these genetic backgrounds. Alternatively, *clf-59* may produce a "hyperactive" protein that can elevate H3K27me3 levels without increased occupancy at *FLC*. It is intriguing that *clf-59* can suppress *FLC* expression in non-vernalized plants to a level comparable to that found in wild type after vernalization in the absence of proteins known to mediate the vernalization-induced silencing of *FLC* including VRN2, VIN3, and VIL1. Future studies addressing the unique biochemical properties of the *clf-59* protein may further our knowledge of *FLC* regulation and PcG targeting in general by providing a better understanding of the molecular events that either recruit or exclude PcG genes to *FLC* chromatin.
Materials and Methods

Plant Growth Conditions and Mutant Stocks

Plants were grown under cool-white fluorescent lights at 22°C in long day photoperiods (16hr light: 8hr dark). Cold treatments were carried out at 4°C under cool-white fluorescent lights. The clf-59 mapping population was created by crossing ld-1 to ld-3 clf-59. The ld-1 and ld-3 mutants are from the Col and Ws-2 backgrounds respectively (Lee et al., 1994b; Michaels and Amasino, 2001). F2 plants segregated 1:2:1 for the fully suppressed, partially suppressed, and the parental delayed-flowering phenotypes respectively. The latest flowering F2 plants were used to map the locus as these plants were known to be homozygous Col at the locus of interest. A PCR-based dCAPS marker (Michaels and Amasino, 1998) was designed to detect the clf-59 mutation in both genetic studies and in transgenic plants. The marker was designed such that the wild-type CLF sequence contained an NcoI restriction site at the lesion that was absent in the clf-59. Primer sequences are listed in Supplemental Table 1. Other lines used in this work have been previously described. The fca-1 allele is from the Landsberg erecta (Ler) but contains a Col allele of FLC (Sanda and Amasino, 1996). Alleles of other autonomous-pathway mutants used in this work are in the Columbia background: fpa-7 (Michaels and Amasino, 2001), fca-9 (Bezerra et al., 2004), ld-1 (Redei, 1962), fld-3 (He et al., 2003), and fve-4 (Michaels and Amasino, 2001). clf-28 is from the Salk T-DNA collection (SALK_139371), and clf-52 is a T-DNA allele from the Ws-2 background (Noh and Amasino, 2003). The Ws-2 line with an introgressed FRI and the CLF:GFP line have been described previously (Noh and Amasino, 2003; Schubert et al., 2006). CLF:GFP is under the control of the CaMV 35S promoter as described. CLF:GFP segregates as a single locus and all lines containing this construct carried homozygous null mutations in the endogenous CLF gene. The CLF genomic region was amplified out of both ld-3 and ld-3 clf-59 using PCR. DNA fragments were then cloned into the binary vector pPZP221B using BamHI sites engineered into the primers. These primers and primers used to clone and make site-directed mutants in MEA and SWN are listed in Supplementary Table 1.
Analysis of RNA Abundance

Tissue was harvested from 7-day-old seedlings. RNA was isolated using TRI reagent (Sigma). For Northern analysis 12μg of total RNA was run on a 1% agarose denaturing formaldehyde gel and transferred to a nylon filter (Hybond). A DNA probe was used that was complementary to the 3’UTR of FLC. Real-time PCR was performed with the 7000 Real Time PCR System (Applied Biosystems) using the DyNAmo™ Flash SYBR® Green qPCR Kit (Finnzymes). The PCR parameters were: one cycle of 15 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 58°C, and 45 s at 72°C. The constitutively expressed gene encoding the A3 subunit of protein phosphotase 2A was used as a control (Czechowski et al., 2005). PCR primer sequences are listed in Supplementary Table 1. All results presented are an average of at least three biological replicates.

ChIP

Tissue was harvested from 7-day-old seedlings. Chromatin samples were prepared as described (Gendrel et al., 2005). Antibodies used in this work were as follows: Anti-GFP (Invitrogen cat# A-11122); Anti-H3K27me3 (Upstate cat# 07-449). Real-time PCR was done as described above. Changes at FLC chromatin are shown relative to an AG control. Because AG has abundant H3K27me3, it is possible that this mark at AG may behave similarly to that at FLC. However, observed patterns of H3K27me3 and CLF localization at FLC were also evident relative to an Actin control. Actin does not contain high levels of H3K27me3 (data not shown). PCR primer sequences are listed in Supplementary Table 1. All ChIP results presented are an average of at least two biological replicates unless noted otherwise.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: CLF, At2g23380; FLC, At5g10140; FRI, At4g00650; LD, At4g02560; FCA, At4g16280; FLD, At3g10390; FPA, At2g43410; FVE, At2g19520; AG, At4g18960; VIL1, At3g24440; VRN2, At4g16845; VIN3, At5g57380; VRN1, At3g18990; MEA, At1g02580; SWN, At4g02020
Supplemental Material

Supplemental Table 1  Sequences of oligos used in cloning and gene expression analysis.
Acknowledgements

We would like to thank Dr. Scott Michaels for identifying the clf-59 mutant and for helpful discussions. We would also like to thank Drs. Justin Goodrich and Daniel Schubert for supplying constructs and seed used in this work, and the Salk Institute Genomic Analysis Laboratory for providing the Arabidopsis T-DNA insertion lines.

References

Andersen, S.U., Algreen-Petersen, R.G., Hoedl, M., Jurkiewicz, A., Cvitanich, C., Braunschweig, U., Schauser, L., Oh, S.A., Twell, D., and Jensen, E.O. (2007). The conserved cysteine-rich domain of a tesmin/TSO1-like protein binds zinc in vitro and TSO1 is required for both male and female fertility in Arabidopsis thaliana. Journal of experimental botany 58, 3657-3670.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nature genetics 36, 162-166.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. Nature 427, 164-167.

Baurle, I., Smith, L., Baulcombe, D.C., and Dean, C. (2007). Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. Science (New York, N.Y) 318, 109-112.

Bezerra, I.C., Michaels, S.D., Schomburg, F.M., and Amasino, R.M. (2004). Lesions in the mRNA cap-binding gene ABA HYPERSENSITIVE 1 suppress FRIGIDA-mediated delayed flowering in Arabidopsis. Plant J 40, 112-119.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in Arabidopsis. Plant Cell 1, 37-52.

Carrington, E.A., and Jones, R.S. (1996). The Drosophila Enhancer of zeste gene encodes a chromosomal protein: examination of wild-type and mutant protein distribution. Development (Cambridge, England) 122, 4073-4083.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development (Cambridge, England) 131, 5263-5276.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant physiology 139, 5-17.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111, 185-196.
De Lucia, F., Crevillen, P., Jones, A.M., Greb, T., and Dean, C. (2008). Inaugural Article: A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of FLC during vernalization. Proceedings of the National Academy of Sciences of the United States of America.

Dennis, E.S., and Peacock, W.J. (2007). Epigenetic regulation of flowering. Current opinion in plant biology 10, 520-527.

Finnegan, E.J., and Dennis, E.S. (2007). Vernalization-Induced Trimethylation of Histone H3 Lysine 27 at FLC Is Not Maintained in Mitotically Quiescent Cells. Curr Biol 17, 1978-1983.

Gendall, A.R., Levy, Y.Y., Wilson, A., and Dean, C. (2001). The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107, 525-535.

Gendrel, A.V., Lippman, Z., Martienssen, R., and Colot, V. (2005). Profiling histone modification patterns in plants using genomic tiling microarrays. Nature methods 2, 213-218.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature 386, 44-51.

Greb, T., Mylne, J.S., Crevillen, P., Geraldo, N., An, H., Gendall, A.R., and Dean, C. (2007). The PHD finger protein VRN5 functions in the epigenetic silencing of Arabidopsis FLC. Curr Biol 17, 73-78.

Grossniklaus, U., Vlle-Calzada, J.P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis. Science (New York, N.Y. 280, 446-450.

Hauser, B.A., He, J.Q., Park, S.O., and Gasser, C.S. (2000). TSO1 is a novel protein that modulates cytokinesis and cell expansion in Arabidopsis. Development (Cambridge, England) 127, 2219-2226.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of Flowering Time by Histone Acetylation in Arabidopsis. Science (New York, N.Y.

Hobert, O., Jallal, B., and Ullrich, A. (1996). Interaction of Vav with ENX-1, a putative transcriptional regulator of homeobox gene expression. Molecular and cellular biology 16, 3066-3073.

Jiang, D., Gu, X., and He, Y. (2009). Establishment of the Winter-Annual Growth Habit via FRIGIDA-Mediated Histone Methylation at FLOWERING LOCUS C in Arabidopsis. Plant Cell.

Jiang, D., Wang, Y., Wang, Y., and He, Y. (2008). Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. PLoS ONE 3, e3404.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of FRIGIDA, a major determinant of natural variation in arabidopsis flowering time [In Process Citation]. Science (New York, N.Y 290, 344-347.

Ketel, C.S., Andersen, E.F., Vargas, M.L., Suh, J., Strome, S., and Simon, J.A. (2005). Subunit contributions to histone methyltransferase activities of fly and worm polycomb group complexes. Molecular and cellular biology 25, 6857-6868.
Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994). The Phenotype of Some Late-Flowering Mutants is Enhanced by a Locus on Chromosome 5 that is not Effective in the Landsberg erecta Wild-Type. Plant Journal 6, 911-919.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes & development 16, 2893-2905.

Lee, I., and Amasino, R.M. (1995). Effect of Vernalization, Photoperiod, and Light Quality on the Flowering Phenotype of Arabidopsis Plants Containing the FRIGIDA Gene. Plant Physiol. 108, 157-162.

Lee, I., Michaels, S.D., Masshardt, A.S., and Amasino, R.M. (1994a). The Late-Flowering Phenotype of FRIGIDA and LUMINIDEPENDENS is Suppressed in the Landsberg erecta Strain of Arabidopsis. Plant Journal 6, 903-909.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., and Amasino, R.M. (1994b). Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in Arabidopsis. Plant Cell 6, 75-83.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science (New York, N.Y 297, 243-246.

MacKnight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997). FCA, a Gene Controlling Flowering Time in Arabidopsis, Encodes a Protein Containing RNA-Binding Domains. Cell 89, 737-745.

Michaels, S., and Amasino, R. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous-pathway mutations, but not responsiveness to vernalization. Plant Cell 13, 935-942.

Michaels, S.D., and Amasino, R.M. (1998). A robust method for detecting single-nucleotide changes as polymorphic markers by PCR. Plant J 14, 381-385.

Michaels, S.D., and Amasino, R. (1999). Flowering Locus C Encodes a Novel MADS Domain Protein That Acts as a Repressor of Flowering. The Plant cell 11, 949-956.

Michaels, S.D., Himelblau, E., Kim, S.Y., Schomburg, F.M., and Amasino, R.M. (2005). Integration of flowering signals in winter-annual Arabidopsis. Plant physiology 137, 149-156.

Mizukami, Y., and Ma, H. (1992). Ectopic expression of the floral homeotic gene AGAMOUS in transgenic Arabidopsis plants alters floral organ identity. Cell 71, 119-131.

Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111, 197-208.

Mylne, J.S., Barrett, L., Tessadori, F., Mesnage, S., Johnson, L., Bernatavichute, Y.V., Jacobsen, S.E., Fransz, P., and Dean, C. (2006). LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic
silencing of FLC. Proceedings of the National Academy of Sciences of the United States of America 103, 5012-5017.

Napp-Zinn, K. (1979). On the genetical basis of vernalization requirement in Arabidopsis thaliana (L.) Heynh. In La Physiologie de la Floraison, P. Champagnat and R. Jaques, eds (Paris: Coll. Int. CNRS), pp. 217-220.

Noh, Y.S., and Amasino, R.M. (2003). PIE1, an ISWI family gene, is required for FLC activation and floral repression in Arabidopsis. Plant Cell 15, 1671-1682.

Rea, S., Eisenhaber, F., O’Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406, 593-599.

Redei, G.P. (1962). Supervital mutants in Arabidopsis. Genetics 47, 443-460.

Sanda, S.L., and Amasino, R.M. (1996). Interaction of FLC and late-flowering mutations in Arabidopsis thaliana. Molecular and General Genetics 251, 69-74.

Schmitz, R.J., and Amasino, R.M. (2007). Vernalization: a model for investigating epigenetics and eukaryotic gene regulation in plants. Biochimica et biophysica acta 1769, 269-275.

Schmitz, R.J., Sung, S., and Amasino, R.M. (2008). Histone arginine methylation is required for vernalization-induced epigenetic silencing of FLC in winter-annual Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America 105, 411-416.

Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., and Goodrich, J. (2006). Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. The EMBO journal 25, 4638-4649.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The FLF MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell 11, 445-458.

Song, J.Y., Leung, T., Ehler, L.K., Wang, C., and Liu, Z. (2000). Regulation of meristem organization and cell division by TSO1, an Arabidopsis gene with cysteine-rich repeats. Development (Cambridge, England) 127, 2207-2217.

Sung, S., and Amasino, R.M. (2004). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427, 159-164.

Sung, S., and Amasino, R.M. (2005). Remembering winter: toward a molecular understanding of vernalization. Annual review of plant biology 56, 491-508.

Sung, S., Schmitz, R.J., and Amasino, R.M. (2006a). A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes & development 20, 3244-3248.

Sung, S., He, Y., Eshoo, T.W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S.E., and Amasino, R.M. (2006b). Epigenetic maintenance of the vernalized state in Arabidopsis thaliana requires LIKE HETEROCHROMATIN PROTEIN 1. Nature genetics 38, 706-710.

Wang, D., Tyson, M.D., Jackson, S.S., and Yadegari, R. (2006). Partially redundant functions of two SET-domain polycomb-group proteins in controlling initiation of seed development in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 103, 13244-13249.
Wood, C.C., Robertson, M., Tanner, G., Peacock, W.J., Dennis, E.S., and Helliwell, C.A. (2006). The Arabidopsis thaliana vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proceedings of the National Academy of Sciences of the United States of America 103, 14631-14636.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A., and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. Nature 346, 35-39.
Figure Legends

Figure 1. clf-59 suppresses FLC-mediated late flowering.
(A) The average total leaf number at flowering, a measure of flowering time in wild type, ld-3 and ld-3 combined with homozygous and heterozygous clf-59 mutants.
(B) Images of the ld-3 mutant (left) and ld-3 carrying a transgenic copy of clf-59 (left).
(C,D) The average total leaf number at flowering of plants containing clf-59 combined with other FLC-mediated late-flowering backgrounds including [C] fca-1 and FRI-Col and [D] fve-4 and fld-3. Data in [D] represent the average of at least 8 individual T1 plants.
(E) Northern blot showing the suppression of FLC expression in clf-59 mutants. 18S ribosomal RNA is shown as a loading control.
(A-D) All leaf counts include both rosette and cauline leaves and represent the average of at least 8 plants. Error bars represent standard error. In A,C clf-59/+ indicates a heterozygote. In B,D a "+" indicates that clf-59 was added transgenically.

Figure 2. Amino acid alignment of the Cys-rich domain of CLF with other E(z)-like proteins. Sequence alignments were done using ClustalW. A "*" indicates the location of a conserved cysteine. Abbreviations are as follows: At, Arabidopsis thaliana; Zm, Zea maize; Os, Oryza sativa; Dm, Drosophila melanogaster; Hs, Homo sapiens.

Figure 3. clf-59 mutants do not display phenotypes seen in clf loss-of-function mutants.
(A,B) Northern blot showing the expression of AG in seedlings [A] and inflorescences [B]. 18S ribosomal RNA is shown as a loading control.
(C,D) Images of rosettes [C] and flowers [D] in Ws, ld-3, ld-3 clf-59 and clf-52.

Figure 4. Flowering time and FLC expression in clf loss-of-function mutants.
(A) Average total leaf number of clf-28 in Col and FRI-Col backgrounds. Data represent the average of at least 8 plants. Error bars represent standard error.
(B) Quantitative reverse transcription PCR (qRT-PCR) showing FLC expression. The inset is shown to highlight the difference between Col and clf-28. All data represent the average of at least 3 experiments and error bars represent standard error.
Figure 5. H3K27me3 accumulation at *FLC* chromatin in different genetic backgrounds. 
(A,C) Real-time PCR on ChIP samples. *FLC* 5’ is near the transcriptional start site. *FLC*-I is in the first intron. *APETALA1 (AP1)*, *AG*, and *Actin* were used as controls. 
(B,D,E) Real-time PCR on ChIP samples showing H3K27me3 levels near the transcriptional start site of *FLC*. (E) NV and V indicate non-vernalized and vernalized samples respectively. Graphs represent the average of at least three experiments. Error bars represent standard error. Primer sequences can be found in Supplementary Table 1. Real-time data shown is relative to an *AG* control.

Figure 6. The interaction of *clf-59* with vernalization-insensitive mutants. 
(A) Flowering-time data showing the effect of *clf-59* on *vrn1* and *vin3* mutants. Arrows indicate that greater than 70 leaves were produced prior to flowering. 
(B) Flowering time of *fca-1* and *fca-1 vrn2-1* T1 plants transformed with either wild-type *CLF* or *clf-59*. Data represent all T1 plants generated and are shown in part to demonstrate the high transformation efficiency of the *clf-59* transgene. Similar efficiency was observed in other transformations such as those represented in Figure 1 B and D. 
(C) Quantitative reverse transcription PCR (qRT-PCR) showing *FLC* expression. Data represent the average of at least three experiments. Error bars represent standard error. 
(D,E) Images of pleiotropic phenotypes seen in *FRI vill1 clf-59* plants. [E] Upper and lower pictures show adaxial and abaxial angles of the same plants, respectively.

Figure 7. CLF localization at FLC. 
(A-C) Real-time PCR on ChIP samples showing the enrichment of CLF near the transcriptional start site of *FLC* under non-vernalized [A,B] and vernalized [C] conditions. Real-time data shown is relative to an *AG* control. Graphs in [A] and [C] represent the average of three experiments. Data in [B] represent a single experiment. Error bars represent standard error. Samples were standardized to a WS line which does not contain GFP.
(D) Flowering time of lines used in [A,C] both with (V) and without (NV) vernalization. The arrow indicates that greater than 60 leaves were produced prior to flowering. Leaf counts represent the average of at least 10 plants. Error bars represent standard error.
Figure 1. clf-59 suppresses FLC-mediated late flowering.
(A) The average total leaf number at flowering, a measure of flowering time in wild type, ld-3 and ld-3 combined with homozygous and heterozygous clf-59 mutants.
(B) Images of the late-flowering ld-3 mutant (left) and a ld-3 plant carrying a transgenic copy of clf-59 (right).
(C,D) The average total leaf number at flowering of plants containing clf-59 combined with other FLC-mediated late-flowering backgrounds including [C] fca-1 and FRI-Col and [D] fve-4 and fld-3. Data in [D] represent the average of at least 8 individual T1 plants.
(E) Northern blot showing the suppression of FLC expression under clf-59 conditions. 18S RNA is shown as a loading control.
(A-D) All leaf counts include both rosette and cauline leaves and represent the average of at least 8 plants. Error bars represent standard error. In A,C clf-59/+ indicates a heterozygote. In B,D a "+" indicates that clf-59 was added transgenically.
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Figure 3

Clf-59 mutants do not display phenotypes seen in off loss-of-function mutants.

(A,B) Northern blot showing the expression of AG in seedlings [A] and inflorescences [B]. 18S ribosomal RNA is shown as a loading control.

(C,D) Images of rosettes [C] and flowers [D] in Ws, ld-3, ld-3 clf-59 and clf-52.
Figure 4 Flowering time and FLC expression in clf loss-of-function mutants.
(A) Average total leaf number of clf-28 in Col and FRI-Col backgrounds. Data represent the average of at least 8 plants. Error bars represent standard error.
(B) Real-time PCR showing FLC expression. The inset is shown to highlight the difference between Col and clf-28. All data represent the average of at least 3 experiments and error bars represent standard error.
Figure 5

**A**

|        | Col | FRI/Col | FRI/Col clf-59 |
|--------|-----|---------|----------------|
| FLC 5' |     |         |                |
| FLC-I  |     |         |                |
| AP1    |     |         |                |
| AG     |     |         |                |
| ACTIN  |     |         |                |

**B**

|        | Col | FRI | FRI clf-59 |
|--------|-----|-----|------------|
| Relative Level H3K27m3 |     |     |            |

**C**

|         | Col | fca-9 | fld-3 | Id-1 |
|---------|-----|-------|-------|------|
| FLC-I   |     |       |       |      |
| ACTIN   |     |       |       |      |

**D**

|        | Col | FRI | Fe-4 | fld-3 | fpa-7 | Id-1 | fca-9 |
|--------|-----|-----|------|-------|-------|------|-------|
| Relative Level H3K27m3 |     |     |      |       |       |      |       |

**E**

|        | NV | V  |
|--------|----|----|
| Relative Level H3K27m3 |     |    |

**Figure 5** H3K27m3 accumulation at FLC chromatin in different genetic backgrounds. **(A,C)** RT-PCR using ChIP samples. FLC 5' is near the transcriptional start site. FLC-I is in the first intron. APETELA1 (AP1), AG, and Actin were used as controls. **(B,D,E)** Real-time PCR on ChIP samples showing H3K27m3 levels at the transcriptional start site of FLC. [E] NV and V indicate non-vernalized and vernalized samples, respectively. Graphs represent the average of at least three experiments. Error bars represent standard error. Primer sequences can be found in Supplementary Table 1. Real-time data shown is relative to an AG control. Observed patterns were also evident relative to an Actin control (data not shown).
Figure 6 The interaction of clf-59 with vernalization-insensitive mutants.

(A) Flowering-time data showing the effect of clf-59 on vm1 and vin3 mutants. Arrows indicate that greater than 70 leaves were produced prior to flowering.

(B) Flowering time of fca-1 and fca-1 vm2-1 T1 plants transformed with either wild-type CLF or clf-59. Data represent all T1 plants generated and are shown in part to demonstrate the high transformation efficiency of the clf-59 transgene. Similar efficiency was observed in other transformations such as those represented in Figure 1 B and D.

(C) Real-time PCR showing FLC expression. Data represent the average of at least three experiments. Error bars represent standard error.

(D,E) Images of pleiotropic phenotypes seen in FRI vil1 clf-59 plants. [D] Upper and lower pictures show adaxial and abaxial angles of the same plants, respectively.
Figure 7 CLF localization at FLC.
(A-C) Real-time PCR on ChIP samples showing the enrichment of CLF near the transcriptional start site of FLC under non-vernalized [A,B] and vernalized [C] conditions. Real-time data shown is relative to an AG control. Observed patterns were also evident relative to an Actin control (data not shown). Graphs in [A] and [C] represent the average of three experiments. Data in [B] represent a single experiment. Error bars represent standard error. Bars were normalized to a WS line which does not contain GFP.

(D) Flowering time of lines used in [A,B] both with (V) and without (NV) vernalization. The arrow indicates that greater than 60 leaves were produced prior to flowering. Leaf counts represent the average of at least 10 plants. Error bars represent standard error.