A Single Amino Acid Change in the Enhancer of Zeste Ortholog CURLY LEAF Results in Vernalization-Independent, Rapid Flowering in Arabidopsis[1][C][W][OA]

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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 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 trimethylation 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*; thus, our work provides insight into the molecular role that *FRI* plays in delaying the onset of flowering.

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 (for review, see 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), Wassilewskija (Ws), and Landsberg *erecta*, 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 that, in contrast to *FRI*, act as repressors of *FLC*. *LUMINIDEPENDENS* (*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

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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 (for review, see 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 trimethylation on Lys-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 that contain orthologs of *Drosophila melanogaster* Polycomb Repressive Complex 2 (PRC2) components and other plant-specific proteins, such as *VERNALIZATION INSENSITIVE3* (*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 *CLF* 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 semidominant manner (Fig. 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 nontransformed plants (an example of a transgenic versus parental line is shown in Fig. 1B). An introduced copy of wild-type *CLF* did not alter flowering time (data not shown). Thus, this semidominant 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 Col line that contains a functional copy of *FRI* (*FRI*-Col; Lee et al., 1994a); in both cases, *clf-59* suppressed late flowering in a semidominant manner (Fig. 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; Fig. 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* (Fig. 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 (Fig. 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 Cys-rich region precedes the SET domain. clf-59 contains a single amino acid substitution within this region that converts a conserved Pro to a Ser (P704S; Fig. 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

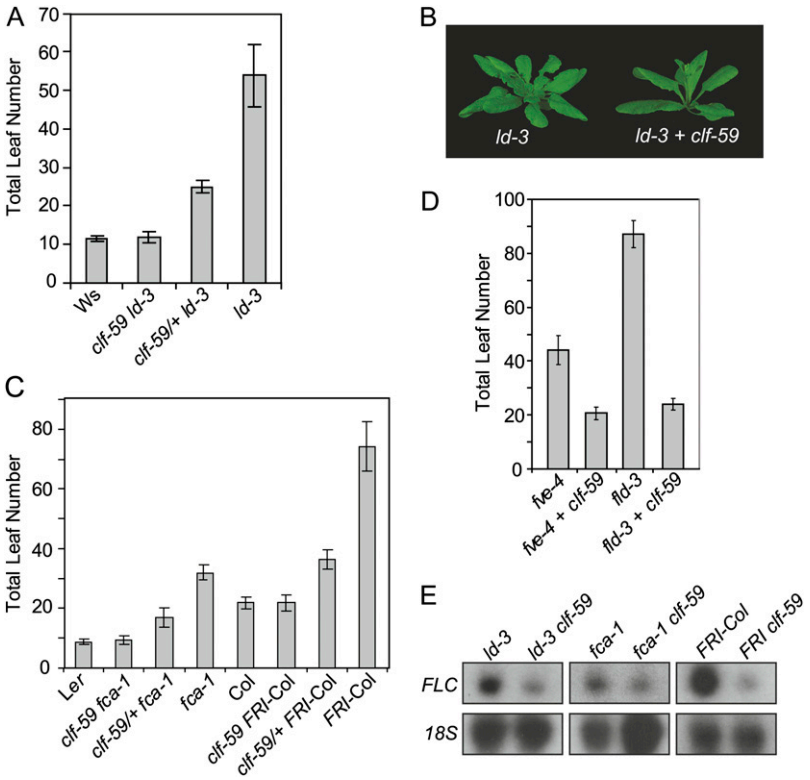

Figure 1. *clf-59* suppresses *FLC*-mediated late flowering. A, The average total leaf number at flowering, a measure of flowering time in the wild type, *ld-3*, and *ld-3* combined with homozygous and heterozygous *clf-59* mutants. B, Images of the *ld-3* mutant (left) and *ld-3* carrying a transgenic copy of *clf-59* (left). C and D, The average total leaf number at flowering of plants containing *clf-59* combined with other *FLC*-mediated late-flowering backgrounds, including *fca-1* and *FRI*-Col (C) and *fve-4* and *fld-3* (D). Data in D represent the average of at least eight 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. In A to D, all leaf counts include both rosette and cauline leaves and represent the average of at least eight plants. Error bars represent SE. In A and C, *clf-59*/+ indicates a heterozygote. In B and D, a "+" indicates that *clf-59* was added transgenically. [See online article for color version of this figure.]

(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 nontransformed 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.

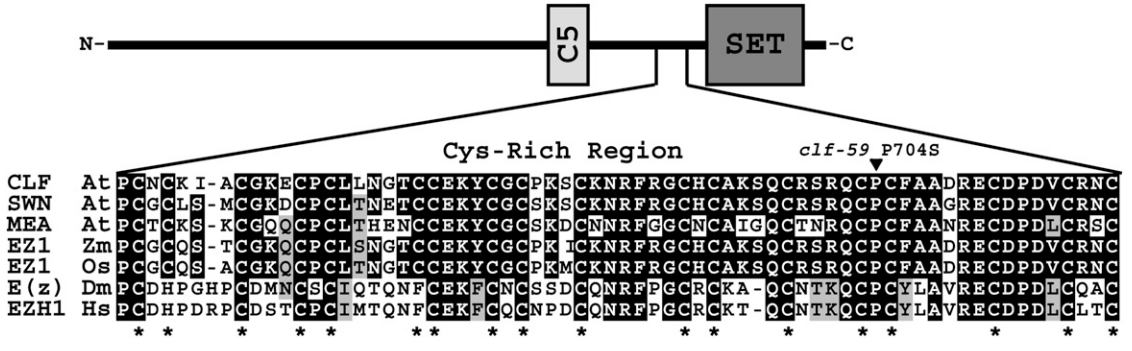

Figure 2. Amino acid alignment of the Cys-rich domain of CLF with other E(z)-like proteins. Sequence alignments were done using ClustalW. An asterisk indicates the location of a conserved Cys. Abbreviations are as follows: At, Arabidopsis; Zm, *Zea mays*; Os, *Oryza sativa*; Dm, *D. melanogaster*; Hs,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

clf-59 Mutants Do Not Display Phenotypes Seen in Either *clf* or *ag* 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 hypersuppression *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 (Fig. 3A) or inflorescence tissue (Fig. 3B) is indistinguishable from the wild type nor are there any *ag* reduction-of-function phenotypes in *clf-59* mutants (Fig. 3, C and 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* (Fig. 4A) and, unlike *clf-59*, did not have a significant effect on *FLC* expression (Fig. 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 (Fig. 4, A and B). Such *FLC* derepression 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 derepression of *FT* (Jiang et al., 2008), a strong promoter of flowering that when overexpressed 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); thus, we have focused our study on regions of *FLC* near the transcriptional start site. Levels of H3K27me3 were analyzed in nonvernalized *FRI clf-59*, Col, and *FRI*-Col using chromatin immunoprecipitation (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 (Fig. 5, A and B).

When assaying H3K27me3 in *clf-59* plants, a marked difference in H3K27me3 levels was observed between Col and *FRI*-Col (Fig. 5, A and 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

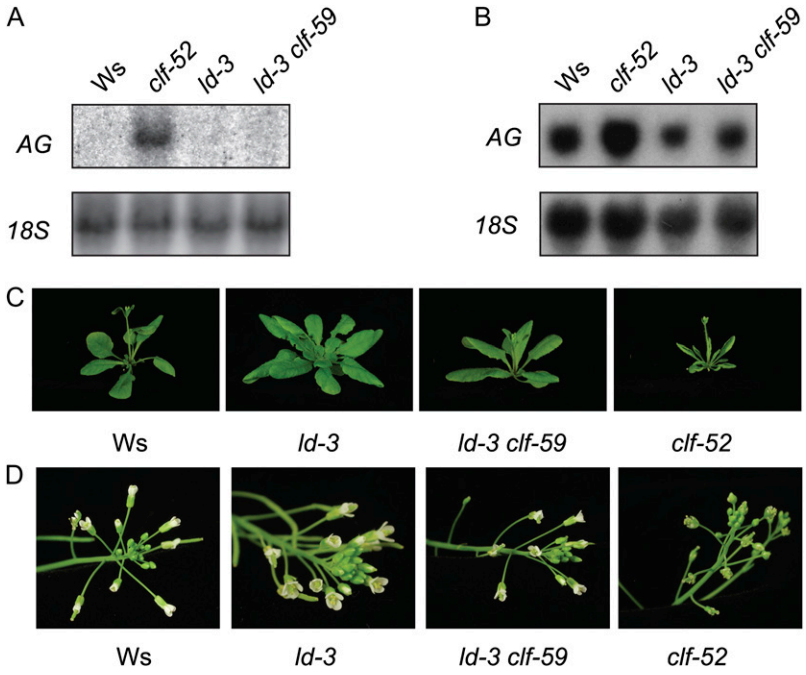

Figure 3. *clf-59* mutants do not display phenotypes seen in *clf* loss-of-function mutants. A and B, Northern blot showing the expression of *AG* in seedlings (A) and inflorescences (B). 18S ribosomal RNA is shown as a loading control. C and D, Images of rosettes (C) and flowers (D) in Ws, *ld-3*, *ld-3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

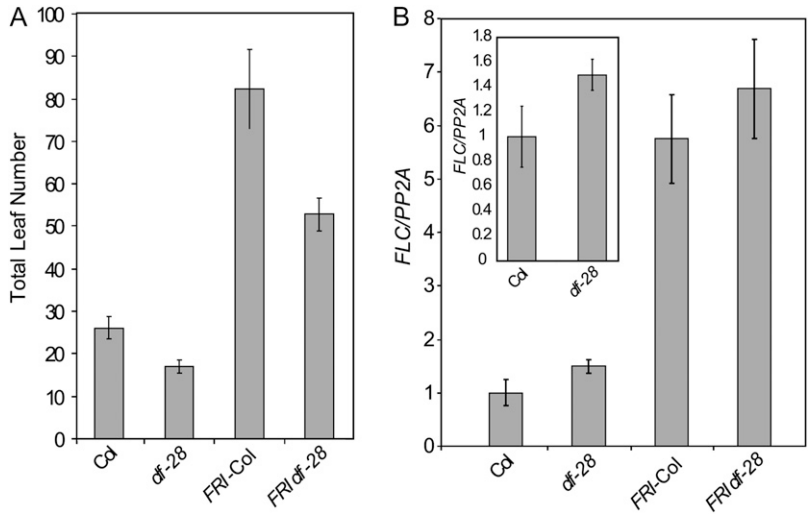

Figure 4. Flowering time and *FLC* expression in *clf* loss-of-function mutants. A, Average total leaf number of *clf-28* in Col and *FRI*-Col backgrounds. Data represent the average of at least eight plants. Error bars represent SE. B, Quantitative reverse transcription PCR showing *FLC* expression. The inset is shown to highlight the difference between Col and *clf-28*. All data represent the average of at least three experiments, and error bars represent SE.

levels of H3K27me3 at *FLC*, which are comparable to levels seen in *FRI*-Col (Fig. 5, C and 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 nonvernalized 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 (Fig. 5E). In both vernalized and nonvernalized plants, *FLC* had higher levels of H3K27me3 in Col than in *FRI*-Col. However, it should be noted that the 30-d cold treatment given in these experiments is not saturating with respect to the acceleration of

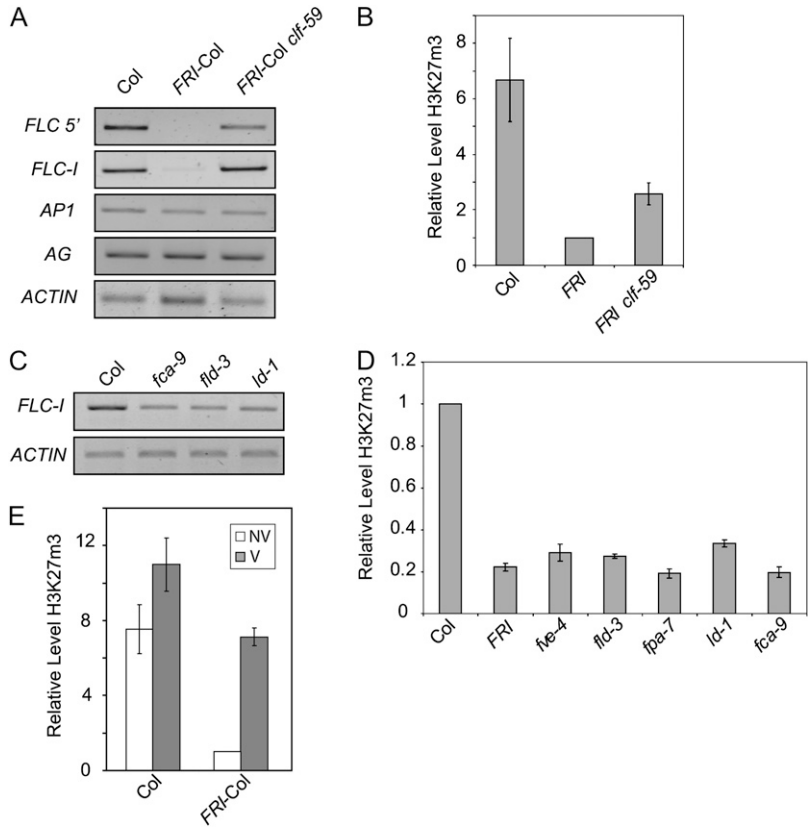

Figure 5. H3K27me3 accumulation at *FLC* chromatin in different genetic backgrounds. A and C, Real-time PCR on ChIP samples. *FLC 5′* is near the transcriptional start site. *FLC*-I is in the first intron. *APETALA1* (*AP1*), *AG*, and *Actin* were used as controls. B, D, and E, Real-time PCR on ChIP samples showing H3K27me3 levels near the transcriptional start site of *FLC*. E, NV and V indicate nonvernalized and vernalized samples, respectively. Graphs represent the average of at least three experiments. Error bars represent SE. Primer sequences can be found in Supplemental Table S1. Real-time data shown are relative to an *AG* control.

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 *VERNALIZATION2* (*VRN2*; Gendall et al., 2001; Bastow et al., 2004; Sung and Amasino, 2004) as well as *VRN1* and the PHD domain proteins *VIN3* and *VIN3-LIKE1* (*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* (Fig. 6, A and 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 (Fig. 6C), *FRI vil1 clf-59* plants did display several pleiotropic phenotypes, including pale green leaves and downward leaf curling (Fig. 6, D and 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 (Fig. 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 (Fig. 7A). Such a reduction in CLF occupancy likely contributes to the lower levels of H3K27me3 at *FLC* seen in *FRI*-containing backgrounds (Fig. 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 the wild type (Fig. 7B). Thus,

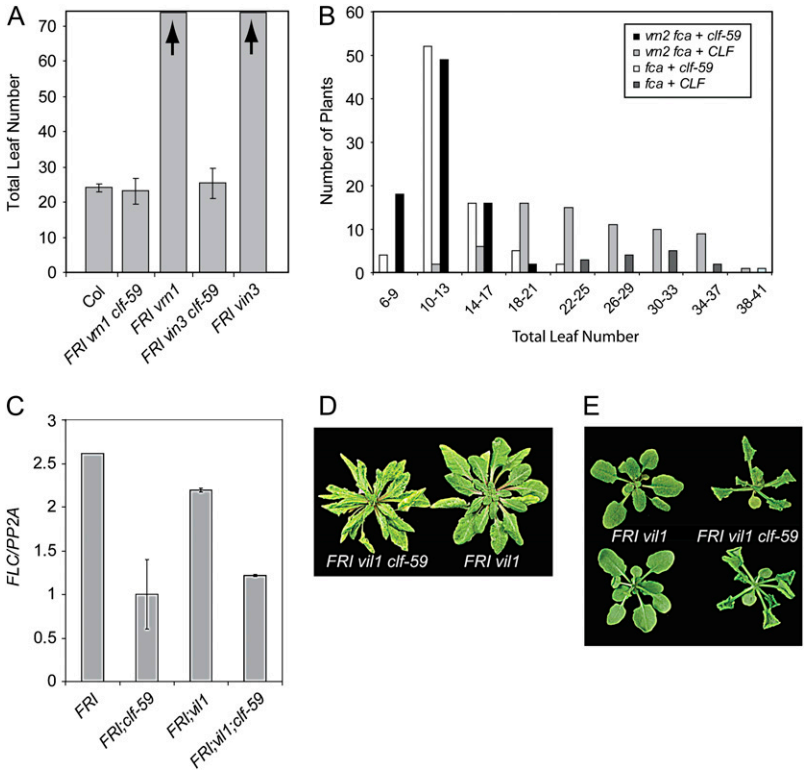

Figure 6. The interaction of *clf-59* with vernalization-insensitive mutants. A, Flowering-time data showing the effect of *clf-59* on *vrn1* and *vin3* mutants. Arrows indicate that >70 leaves were produced prior to flowering. B, Flowering time of *fca-1* and *fca-1 vrn2-1* T1 plants transformed with 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 showing *FLC* expression. Data represent the average of at least three experiments. Error bars represent SE. D and E, Images of pleiotropic phenotypes seen in *FRI vil1 clf-59* plants. E, Top and bottom pictures show adaxial and abaxial angles of the same plants, respectively. [See online article for color version of this figure.]

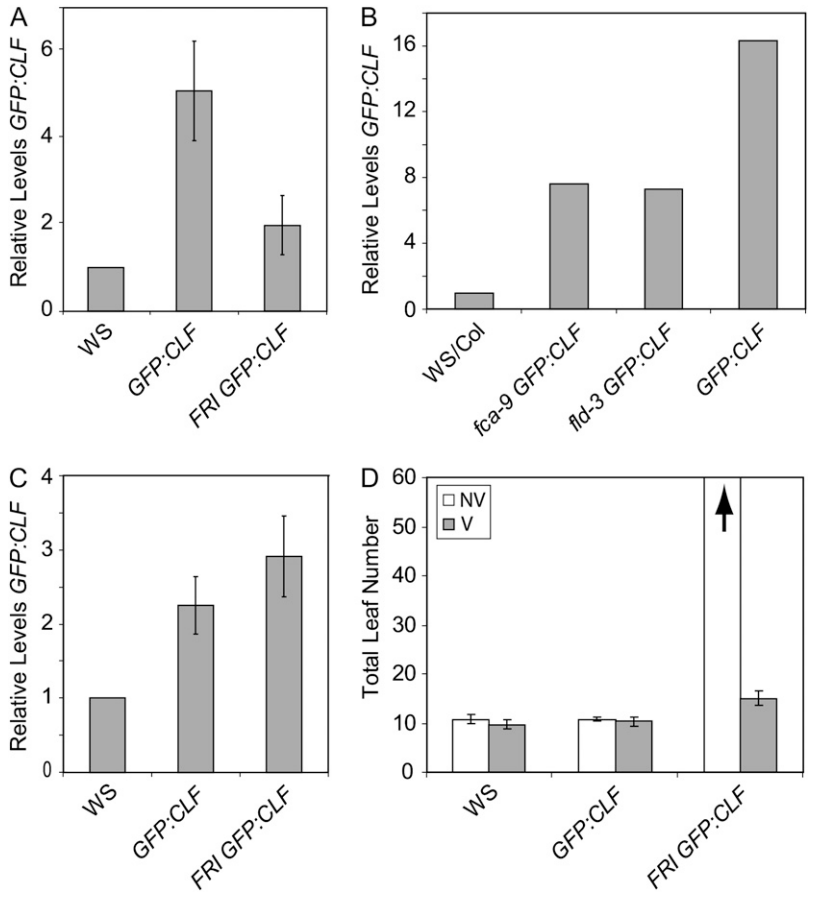

Figure 7. CLF localization at FLC. A to C, Real-time PCR on ChIP samples showing the enrichment of CLF near the transcriptional start site of *FLC* under non-vernalized (A and B) and vernalized (C) conditions. Real-time data shown is relative to an *AG* control. Graphs in A and C represent the average of three experiments. Data in B represent a single experiment. Error bars represent SE. Samples were standardized to a Ws line that does not contain GFP. D, Flowering time of lines used in A and C both with (V) and without (NV) vernalization. The arrow indicates that >60 leaves were produced prior to flowering. Leaf counts represent the average of at least 10 plants. Error bars represent SE.

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-d cold exposure, a difference in CLF occupancy at *FLC* could no longer be detected between plants that contain and those that lack *FRI* (Fig. 7C). This 35-d cold exposure was sufficient to substantially hasten flowering time in *FRI*-Ws (Fig. 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 Pro-to-Ser 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 Cys residues 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 hyperrepression of *FLC* expression with no noticeable effect on *AG*. In contrast, *clf* loss-of function mutants cause derepression of several genes, including *AG*, *FLC*, and *FT* (Goodrich et al., 1997; Jiang et al., 2008), and it is likely that *FT* derepression 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* derepression 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* derepression than we observe. In a delayed-flowering background, the difference is even greater: Jiang et al. (2008) report extensive derepression in an *fca clf* double mutant, whereas we do not observe derepression in *FRI clf*. Therefore, with respect to *FLC* derepression, *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* derepression 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 derepression when CLF is genetically removed.

In this work, we present data that are 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; indeed, this is consistent with the lack of *FLC* derepression 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 articles 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; 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 nonvernalized plants to a level comparable to that found in the 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 (16 h light: 8 h 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 derived cleaved amplified polymorphic sequence 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 *Nco*I restriction site at the lesion that was absent in the *clf-59*. Primer sequences are listed in Supplemental Table S1. Other lines used in this work have been previously described. The *fca-1* allele is from the Landsberg *erecta* but contains a Col allele of *FLC* (Sanda and Amasino, 1996). Alleles of other autonomous pathway mutants used in this work are in the Col 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 cauliflower mosaic virus 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 *Bam*HI sites engineered into the primers. These primers and primers used to clone and make site-directed mutants in MEA and SWN are listed in Supplemental Table S1.

Analysis of RNA Abundance

Tissue was harvested from 7-d-old seedlings. RNA was isolated using TRI reagent (Sigma-Aldrich). For northern analysis, 12 μg 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′ untranslated region 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 as follows: 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 phosphatase 2A was used as a control (Czechowski et al., 2005). PCR primer sequences are listed in Supplemental Table S1. All results presented are an average of at least three biological replicates.

ChIP

Tissue was harvested from 7-d-old seedlings. Chromatin samples were prepared as described (Gendrel et al., 2005). Antibodies used in this work were as follows: anti-GFP (Invitrogen catalog no. A-11122) and anti-H3K27me3 (Upstate catalog no. 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 Supplemental Table S1. All ChIP results presented are an average of at least two biological replicates unless noted otherwise.

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; and SWN, At4g02020.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Sequences of oligos used in cloning and gene expression analysis.

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