Vernalization-Mediated Epigenetic Silencing by a Long Intronic Noncoding RNA

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Vernalization is an environmentally-induced epigenetic switch in which winter cold triggers epigenetic silencing of floral repressors and thus provides competence to flower in spring. In *Arabidopsis*, winter cold triggers enrichment of tri-methylated histone H3 Lys^{27} at chromatin of the floral repressor, *FLOWERING LOCUS C* (*FLC*), and results in epigenetically stable repression of *FLC*. This epigenetic change is mediated by an evolutionarily conserved repressive complex, polycomb repressive complex 2 (PRC2). Here, we show that a long intronic noncoding RNA [termed COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR)] is required for the vernalization-mediated epigenetic repression of *FLC*. COLDAIR physically associates with a component of PRC2 and targets PRC2 to *FLC*. Our results show that COLDAIR is required for establishing stable repressive chromatin at *FLC* through its interaction with PRC2.

Developmental fates of cells are determined by innate genetic programs and interactions with their environments. A major environmental cue that plants sense to monitor seasonal change is winter cold, as exemplified by vernalization (*1*). Vernalization is the process by which certain plants acquire competence to flower rapidly in spring by sensing prolonged exposure to winter cold (*1*). In *Arabidopsis*, the stability of the vernalized state results from the stable repression of a potent floral repressor, *FLOWERING LOCUS C* (*FLC*) (*2*). A prolonged exposure to cold induces a plant homeo domain (PHD) finger–containing protein, VERNALI-ZATION INSENSITIVE 3 (VIN3) (*3*), and VIN3 becomes associated with an evolutionarily conserved repressive complex, polycomb repressive complex 2 (PRC2) (*4*, *5*). PRC2 mediates histone H3 Lys^{27} trimethylation (H3K27me3) through its core component, a histone methyltransferase, Enhancer of Zeste [E(z)] (*2*, *6*). During and after vernalization, PRC2 occupancy at *FLC* increases and correlates with an increased level of H3K27me3 at *FLC* chromatin, which is required for the stable maintenance of *FLC* repression (*2*, *5*, *7*, *8*). In *Arabidopsis*, there are three homologs of E(z). These include CURLY LEAF (CLF), SWINGER (SWN), and MEDEA, which are involved in several developmental programs in *Arabidopsis* (*6*). However, the molecular determinants for the increased recruitment of PRC2 and subsequent establishment of H3K27me3-enriched repressive chromatin at *FLC* by vernalization are not known. We identified a long intronic, noncoding transcript that plays role in the vernalization-mediated epigenetic silencing of *FLC* through the recruitment of PRC2 to *FLC* locus.

Several long noncoding RNAs (ncRNAs) have been shown to target repressive histone-modifying activities and epigenetically silence transcription through a molecular interaction with specific chromatin domains (*9*, *10*). Notably, PRC2-mediated silencing includes the interaction with such ncRNAs (*11*, *12*). A group of related antisense ncRNAs (termed COOLAIR) from *FLC* have been proposed to be involved in vernalization-mediated *FLC* repression (*13*). However, the importance of COOLAIR in the vernalization process has yet to be demonstrated. We independently hypothesized that long ncRNAs may play a role in vernalization-mediated repression of *FLC*, and we identified a candidate ncRNA from *FLC* that is distinct from COOLAIR and that we have designated COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR) (Fig. 1A and fig S1A). We found COLDAIR by using a "tiling" reverse transcription polymerase chain reaction (RT-PCR) approach with more than 100 pairs of oligonucleotide primers to cover the entire *FLC* genomic region during the course of vernalization (Fig. 1A). From this tiling RT-PCR approach, we observed a contiguous batch of RT-PCR products from the first intron of *FLC* that transiently appear during the cold exposure (Fig. 1, A and B).

Unlike COOLAIR, COLDAIR is in the sense direction relative to *FLC* mRNA transcription (Fig. 1A and fig. S1B). COLDAIR contains a 5′ cap structure (fig. S1C), but we did not observe significant levels of COLDAIR in a polyadenylate[poly(A)]-rich fraction of RNA (fig.S1D), suggesting that COLDAIR is not likely to be polyadenylated. Thus, the 3′ ends of COLDAIR were determined by a tiling 5′ rapid amplification of cDNA ends RT-PCR approach (fig S1F). The approximate size of COLDAIR is 1100 bases long (fig. S1, A, F, and G).

The increased level of COLDAIR was observed only during cold exposure with the maximum expression at 20 days of cold, and COLDAIR levels returned to the prevernalized level after more than 30 days of cold (Fig. 1B and fig S2). This transient induction of COLDAIR during cold exposure is similar to that observed for COOLAIR (*13*); however, the induction of

COOLAIR peaked at 10 days of cold exposure, earlier than the COLDAIR peak (Fig. 1B).

Deletion of the vernalization response element (VRE) in the first intron of *FLC* impairs vernalization-mediated *FLC* repression without compromising the floral repressor function of *FLC* (*14*). The transcription start site of COLDAIR is within the VRE region (Fig. 1A). To test whether the 5′ region of the VRE (excluding the COLDAIR transcribed region) is sufficient to mediate cold-inducible transcription, we generated transgenic lines in which luciferase is driven by 109 base pairs (bp) of the VRE 5′ region (Fig. 1C). We observed strong luciferase expression after 20 days of cold treatment in multiple transgenic lines (Fig. 1, C and D), confirming the presence of a cryptic promoter in VRE. In addition, luciferase expression remained robust as long as plants were kept in cold (Fig. 1, C and D). Endogenous COLDAIR was induced by ~10 to 20 days of cold (Fig. 1, B and D). However, after 20 days, *FLC* becomes repressed through chromatin changes, and thus COLDAIR promoter regions would become inaccessible to the transcription machinery. Consistent with this interpretation, COLDAIR was expressed well beyond 20 days of cold in *vin3-like 1* (*vil1*)/*vernalization 5* (*vrn5*) and *vernalization 1* (*vrn1*) mutants in which *FLC* repression by vernalization is impaired (fig. S3).

A group of long ncRNAs is transcribed by RNA polymerase V (RNAPV) together with RNA polymerase IV (RNAPIV) to mediate the silencing of constitutively silenced loci in *Arabidopsis* (*15*, *16*). This class of ncRNAs also has a 5′ cap but lacks a 3′ poly(A) tail similar to COLDAIR. However, neither RNAPIV nor RNAPV appears to be involved in the transcription of COLDAIR (fig. S4). Instead, we observed that, although the enrichment of RNA polymerase II (RNAPII) diminished at the *FLC* promoter as plants were exposed to cold, the enrichment of RNAPII at the COLDAIR promoter region transiently increased when expression of COLDAIR peaked at 20 days of cold (Fig. 1E). Thus, it is likely that RNAPII is responsible for the transcription of COLDAIR.

The PRC2 complex, including E(z), interacts with ncRNAs in vitro and in vivo (*11*, *17*). We tested for direct interactions between COLDAIR and the protein components of PRC2 {CURLY LEAF (CLF) and SWINGER (SWN) [E(z) homologs] and VERNALIZATION 2 (VRN2) [Su(z)12 homolog]} recombinant proteins by using in vitro RNA binding assays (Fig. 2, A and B, and fig. S5A). Both E(z) homologs, CLF and SWN, bound to COLDAIR in vitro through the CXC domain (Fig. 2, A and C). However, recombinant CLF protein also bound to antisense COLDAIR (fig. S5B), suggesting non–sequence-specific binding. The non–sequence-specific binding to single-stranded nucleotides (including RNA) by E(z) has been reported previously (*18*). To address the specificity of the interaction between COLDAIR and native CLF protein, we used nuclear extracts prepared from *GFP::CLF*-tagged transgenic lines (where GFP is green fluorescent protein)

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(8) to perform RNA binding assay. Unlike that observed for recombinant CLF proteins, we detected the CLF association only with the sense strand of COLDAIR (Fig. 2D), confirming the specific interaction of COLDAIR with native PRC2.

We also addressed the specific in vivo association of COLDAIR with PRC2 during the course of vernalization by RNA immunoprecipitation (RIP) assays using the *GFP::CLF*-tagged lines (Fig. 2E). Indeed, we retrieved COLDAIR from precipitates during the course of vernalization (Fig. 2E). We observed the strongest association of COLDAIR with CLF when COLDAIR expression was maximal at 20 days of cold. However, this is not due to the expression level of COLDAIR, because we normalized fold enrichments to reflect RNA populations in input material (19). Thus, there is an increased association of COLDAIR with PRC2 specifically during the cold exposure. We also examined the enrichment of COOLAIR from the RIP assays and did not observe any significant enrichment of COOLAIR (Fig. 2E). Thus, COLDAIR, but not COOLAIR, interacts with PRC2.

To address the biological function of COLDAIR, we used RNA interference (RNAi) to knock down expression of COLDAIR in the *GFP::CLF* line (Fig. 3). Many of the RNAi-expressing transgenic lines showed late flowering after vernalization (Fig. 3A), suggesting that COLDAIR knockdown compromises the vernalization response. We chose two representative COLDAIR knockdown lines for further analysis. COLDAIR knockdown lines have greatly reduced levels of COLDAIR during the course of vernalization compared with those of the parental line (fig. S6). Parental lines showed accelerated flowering, whereas COLDAIR knockdown lines exhibited late flowering after vernalization treatment (Fig. 3, A and B). The reduced vernalization responses observed in COLDAIR knockdown lines are consistent with levels of *FLC* expression during the course of vernalization (Fig. 3C).

Repression of *FLC* was still observed in COLDAIR knockdown lines during the cold exposure (Fig. 3C). However, after plants were returned to a warm growth temperature, *FLC* repression was not maintained (Fig. 3C), indicating that COLDAIR is required to establish a silenced state that is stable. The transient repression of *FLC* in the COLDAIR knockdown lines is similar to that observed in mutations in other components of PRC2 and in other genes that are required for the stable maintenance of repressed *FLC* by vernalization (4, 14, 20, 21).

After longer vernalization, COLDAIR knockdown lines showed substantially earlier flowering than nonvernalized plants (Fig. 3B), although the level of *FLC* was significantly higher (Fig. 3, C and D). Other *FLC*-related floral repressor genes, including *FLOWERING LOCUS M* (*FLM*)/*MADS AFFECTING FLOWERING* (*MAF1*), *MAF2*, and *MAF3*, are also repressed in response to vernalization (22). In COLDAIR knockdown lines, repression of *FLM/MAF1*, *MAF2*, and *MAF3* by vernalization still occured as in the parental lines (Fig. 3D), and thus it is likely that repression of these *FLC*-related genes causes the earlier flowering in COLDAIR knockdown lines after longer vernalization. This also indicates that COLDAIR knockdown specifically affects *FLC* expression but not expression of these *FLC*-related genes.

COLDAIR knockdown lines exhibited much later flowering than the parental line without vernalization (Fig. 3, A and B), consistent with higher levels of *FLC* expression in COLDAIR knockdown lines before vernalization treatment (Fig. 3, B and C). It has been reported that levels of *FLC* mRNA expression increase in *clf* mutants, consistent with the findings that *FLC* chromatin has a basal level of PRC2 occupancy before vernalization (8, 23). However, late flowering is not observed in *clf* mutants because the loss of *CLF* also causes the derepression of floral activators, such as *FLOWERING LOCUS T* (*FT*) and *AGAMOUS* (*AG*), resulting in early flowering (23). The delay in flowering observed in COLDAIR knockdown lines before vernalization further confirms that COLDAIR is necessary for the activity of PRC2 to be directed to *FLC* chromatin.

As noted above, there is a basal level of CLF occupancy at *FLC* before vernalization, and the level of occupancy increases after vernalization

Fig. 1. COLDAIR, an intronic long ncRNA of the *FLC*. (**A**) Schematic representation of transcription start sites for COLDAIR and COOLAIR and the location of VRE at the *FLC* genomic region. (**B**) Transcript expression patterns of COOLAIR, COLDAIR, VIN3, and FLC during the course of vernalization. Relative levels of mRNA expressions were calculated compared with those of the control, *PP2A*. Maximum expression for each gene is set as 100%, and relative levels are shown. Mean ± SD of quantitative RT-PCR data are shown (*n* = 3). NV, nonvernalized. V10, 10 days of vernalization treatment. V20, 20 days of vernalization treatment. V30, 30 days of vernalization treatment. (**C**) Luciferase expression in COLDAIR-promoter::*Luciferase* transgenic lines during the course of vernalization. (**D**) Expression patterns of luciferase (left) and COLDAIR (right) transcripts in two stable representative COLDAIR-promoter::*Luciferase* transgenic lines (1 and 2) and nontransgenic line (NT). Mean ± SD of quantitative RT-PCR data compared with the control, *PP2A*, are shown (*n* = 3). ND, not detectable. (**E**) Transient increase in RNAPII occupancy at the COLDAIR promoter region. Chromatin immunoprecipitation (ChIP) using RNAPII antibody (8WG16). Relative occupancies of RNAPII at *FLC* regions were calculated by comparison to occupancy of RNAPII at the *UBQ10* promoter region. Relative locations of P1 to P3 are shown in (A). Mean ± SD of quantitative ChIP-PCR data are shown (*n* = 3). V40, 40 days of vernalization treatment; V40T10, 40 days of vernalization treatment followed by 10 days of normal growth temperature.
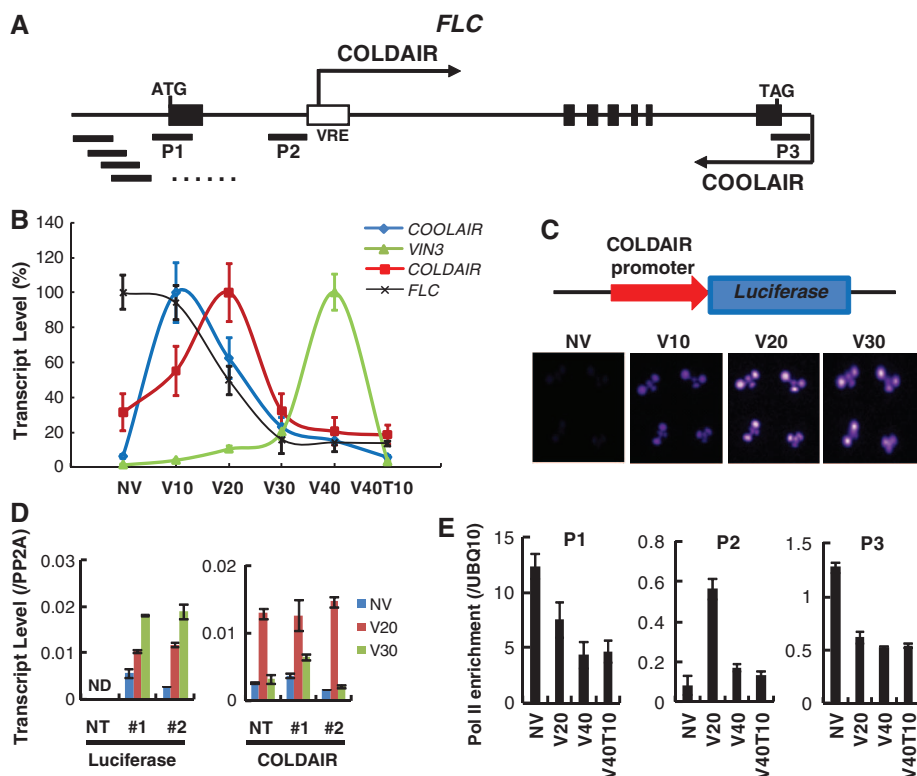
(Fig. 4, B) (5). In COLDAIR knockdown lines, the enrichment of CLF at *FLC* chromatin was greatly reduced (Fig. 4B). The relative enrichment of CLF at *FLC* chromatin was lower before vernalization, and the increased enrichment of CLF by vernalization was not observed in COLDAIR knockdown lines (Fig. 4B). This is consistent with elevated levels of *FLC* transcripts

in COLDAIR knockdown lines during the course of vernalization, showing that COLDAIR is necessary for the recruitment of CLF to *FLC* chromatin.

Consistent with the increased occupancy of CLF at *FLC* chromatin, the levels of H3K27me3 also increased at *FLC* chromatin as a result of vernalization (Fig. 4C) (7, 8, 24, 25). In COLD-

AIR knockdown lines, however, cold-mediated H3K27me3 enrichment was largely impaired (Fig. 4C). In addition, vernalization results in the stable reduction of the levels of histone H3 Lys[4] trimethylation (H3K4me3), a histone mark associated with active chromatin, at *FLC* chromatin (Fig. 4 D) (14). In COLDAIR knockdown lines, the levels of H3K4me3 at *FLC* chromatin
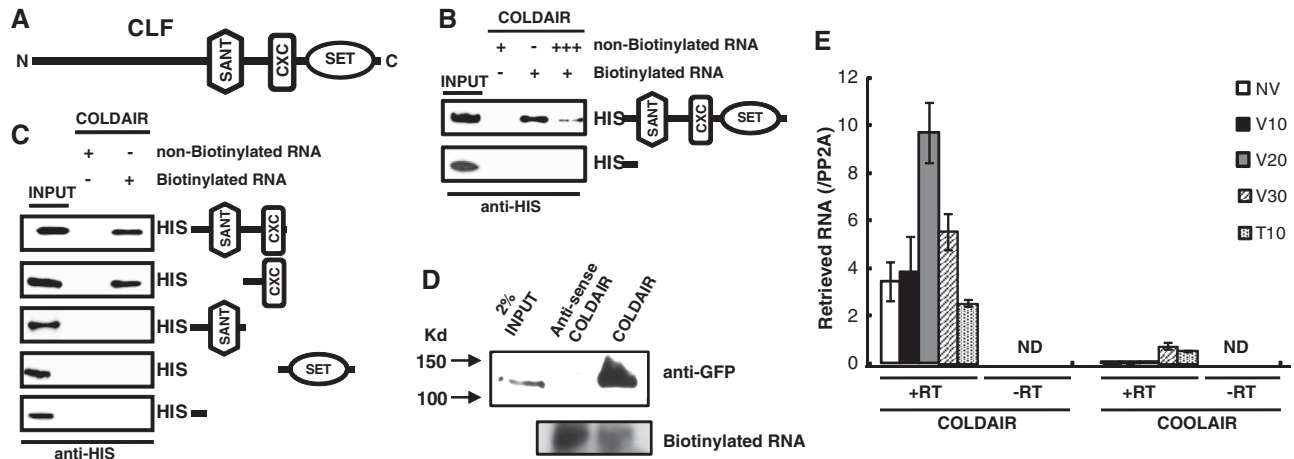

Fig. 2. COLDAIR associates with PRC2 during the cold exposure. (**A**) Domain structure of CLF protein. (**B**) In vitro transcribed (IVT) biotinylated COLDAIR RNA retrieves recombinant CLF protein. (**C**) In vitro RNA binding assays using domains of CLF in isolation. IVT biotinylated COLDAIR RNA retrieves CXC domain. (**D**) IVT COLDAIR RNA retrieves CLF-containing PRC2 from nuclear extracts. (**E**) CLF immunoprecipitation retrieves COLDAIR RNA but not COOLAIR RNA during the course of vernalization. Data (mean ± SD of quantitative PCR; *n* = 3) are relative to the background level of RNA precipitation (*PP2A*). +RT, with reverse transcription of precipitates. −RT, without reverse transcription of precipitates. T10, 30 days of vernalization treatment followed by 10 days of normal growth temperature.
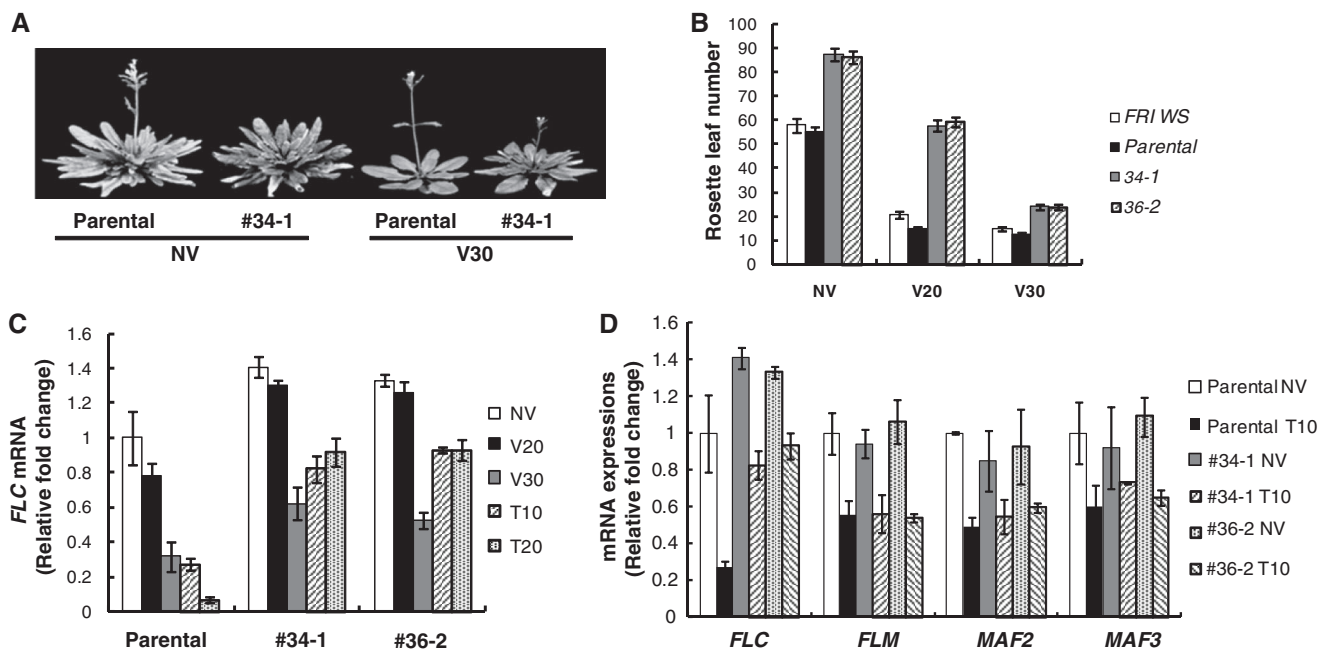

Fig. 3. COLDAIR is required for proper repression of *FLC* during the course of vernalization. (**A**) Flowering behaviors of the parental line and a representative COLDAIR knockdown line. (**B**) Flowering times are determined by the rosette leaf number at the timing of flowerings of *FRI* in Ws, the parental line, and two representative COLDAIR knockdown lines (34-1, 36-2). (**C**) Changes in *FLC* mRNA during the course of vernalization in the parental line and two representative COLDAIR knockdown lines. Data (fold change; mean ± SD of quantitative RT-PCR; *n* = 3) are relative to the *FLC* mRNA level in the parental line before vernalization. T20, 30 days of vernalization treatment followed by 20 days of normal growth temperature. (**D**) Changes in *FLC*, *FLM*, *MAF2*, and *MAF3* mRNA during the course of vernalization in the parental line and two representative COLDAIR knockdown lines. Data (fold change; mean ± SD of quantitative RT-PCR; *n* = 3) are relative to the *FLC*, *FLM*, *MAF2*, and *MAF3* mRNA levels in the parental line before vernalization.

transiently decreases when plants are kept in cold but return to the prevernalized levels once plants are returned to a warm temperature (Fig. 4D). The transient reduction in the level of H3K4me3 at *FLC* chromatin is also observed in vernalization mutants that are involved in the maintenance of the repression (*14*). Thus, the intronic ncRNA COLDAIR mainly plays a role in the recruitment of PRC2 to *FLC* chromatin to establish the stable silencing of *FLC* by vernalization (fig. S7).

Transiently increased interaction between COLDAIR and PRC2 during vernalizing cold suggests that COLDAIR mainly acts to recruit PRC2 to *FLC* during the cold (fig. S7). It remains to be determined whether COLDAIR participates in maintaining the PRC2 association with *FLC* after vernalization treatment. Alternatively, for-

mation of the PHD-PRC2 complex may contribute to the maintenance after the cold (fig. S7).

In animals, a long intergenic noncoding RNA, HOTAIR, is transcribed from *HOXC* locus and targets PRC2 to silence *HOXD* and other loci (*11*, *12*). We observed a similar interaction of a long ncRNA with PRC2 in plants, suggesting that the interaction of long ncRNA with PRC2 appears to be an evolutionarily conserved mechanism. Unlike HOTAIR, COLDAIR is transcribed from an intron of the target gene itself, *FLC*. *FLC* harbors a cryptic promoter for COLDAIR ncRNA within its first intron, and this promoter becomes active when *FLC* is being repressed. Deep RNA sequencing efforts have revealed transcripts that originated from intergenic regions as well as from previously known intronic regions in eu-

karyotes (*26–28*). Although some intronic transcripts represent alternative splicing forms (*28*), our identification of an intronic long ncRNA that plays a regulatory role in gene repression suggests that intronic transcripts can function as a part of targeting mechanisms for transcriptional regulatory machineries in eukaryotes.

References and Notes

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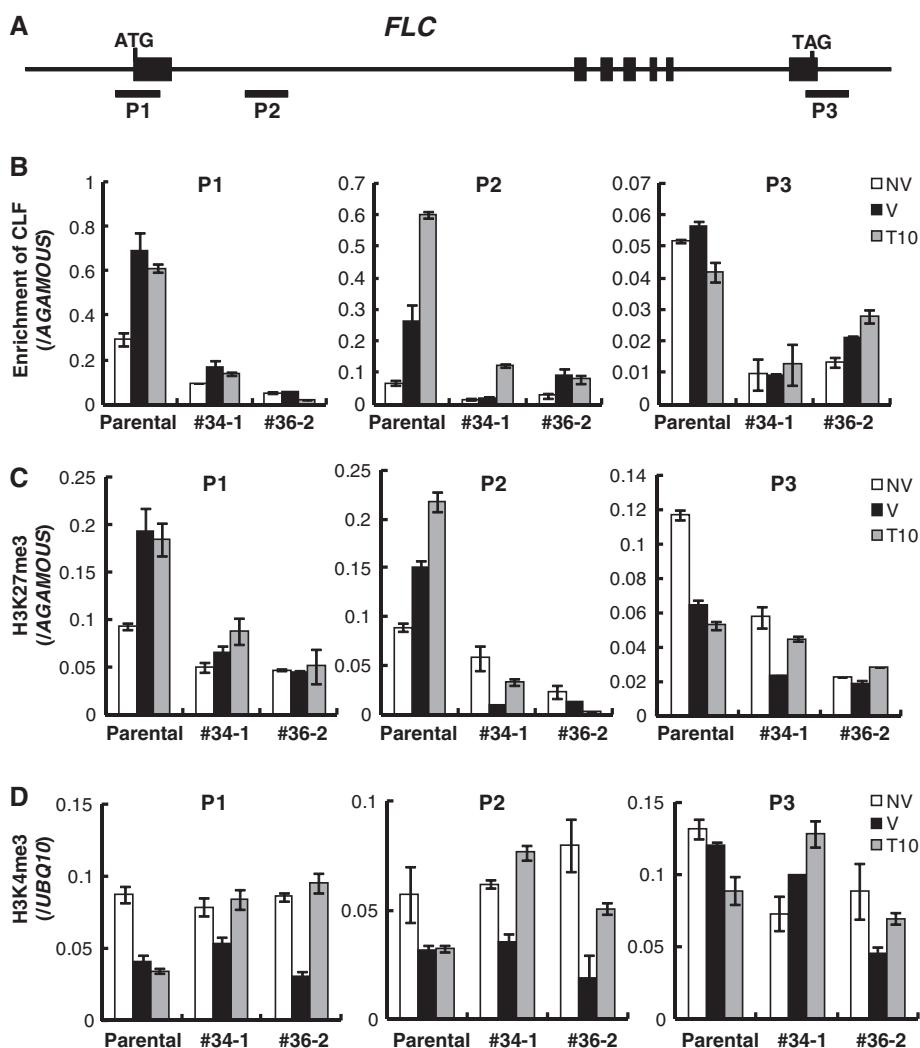
Fig. 4. Dynamic changes at *FLC* chromatin during the course of vernalization. (**A**) Schematic representation of the *FLC* genomic region. Relative positions of primer sets used for ChIP assays are shown (P1 to P3). (**B**) Changes in occupancy of CLF at *FLC* chromatin during the course of vernalization. (**C**) Changes in enrichment of H3K27me3 at *FLC* chromatin during the course of vernalization. (**D**) Changes in enrichment of H3K4me3 at *FLC* chromatin during the course of vernalization. Data (mean ± SD of quantitative ChIP-PCR; $n = 3$) is relative to the occupancy of CLF at *AGAMOUS* chromatin (B), the level of H3K27me3 enrichment at *AGAMOUS* chromatin (C), and the level of H3K4me3 enrichment at *UBQ10* chromatin (D).