Repression of **FLOWERING LOCUS C** and **FLOWERING LOCUS T** by the *Arabidopsis* Polycomb Repressive Complex 2 Components

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**Abstract**

Polycomb group (PcG) proteins are evolutionarily conserved in animals and plants, and play critical roles in the regulation of developmental gene expression. Here we show that the *Arabidopsis* Polycomb repressive complex 2 (PRC2) subunits CURLY LEAF (CLF), EMBRYONIC FLOWER 2 (EMF2) and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) repress the expression of **FLOWERING LOCUS C** (**FLC**), a central repressor of the floral transition in *Arabidopsis* and **FLC** relatives. In addition, CLF directly interacts with and mediates the deposition of repressive histone H3 lysine 27 trimethylation (**H3K27me3**) into **FLC** and **FLC** relatives, which suppresses active histone H3 lysine 4 trimethylation (**H3K4me3**) in these loci. Furthermore, we show that during vegetative development CLF and FIE strongly repress the expression of **FLOWERING LOCUS T** (**FT**), a key flowering-time integrator, and that CLF also directly interacts with and mediates the deposition of **H3K27me3** into **FT** chromatin. Our results suggest that PRC2-like complexes containing CLF, EMF2 and FIE, directly interact with and deposit into **FT**, **FLC** and **FLC** relatives repressive trimethyl H3K27 leading to the suppression of active **H3K4me3** in these loci, and thus repress the expression of these flowering genes. Given the central roles of **FLC** and **FT** in flowering-time regulation in *Arabidopsis*, these findings suggest that the CLF-containing PRC2-like complexes play a significant role in control of flowering in *Arabidopsis*.

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**Introduction**

The transition from a vegetative to a reproductive phase (i.e., flowering) is a major developmental switch in the plant life cycle that must be properly timed to ensure maximal reproductive success. In *Arabidopsis thaliana*, this transition is genetically controlled by several pathways, including the autonomous pathway, the photoperiod pathway and the vernalization pathway, which form a regulatory network [1,2]. This network integrates the endogenous developmental state of the plant with environmental cues (e.g., day length and temperature) to precisely control the timing of the floral transition [1,2].

A key component in this regulatory network in *Arabidopsis* is **FLC**, a MADS box transcription factor that quantitatively inhibits the floral transition [3,4]. **FLC** expression is delicately controlled by various activators and repressors. The autonomous pathway, which includes **FVE** [5,6], **FCA** [7] and **FLOWERING LOCUS D** (**FLD**) [8], constitutively represses **FLC** expression to promote flowering, whereas **FRIGIDA** (**FRI**) activates **FLC** expression to delay flowering [9]. The vernalization pathway also represses **FLC** expression in response to a prolonged cold exposure (a typical winter) to accelerate flowering in *Arabidopsis* [10,11]. Besides **FLC**, in the *Arabidopsis* genome there are five close **FLC** relatives including **FLOWERING LOCUS M** (**FLM**), **MADS AFFECTING FLOWERING 2** (**MAF2**), **MADS AFFECTING FLOWERING 3** (**MAF3**), **MADS AFFECTING FLOWERING 4** (**MAF4**) and **MADS AFFECTING FLOWERING 5** (**MAF5**); these **FLC** relatives also appear to repress the floral transition [12,13].

Chromatin modification plays an important role in the regulation of **FLC** expression. Activation of **FLC** expression in the presence of **FRI** is associated with the **H3K4** trimethylation and also requires deposition of the histone variant H2A.Z in **FLC** chromatin [14,15,16]. The autonomous-pathway represses **FLC** expression partly through generating repressive histone modifications in **FLC** chromatin. **FLD** is involved in the **H3K4** demethylation and deacetylation of **FLC** chromatin [8,17,18]; **FC1** functions closely with **FLD** and is involved in **H3K4** demethylation in **FLC** chromatin [18]; **FVE** is partly involved in the histone deacetylation of **FLC** chromatin [5,8]. In addition, histone H4 dimethylation at arginine 3 (**H4R3**) in **FLC** chromatin by Type I and Type II arginine methyltransferases is also associated with **FLC** repression [19,20,21]. Furthermore, small RNA-mediated repressive histone modifications are also linked to **FLC** repression [22,23]. Recent studies also reveal that vernalization leads to repressive histone modifications in **FLC** chromatin such as increased trimethylation of histone H3 at lysine 9 and **H3K27**, and **H4R3** dimethylation [24,25,26,27].

**FLC** inhibits the floral transition partly by reducing expression of a key flowering-time integrator, **FT** [28]. **FT** was first identified as a component of the photoperiod pathway, which promotes
flowering in response to increased day length [29,30,31]. In the presence of light, FT expression is activated by CO/NL2 (CO), another component in the photoperiod pathway [31]. FT is expressed in the vasculature [32], and subsequently, FT proteins are translocated from veins to the shoot apex to promote flowering [33,34,35]. FLC binds to the FT locus and represses its expression, and thus antagonizes the activation by CO [28]. Hence, FT acts as a flowering-time integrator that integrates signals from the photoperiod pathway and the FLC-mediated flowering pathways to promote the Arabidopsis flowering. Recent studies indicate that chromatin modification may play a role in the regulation of FT expression. It has been shown that LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) directly interacts with FT chromatin and represses FT expression [36,37,38]; in addition, recent whole-genome analysis of H3K27 trimethylation in Arabidopsis has revealed that this repressive mark is associated with FT chromatin [39]. However, how H3K27me3 is deposited is less clear.

Repressive H3K27me3 is deposited by the PRC2 complex in Drosophila. PRC2 is composed of four core proteins including Enhancer of zeste (E(z); an H3K27 methyltransferase), Extra sex comb (Esc), Suppressor of zeste 12 (Su(z)12) and p55, and deposits trimethyl H3K27 to silence the expression of homeotic genes in Drosophila (reviewed in [40]). Homologs of Drosophila PRC2 components have also been identified in Arabidopsis, and play important roles in the control of plant developmental processes such as floral induction, flower organogenesis, seed development and sporophyte development (reviewed in [41,42]). To date, a PRC2-like complex composed of MEDEA (MEA), FIE, FERTILIZATION INDEPENDENT SEED 2 and MULTICOPY SUPPRESSOR OF IRA1 (MSI1), which are relatives of E(z), Esc, Su(z)12 and p55 respectively, has been biochemically characterized [43,44]. This complex represses the MADS box gene PHERES1 during seed development and thus controls this developmental process [45,46].

Recent studies have also shown that CLF, an Arabidopsis homolog of E(z), directly mediates the repression of AGAMOUS (AG) via H3K27 trimethylation and thus controls floral organogenesis [47,48]. CLF plays multiple roles in plant development, and also directly represses the expression of SHOOTMERISTEMLESS (STM) and a flowering gene, AGAMOUS LIKE 19 (AGL19), during vegetative development [48,49]. Recent studies also reveal that VERNALIZATION 2 (VRN2), a homolog of Su(z)12, plays an important role in the vernalization-mediated FLC repression [50]. VRN2 is required for FLC repression by vernalization treatment [50]; VRN2 forms a complex with CLF, SWINGER (SWN; another homolog of E(z)), FIE and VERNALIZATION INSENSITIVE 3 to repress FLC expression in response to vernalization treatment [51]. In addition, EMF2, a relative of VRN2 and Su(z)12, also plays an important role in sporophyte development, and maintains vegetative development by repressing the floral induction [52,53,54]. However, the underlying mechanisms of the EMF2-mediated floral repression are unclear [54].

Here we report that Arabidopsis PRC2-like complex subunits CLF, EMF2 and FIE repress the expression of FLC and FLC relatives including MAF4 and MAF5, and that CLF directly binds to and mediates the deposition of H3K27me3 in FLC, MAF4 and MAF5 chromatin. Furthermore, we show that during vegetative development CLF and FIE strongly repress FT expression, and that CLF also directly interacts with and mediates the deposition of H3K27me3 in FT chromatin. These results imply that PRC2-like complexes containing CLF, EMF2 and FIE deposit repressive H3K27me3 in and directly repress the expression of these flowering genes, and thus control the flowering program in Arabidopsis.

Results

PRC2 Subunits CLF, EMF2 and FIE Repress the Expression of FLC, MAF4 and MAF5 in Vegetative Development

Arabidopsis PRC2-like complex components including VRN2, FIE, SWN and CLF are required for the vernalization-mediated FLC repression [50,51]. We sought to investigate PRC2-mediated FLC repression in Arabidopsis plants grown in normal conditions (i.e., without vernalization treatment). In addition, the expression of FLC relatives such as FLM, MAF4 and MAF5, like FLC expression, is also regulated by chromatin modification [14,15]; hence, it was also of interest to investigate whether PRC2-like complexes repress the expression of FLC relatives. First, we addressed the role of CLF in the regulation of FLC and FLC relatives. Transcript levels of these genes were examined in seedlings of the clf-61 mutant carrying a lesion in the SET domain of CLF [48]. We found that FLC, MAF4 and MAF5 were de-repressed in clf, whereas transcripts of FLM, MAF2 and MAF3 in clf remained at levels similar to wild-type Col (Figure 1A); hence, CLF plays an essential role in repressing the expression of FLC, MAF4 and MAF5 during vegetative development.

Secondly, we investigated the role of FIE in the regulation of FLC and FLC relatives using FIE-suppressed seedlings [50] (note that fie alleles can not be transmitted through the female gamete [56]). Consistent with a recent report [51], in FIE-suppressed seedlings FLC expression was de-repressed (Figure 1B); furthermore, we found that MAF4 and MAF5 were also de-repressed, whereas FLM, MAF2 and MAF3 in these seedlings were expressed at levels similar to those in the wild type (Figure 1B). Hence, like CLF, FIE also selectively represses the expression of FLC, MAF4 and MAF5.

CLF has been shown to directly interact with EMF2 and these two proteins may be part of a PRC2-like complex involved in the regulation of vegetative development in Arabidopsis [57]. We therefore examined transcript levels of FLC and FLC relatives in emf2 seedlings. Indeed, FLC, MAF4 and MAF5, but not FLM, MAF2 or MAF3, were de-repressed in emf2 (Figure 1C). Hence, like CLF and FIE, EMF2 also selectively represses FLC, MAF4 and MAF5 expression during vegetative development. Together, these data suggest that there is a CLF-containing PRC2-like complex composed of at least EMF2 and FIE, which acts to repress FLC, MAF4 and MAF5 expression during vegetative development.

CLF and FIE also Repress FT Expression in Vegetative Development

The de-repression of FLC and MAFs in clf, emf2 and FIE-suppressed plants was expected to lead to late flowering because the elevated expression of these genes alone causes late flowering [3,4,13]; however, these mutant plants all are early-flowering [47,52,55]. These early-flowering phenotypes are likely due to increased or ectopic expression of genes that promote flowering. CLF and EMF2 have been shown to repress the expression of the flowering promoter AGL19 [49]; furthermore, ectopic expression of AG in clf and emf2 may also partly contribute to the early-flowering phenotypes [47,54]. In addition, a very recent report shows that FT expression is upregulated in 21-day-old clf mutant plants grown under continuous light [58], indicating that FT de-repression may partly account for the early-flowering phenotype of clf. We examined FT mRNA levels in young Col and clf seedlings to address whether FT is also de-repressed in clf mutants before the floral transition. Indeed, FT expression was greatly de-repressed in clf seedlings (Figure 2A). These data together with recent findings [58] suggest that CLF represses FT expression throughout vegetative development.

Recently, it has been shown that FT mRNA levels are higher in emf2 relative to Col [54,58], but the role of EMF2 in FT repression
is unclear [54]. We also examined FT mRNA levels in emf2 seedlings. Consistent with the recent reports [54, 58], FT expression was de-repressed in emf2 (Figure 2B). Because FIE may be part of the PRC2-like complexes containing EMF2 and CLF [41], we examined FT transcript levels in FIE-suppressed seedlings to determine whether FIE is also involved in FT repression, and found that FT is strongly de-repressed in these seedlings compared to the control Col-gl1 seedlings (Figure 2C). Taken together, these data suggest that a PRC2-like complex containing CLF, EMF2 and FIE, represses FT expression in vegetative development to repress the floral transition.

Interestingly, although these PRC2 subunits repress both FLC and FT expression and FLC directly represses FT expression, loss or suppression of the functions of these subunits leads to a greater FT derepression compared to FLC derepression (Figure 1 and Figure 2; also refer to Figure 3), suggesting that PRC2-like complexes have a repressive effect on FT expression much stronger than that on FLC expression.

**CLF Acts in Partial Redundancy with Part of the Autonomous Pathway to Repress FLC Expression in the Absence of Vernalization**

The autonomous pathway constitutively represses FLC expression to promote flowering, and part of this pathway is involved in the generation of repressive histone modifications in FLC

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**Figure 1.** PRC2 subunits CLF, EMF2 and FIE repress the expression of FLC and FLC relatives. (A) Analysis of the expression of FLC and FLC relatives in clf seedlings by RT-PCR. ACTIN2 (ACT2) served as an internal control. (B) Analysis of the expression of FLC and FLC relatives in seedlings of Col-gl1 in which FIE is co-suppressed [55]. (C) Analysis of the expression of FLC and FLC relatives in emf2 seedlings. emf2 homozygotes were isolated from a selfed population of an emf2 heterozygote. “Control” is a mixture of wild-type like seedlings consisting of Col and emf2 heterozygotes.

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**Figure 2.** PRC2 subunits CLF, EMF2 and FIE repress FT expression. (A) Analysis of FT expression in clf seedlings by RT-PCR. ACT2 served as an internal control. (B) Analysis of FT expression in emf2 seedlings. The control is as described in Figure 1C. (C) Analysis of FT expression in seedlings of Col-gl1 in which FIE is co-suppressed.

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chromatin [59]. The autonomous-pathway repressor FCA directly binds to the FLC locus and is involved in the H3K4 demethylation of FLC chromatin [18]. Recent studies in mouse embryonic stem cells have suggested the coordinated regulation of H3K4 demethylation and PRC2-mediated repressive histone modifications in maintaining transcriptional gene repression [60]. Hence, it was of interest to examine the genetic interaction of clf with fca. We introduced clf into the fca mutant, and quantified FLC transcripts in clf, fca and clf:fca seedlings by real-time quantitative PCR. Consistent with previous findings [7], FLC was highly expressed in fca mutants (Figure 3A); however, FLC was further de-repressed in clf:fca and FLC mRNA levels in the double mutants were much higher than those in fca or clf (Figure 3A). Hence, CLF acts in partial redundancy with FCA to repress FLC expression in the absence of vernalization.

We further measured flowering times of fca and clf:fca mutants grown in long days. Although FLC was so highly expressed in clf:fca, the double mutants flowered much earlier than fca (Figure 3B). As noted above, FT is de-repressed in clf; hence, it is likely that the early-flowering phenotype of clf:fca is partly due to FT derepression. We quantified FT transcript levels in clf, fca and clf:fca seedlings. FT mRNA levels increased about 200 fold in clf.
relative to Col, whereas FT expression was suppressed in *fea* because of elevated *FLC* expression (Figure 3C). Furthermore, FT expression was partially suppressed in *df/fea*, but FT transcript levels in the double mutant were still higher than those in *fea* (Figure 3C), suggesting that the early-flowering phenotype of *df/fea* is at least partly due to the elevated FT expression.

**CLF Directly Interacts with the FLC, MAF4, MAF5 and FT Chromatin**

As noted above, CLF, EMF2 and FIE repress FLC, MAF4, MAF5 and FT expression, however, it was not known whether these PRC2 subunits acted directly on these genes or indirectly. Using chromatin immunoprecipitation (ChIP), we first examined whether CLF directly interacts with the FLC, MAF4, and MAF5 loci. Specifically, genomic DNA was immunoprecipitated using an antibody recognizing GFP from seedlings of a 35S::GFP-CLF *clf* transgenic line in which GFP:CLF fully functions and its distribution mimics that of the endogenous CLF [48], and subsequently, the genomic DNA was quantified by real-time PCR or examined by FCR if the amounts of DNA in a ChIP sample were too low to be quantified.

We found that both the region (FLC-P2) around the transcription start site (TSS) and 5′ part of Intron I of FLC (FLC-I) were greatly enriched, whereas a 5′ promoter region 1.8 kb upstream from the TSS in FLC was not enriched (Figure 4B and 4C). Moreover, we found that regions in the first introns of MAF4 and MAF5 were also enriched (Figure 4B), whereas MAF3, a close relative of MAF4 and MAF5 located immediately upstream MAF4 (Figure 4A), and Atg5g65090, the gene immediately downstream MAF5 (Atg5g65080), were not enriched (Figure 4C). Together, these data suggest that CLF selectively binds to FLC, MAF4 and MAF5 in *clf* to express the expression of these genes.

To examine whether CLF directly interacts with the FT locus, using ChIP-PCR we checked the middle region of FT (FT-I; see Figure 4A), a region where FLC has been shown to bind [28]. As shown in Figure 4C, FT fragments were strongly enriched in the ChIP samples from the 35S::GFP-CLF *clf* transgenic line. Hence, CLF directly interacts with FT chromatin to repress FT expression during vegetative development.

**Loss of CLF Function Leads to Reduction in Global H3K27 Trimethylation, but not in H3K27 Dimethylation during Vegetative Development**

CLF is a plant homolog of the *Drosophila* El[2], an H3K27 methyltransferase in the Esc-E[c] PRC2 complex [61,62]. Previous studies have shown that El[2] or El[2]2H2, the mammalian homolog of El[2], display PRC2-complex-dependent H3K27 methyltransferase activities on chromatin substrate (reviewed in [63]). It has been shown that CLF is partly required for H3K27me3 in CLF-target genes such as *AG* and *STM* [48]. We compared global histone methylation levels in *clf* and wild-type Col seedlings, including H3K27 dimethylation, H3K27 trimethylation and H3K4 trimethylation. Levels of trimethyl H3K27 were strongly reduced in *clf* relative to Col (Figure 5A), whereas levels of dimethyl H3K27 and trimethyl H3K4 in *clf* were similar to those in Col (Figure 5B and 5C), indicating that CLF is likely to be a histone methyltransferase catalyzing H3K27 trimethylation. Interestingly, lower levels of trimethyl H3K27 were still detected in *clf* mutant seedlings, which may be deposited by PRC2-like complexes containing CLF relatives including SWN and MEA.

**CLF Mediates the Deposition of H3K27me3 in FLC, MAF4, MAF5 and FT**

As noted above, CLF mediates global H3K27 trimethylation during vegetative development; in addition, recent whole-genome analysis of H3K27 trimethylation in *Arabidopsis* has revealed that this modification is associated with FLC chromatin in the absence of vernalization treatment [39], which is likely deposited by a CLF-containing PRC2-like complex. It was of interest to examine the H3K27 trimethylation state in FLC, MAF4 and MAF5 in *clf* seedlings. As shown in Figure 6A, H3K27me3 was enriched in the promoter region FLC-P2 and 5′ part of Intron I of FLC (FLC-I) in Col and loss of CLF activities significantly reduced the levels of trimethyl H3K27, consistent with the derepression of FLC in *clf* (Figure 3A). Furthermore, H3K27me3 was also enriched in MAF4 and MAF5 in the wild type and strongly reduced in *clf* (Figure 6B). In contrast, very little trimethyl H3K27 was detected in the neighboring genes including MAF3 and Atg5g65090 (Figure 6B). In addition, we did not detect trimethyl H3K27 in *FLM* (Figure 6B), another close relative of FLC and MAFs. Together, these data show that CLF mediates the deposition of trimethyl H3K27 selectively in FLC, MAF4 and MAF5, consistent with the selective de-repression of these three genes, but not *FLM* or MAEs in *clf*.

We also found that H3K27me3 was enriched in FT chromatin in Col as reported previously [39], and that H3K27me3 in FT was nearly eliminated in *clf* (Figure 6B), consistent with the drastic de-repression of FT in *clf* (Figure 3C). As described above, CLF, EMF2 and FIE may be part of a PRC2-like complex that represses FT expression. Together, these data suggest that a CLF-containing PRC2-like complex may be responsible for repressing FT expression in the absence of vernalization treatment [39,64] (also see Figure 6A). However, it remains unknown whether FLC chromatin can simultaneously carry these two modifications as it is formally possible that these modifications could occur in two subpopulations of FLC chromatin and not in the same physical region of FLC. To examine whether FLC chromatin comconitantly carries both H3K4me3 and H3K27me3, we performed a sequential ChIP in which FLC chromatin from seedlings was immunoprecipitated first with anti-trimethyl H3K27 and second with anti-trimethyl H3K27. Both the region around TSS (FLC-P2) and 5′ part of Intron I of FLC (FLC-I) in part of the FLC chromatin comconitantly harbor H3K4me3 and H3K27me3 (Figure 7A). Similarly, using sequential ChIP we also found that the 5′ transcribed region (FT-I) and the middle of FT (FT-II) in part of the FT chromatin simultaneously harbor H3K4me3 and H3K27me3 (Figure 7A). In addition, we did not detect any DNA fragments from a heterochromatic locus Ta3 [65] that lacks of H3K4me3 or from a constitutive expressed house-keeping gene ACTIN 2 (ACT2) carrying abundant H3K4me3 (data not shown) but lacking of H3K27me3 (Figure 7A). Together, these data show that part of the FLC and FT chromatin simultaneously possesses the bivalent chromatin marks of active H3K4me3 and repressive H3K27me3.

**CLF-Dependent H3K27 Tri methylation Suppresses H3K4 Tri methylation in its Target-Gene Chromatin**

As noted above, PRC2 subunits repress but do not fully silence FLC and FT expression because both genes are still expressed at low levels in wild-type seedlings. It has been shown that active H3K4me3 is associated with FLC chromatin in *Arabidopsis* accessions which lack of FRI such as Col and Wassilewskija (Ws) in which FLC expression is repressed [14,64], and repressive H3K27me3 is also associated with FLC chromatin in these accessions in the absence of vernalization treatment [39,64] (also see Figure 6A). However, it remains unknown whether FLC chromatin can simultaneously carry these two modifications as it is formally possible that these modifications could occur in two subpopulations of FLC chromatin and not in the same physical region of FLC. To examine whether FLC chromatin comconitantly carries both H3K4me3 and H3K27me3, we performed a sequential ChIP in which FLC chromatin from seedlings was immunoprecipitated first with anti-trimethyl H3K4 and second with anti-trimethyl H3K27. Both the region around TSS (FLC-P2) and 5′ part of Intron I of FLC (FLC-I) in part of the FLC chromatin comconitantly harbor H3K4me3 and H3K27me3 (Figure 7A). Similarly, using sequential ChIP we also found that the 5′ transcribed region (FT-I) and the middle of FT (FT-II) in part of the FT chromatin simultaneously harbor H3K4me3 and H3K27me3 (Figure 7A). In addition, we did not detect any DNA fragments from a heterochromatic locus Ta3 [65] that lacks of H3K4me3 or from a constitutive expressed house-keeping gene ACTIN 2 (ACT2) carrying abundant H3K4me3 (data not shown) but lacking of H3K27me3 (Figure 7A). Together, these data show that part of the FLC and FT chromatin simultaneously possesses the bivalent chromatin marks of active H3K4me3 and repressive H3K27me3.

We further investigated the interaction of H3K27 trimethylation with H3K4 trimethylation in FLC and FT chromatin. The H3K4 trimethylation state in these two loci was examined in *clf* seedlings by ChIP. Levels of trimethyl H3K4 in 5′ genomic FLC including FLC-P1 and FLC-P2 regions and in the 5′ transcribed
Figure 4. CLF binds to the *FLC, MAF4, MAF5* and *FT* loci. (A) Genomic structures of *FLC, FT* and the gene cluster of *MAF3, MAF4* and *MAF5* and the regions examined after ChIP. The transcription start sites are indicated by arrows; black boxes represent exons. (B) Binding of CLF to *FLC, MAF4* and *MAF5* chromatin. DNA fragments of *FLC-P2, MAF4-I* and *MAF5-I*, immunoprecipitated with anti-GFP from seedlings of a 35S:GFP:CLF clf transgenic.
Figure 5. Analysis of histone methylation in the clf mutant by immunoblotting. 
(A) Analysis of H3K27me3 in Col and clf seedlings. Histone extracts from Col and clf were blotted with anti-trimethyl H3K27 (top panel) and anti-H3 (bottom panel). (B) Analysis of H3K27me2 in Col and clf seedlings. Histone extracts were blotted with anti-dimethyl H3K27 (top panel). (C) Analysis of H3K4me3 in Col and clf seedlings. Histone extracts were blotted with anti-trimethyl H3K4 (top panel).

Figure 6. CLF mediates the deposition of H3K27me3 in the FLC, MAF4, MAF5 and FT chromatin. 
(A) Levels of trimethyl H3K27 in FLC chromatin in Col and clf seedlings determined by real-time quantitative PCR. Amounts of DNA fragments after ChIP were quantified and subsequently normalized to an internal control (TUBULIN 2; TUB2). The fold enrichments of the 35S:GFP:CLF clf line over the control (Ws) are shown, and the values shown are means ± SD. (B) Binding of CLF to FT and FLC chromatin analyzed by ChIP-PCR. Two independent immunoprecipitations were shown. “Input” is the total DNA prior to immunoprecipitation (diluted 640 times); “(-)” is the negative control for immunoprecipitation, residual DNA from the rabbit IgG immunoprecipitation. The constitutively expressed TUB2, a nontarget gene of CLF, was used as an internal control for PCR.

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Figure 7. Interaction of the CLF-dependent H3K27 trimethylation with H3K4 trimethylation in its target-gene chromatin. (A) Sequential ChIP analysis of FLC and FT chromatin. The chromatin from wild-typeWs seedlings was immunoprecipitated first with anti-trimethyl H3K4 and second with anti-trimethyl H3K27. Examined regions are as illustrated in Figure 4A. “Input” is the total DNA prior to the first immunoprecipitation (diluted 800 times); Ta3, a heterochromatic locus lacking of H3K4me3 and ACT2, a constitutively expressed locus lacking of H3K27me3, served as negative controls. “(-)” is the negative control for immunoprecipitation, residual DNA from the rabbit IgG immunoprecipitation. (B) Levels of trimethyl H3K4 in the FLC, MAF4 and MAF5 chromatin in clf seedlings relative to Col determined by real-time quantitative PCR. Amounts of DNA fragments from Col and clf seedlings after ChIP were quantified and subsequently normalized to an internal control (TUB2). The fold enrichments of clf over Col are shown, and the values shown are means±SD. (C) Levels of trimethyl H3K4 in FT chromatin in clf seedlings relative to Col determined by real-time quantitative PCR. The fold enrichments of clf over Col are shown, and the values shown are means±SD.

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region of \( FT \) (\( FT-E \)) and the middle of genomic \( FT \) (\( FT-I \)) were increased upon loss of CLF activities (Figure 7B and 7C), consistent with \( FLC \) and \( FT \) derepression in \( clf \). Furthermore, the levels of trimethyl H3K4 in \( MAF4 \) and \( MAF5 \) were also increased in \( clf \) relative to Col (Figure 7B), in line with \( MAF4 \) and \( MAF5 \) derepression in \( clf \). Together, these data suggest that the CLF-dependent H3K27 trimethylation suppresses H3K4 trimethylation in \( FLC \), \( MAF4 \), \( MAF5 \) and \( FT \). Interestingly, the global levels of trimethyl H3K4 in \( clf \) were similar to those in Col (Figure 5C), indicating that CLF-containing PRC2-like complexes only suppresses the H3K4 trimethylation in their target-gene chromatin.

**Discussion**

Our studies reveal that the *Arabidopsis* PRC2-like complex subunits CLF, EMF2 and FIE repress the expression of *FLC* and *FLC* relatives including *MAF4* and *MAF5*, and that CLF directly binds to and mediates the deposition of repressive H3K27me3 in these three loci. Furthermore, we show that during vegetative development CLF and FIE strongly repress *FT* expression, and that CLF directly interacts with and mediates the deposition of H3K27me3 in *FT* chromatin. Our results suggest that CLF-containing PRC2-like (CLF-PRC2) complexes containing EMF2 and FIE directly interact with and deposit into the *FLC*, *MAF4*, *MAF5* and *FT* chromatin repressive trimethyl H3K27 leading to the suppression of active H3K4me3 in these loci, and thus repress the expression of these flowering genes. Given the central roles of *FLC* and *FT* in flowering-time regulation in *Arabidopsis*, these findings suggest that CLF-PRC2 complexes play a significant role in control of the *Arabidopsis* flowering.

**PRC2 Subunits-Mediated Repression of *FLC* and *FLC* Relatives**

Previous studies indicate that a PRC2-like complex containing VRN2, FIE and SWN or CLF might be involved in *FLC* repression in *Arabidopsis* plants grown in normal conditions [51]. In this study, we show that CLF is an essential component for *FLC* repression because CLF directly binds to and mediates chromatin H3K27me3 and H3K4me3, which are often associated with histone deacetylation. SWN, a CLF relative, also may play a role in *FLC* repression because low levels of trimethyl H3K27 in *FLC* derepression. SWN, a CLF relative, may also play a role in *FLC* repression because low levels of trimethyl H3K27 in *FLC* derepression. SWN, a CLF relative, may also play a role in *FLC* derepression [51]. In addition, our studies show that removing CLF and FIE function leads to the synergistic *FLC* derepression, indicating that the CLF-PRC2 complex-mediated H3K27me3 acts in partial redundancy with the *FCA* and *FLD*-mediated chromatin repression in *FLC* suppression in the absence of vernalization. In addition, our studies also suggest that the CLF-dependent H3K27 trimethylation may antagonize H3K4 trimethylation in *FLC* chromatin, indicating that H3K27 trimethylation may antagonize H3K4 derepression. Furthermore, the *Drosophila* PRC2 complex has been shown to be associated with histone deacetylases, suggesting that histone deacetylation is also linked to the PRC2-mediated gene repression [69]. Interestingly, recent studies have shown that FVE can directly interact with a plant retinoblastoma protein (see the discussion below) [5], and future biochemical experiments are required to assess whether FVE is part of a CLF-PRC2 complex.

**A CLF-PRC2 Complex May Act in Concert with the Autonomous-Pathway Repressors to Repress *FLC* Expression in the Absence of Vernalization**

The autonomous pathway includes six classic loci such as *FLC*, *FLD* and *FVE*, and these genes do not form a linear pathway [68]. This pathway is so named because mutations in these genes lead to late flowering in all photoperiods due to the elevated *FLC* expression (reviewed in [2]). FLD, a plant homolog of the human Lysine-Specific Demethylase 1 that has been found in histone deacetylase co-repressor complexes, is involved in the H3K4 demethylation (a mechanism associated with gene repression) and deacetylation of *FLC* chromatin [8,17]. In addition, recent studies have shown that FCA functions closely with *FLD*, and that like *FLD*, it is involved in H3K4 demethylation of *FLC* chromatin [18]. In this study, we have found that removing CLF and FIE function leads to the synergistic *FLC* derepression, indicating that the CLF-PRC2 complex-mediated H3K27me3 acts in partial redundancy with the *FCA* and *FLD*-mediated chromatin repression in *FLC* suppression in the absence of vernalization. In addition, our studies also suggest that the CLF-dependent H3K27 trimethylation may antagonize H3K4 trimethylation in *FLC* chromatin, indicating that H3K27 trimethylation may antagonize H3K4 derepression. Furthermore, the *Drosophila* PRC2 complex has been shown to be associated with histone deacetylases, suggesting that histone deacetylation is also linked to the PRC2-mediated gene repression [69]. Interestingly, recent studies have shown that FVE can directly interact with a plant retinoblastoma protein of which the human homolog has been found to be associated with a histone deacetylase complex [70], and that FVE is indeed involved in the deacetylation of *FLC* chromatin [5]. Taken together, it is likely that a CLF-PRC2 complex may act in concert with the autonomous-pathway repressors such as *FLA* and *FLD*, and histone deacetylases to generate a repressive chromatin environment through histone deacetylation, H3K4 demethylation and H3K27 trimethylation, and thus represses *FLC* expression.

**Recruitment of PRC2 Subunits to the Target Loci**

*FLC*, *FLM* and *MAF2-5* are close relatives and have similar genomic structures [3,13]. Particularly, *MAF2*, *MAF3*, *MAF4* and *MAF5* are arrayed in a gene cluster (a tandem array) located at the bottom of Chromosome 5 [13]; however, CLF represses only *MAF4* and *MAF5*, but not *MAF2* or *MAF3* in this gene cluster. The CLF-dependent H3K27me3 occurs in *MAF4* and *MAF5*, but is absent from *MAF3* and *MAF6* (the gene immediately downstream *MAF5*), suggesting that the H3K27 trimethylation...
in MAF4 and MAF5 is not the result of spreading from the neighboring genes. Furthermore, CLF specifically binds to MAF4 and MAF5, but not to MAF3 or MAF6[39,40]. This suggests that CLF is specifically recruited to the MAF4 and MAF5 loci, indicating that there are cis-regulatory DNA elements in these two genes that may function similarly to Polycomb-group response elements in Drosophila [40] to recruit a PRC2-like complex.

PRC2 Subunits-Mediated FT Repression
PRC2 subunits CLF, EMF2 and FIE all strongly repress FT expression during vegetative development, suggesting that a PRC2-like complex containing CLF, EMF2 and FIE represses FT expression. To date, all known PRC2 complexes in animals and plants contain four core components including p55 or a p55 homolog (reviewed in [40,41]); however, the p55 homolog directly involved in FT repression still remains elusive. FVE, a p55 homolog and an FLC repressor, is not directly involved in FT repression because FT is strongly repressed in fve due to the elevated FLC expression [67], indicating that the PRC2-like complex repressing FT expression might be different from the one involved in FLC repression. Consistent with this notion, we have found that H3K27 trimethylation in FT chromatin is nearly eliminated in elf, whereas low levels of trimethyl H3K27 in FLC chromatin have been detected in elf, indicating that CLF relatives such as SWN may partially substitute for CLF in the deposition of H3K27me3 in the FLC locus, but not in the FT locus.

Our studies suggest that the putative CLF-PRC2 complex directly deposits repressive H3K27me3 in FT chromatin to repress FT expression. FT chromatin can be simultaneously marked with active H3K4me3 and repressive H3K27me3; the CLF-dependent H3K27 trimethylation suppresses, but does not eliminate H3K4 trimethylation in FT chromatin (Figure 7A and 7C), consistent with that FT is repressed but not fully silenced by PRC2 subunits in vegetative development. Recent studies suggest that LHP1 specifically recognizes and binds to H3K27me3 deposited by PRC2-like complexes to maintain stable transcriptional gene repression [37,38]. LHP1 has been shown to directly bind to the FT locus and loss of LHP1 activities leads to FT derepression and early flowering [36,37]. Hence, the CLF-dependent H3K27me3 in FT chromatin may be ‘read’ by LHP1 resulting in stable FT repression during vegetative development.

Possible Role of the CLF-PRC2 Complex-Mediated FT Repression in the Regulation of FT by Photoperiod
The PRC2-mediated transcriptional gene repressing mechanisms are conserved in animals and plants (reviewed in [40,41]). Our studies suggest that during vegetative development, Arabidopsis exploits these evolutionarily conserved ancient gene-repressing mechanisms to control FT expression; specifically, a CLF-PRC2 complex is utilized to repress, but not to fully silence FT expression in vegetative development. In the absence of PRC2 subunits, FT is highly activated; for instance, levels of FT transcripts in elf seedlings are about 200 fold of those in the wild type. It has been shown that in the wild type FT is expressed in vasculature such as veins of leaves where day length is perceived (reviewed in [1]). Previous studies show that loss of CLF activities leads to a strong derepression of AG throughout the leaf including veins and mesophyll cells [47]; hence, loss of CLF-PRC2-complex activities may well lead to FT derepression throughout the leaf including veins. Overexpressing FT via a strong constitutive viral promoter (35S) has been shown to give rise to extremely early flowering independent of the photoperiods [29,30]. Thus, it is critical for plants to keep FT to be expressed at low levels for preventing precocious flowering and for the regulation of FT by the photoperiods. PRC2 subunits, likely functioning in the context of a CLF-PRC2 complex, maintain FT expression at basal lower levels in vegetative development, which may serve to provide some room for the elevated FT expression in response to photoperiods and thus enable the photoperiodic control of flowering time in plants.

Materials and Methods

Plant materials and growth conditions
Arabidopsis thaliana clf-81 [48], fve-9 [7], fve-4 [4], emf2-1 [52,53] and FIE-suppressed plants derived from a homozygous transgenic line [55] were described previously. Plants were grown under cool white fluorescent light in long days (16 h light /8 h night) at about 22° C.

RNA isolation, reverse transcription and quantitative PCR assays
Total RNAs from aerial parts of 7 to 10 day-old seedlings grown in long days were extracted as described previously [17]. cDNAs were reverse-transcribed from total RNAs with Moloney murine leukemia virus reverse transcriptase (Promega).

Real-time quantitative PCR was performed on an ABI Prism 7900HT sequence detection system using SYBR Green PCR master mix (Applied Biosystems) as described previously [17]. Each sample was quantified at least in triplicate and normalized using TUB2 ([At5g62690] as the endogenous control. Primers used are specified in Table S1.

Histone extraction and immunoblotting
Histone protein extraction and Western analysis were performed as described previously [18,71]. Briefly, total histones were extracted from about 10-day-old seedlings, separated in an SDS-PAGE gel, and subsequently were transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad). The protein blots were first probed with anti-trimethyl H3K27, anti-dimethyl H3K27 (Millipore) and anti-trimethyl H3K4 (Abcam), and followed by anti-H3 (Millipore). The chemiluminescent SuperSignal West Pico system (Pierce) was used to develop the protein blots according to the manufacturer’s instructions.

Chromatin immunoprecipitation (ChIP)
The ChIP experiments were performed as described previously [65] using seedlings. Rabbit polyclonal anti-trimethyl-histone H3 (Lys 4) (Abcam), anti-trimethyl-histone H3 (Lys 27) (Upstate) and anti-GFP (Invitrogen) were used in immunoprecipitation experiments. Primers used to amplify FLC, MAF4, MAF5 and FT were performed using SYBR Green PCR master mix (Applied Biosystems). Primers used to amplify FLC-P1, FLC-P2, ACTIN 2, TUB2 and TUB8 were described previously [17,72], and other primers used are specified in Table S1. Each of the immunoprecipitations was repeated independently once, and each sample was quantified in triplicate.

Sequential ChIP analysis
The sequential ChIP experiments were performed as previously described [73] with modifications. Briefly, chromatin from Ws
seedlings was immunopurified with anti-trimethyl H3K4, subsequently eluted in a solution of 500 mM NaCl, 30 mM DTI and 0.1% SDS at 37°C, and was further diluted in a lysis buffer [65] supplemented with 1× Roche protease inhibitor cocktails (EDTA). The eluted chromatin was subsequently immunopurified with anti-trimethyl H3K27; DNA fragments were recovered and purified for PCR analysis.

Supporting Information

Figure S1  FVE represses MAF4 and MAF5 expression. Total RNAs were extracted from Col, fie and fca seedlings grown in long days. MAF4 and MAF3 were de-repressed in fie, but not in fca.

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Table S1

Table S1: Table 1

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Author Contributions

Conceived and designed the experiments: DJ YH. Wrote the paper: DJ YH. .
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