Vernalization-Mediated *VIN3* Induction Overcomes the LIKE-HETEROCHROMATIN PROTEIN1/POLYCOMB REPRESSION COMPLEX2-Mediated Epigenetic Repression[1][C][W][OA]

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VERNALIZATION INSENSITIVE3 (*VIN3*) induction by vernalization is one of the earliest events in the vernalization response of Arabidopsis (*Arabidopsis thaliana*). However, the mechanism responsible for vernalization-mediated *VIN3* induction is poorly understood. Here, we show that the constitutive repression of *VIN3* in the absence of the cold is due to multiple repressive components, including a transposable element-derived sequence, LIKE-HETEROCHROMATIN PROTEIN1 and POLYCOMB REPRESSION COMPLEX2. Furthermore, the full extent of *VIN3* induction by vernalization requires activating complex components, including EARLY FLOWERING7 and EARLY FLOWERING IN SHORT DAYS. In addition, we observed dynamic changes in the histone modifications present at *VIN3* chromatin during the course of vernalization. Our results show that the induction of *VIN3* includes dynamic changes at the level of chromatin triggered by long-term cold exposure.

The transition from vegetative growth to reproductive growth is one of major developmental transitions in the life cycle of plants. Flowering plants have evolved to maximize the reproductive success by optimizing the timing of flowering. The onset of floral transition in flowering plants is affected by various environmental cues, including changing daylength and temperature. Plants use such environment cues to monitor seasonal changes and determine the timing of flowering. In temperate climates, the winter season imposes a prolonged period of cold to plants. In many plant species, exposure to prolonged period of cold provides competence to flower in the following spring through the process known as vernalization (for review, see Sung and Amasino, 2005; Dennis and Peacock, 2007).

While most lab strains of Arabidopsis (*Arabidopsis thaliana*) do not require vernalization treatment to flower rapidly, many naturally occurring accessions of Arabidopsis flower very late unless vernalized (Clarke and Dean, 1994; Lee and Amasino, 1995; Michaels and Amasino, 1999; Gazzani et al., 2003). In Arabidopsis, the vernalization requirement is conferred by two dominant genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*; Lee et al., 1993; Clarke and Dean, 1994; Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). *FLC* encodes a MADS box DNA binding protein that functions as a repressor of the floral integrators, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Michaels and Amasino, 1999; Sheldon et al., 1999; Lee et al., 2000; Samach et al., 2000; Hepworth et al., 2002; Helliwell et al., 2006; Searle et al., 2006). FLC antagonizes the effect of CONSTANS (CO) by directly binding to regulatory regions within *FT* and *SOC1*. It appears that *FRI* contributes to the vernalization requirement solely by activating *FLC*. *FRI* encodes a protein with unknown biochemical function (Johanson et al., 2000). Vernalization results in the stable repression of *FLC* (Michaels and Amasino, 1999; Bastow et al., 2004; Sung and Amasino, 2004) so that floral integrators can be activated when the photoperiod pathway activates CO (Parcy, 2005). Thus, vernalization renders plants to be competent to flower upon exposure to inductive photoperiods in winter annuals and biennials.

Other than *FRI*, another group of genes involved in *FLC* activation have been identified from screens for early flowering in certain genotypes or photoperiod conditions. They include *EARLY FLOWERING5* (*ELF5*), *ELF7*, *ELF8*, *VERNALIZATION INDEPENDENCE3* (*VIP3*), *VIP4*, *EARLY FLOWERING IN SHORT DAYS4* (*ESD4*), *PHOTOPERIOD INDEPENDENT EARLY1* (*PIE1*), *EARLY FLOWERING IN SHORT DAYS* (*EFS*),

and *ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1)/ATX2/ARABIDOPSIS TRITHORAX-RELATED7 (ATXR7;* Reeves et al., 2002; Zhang and van Nocker, 2002; Noh and Amasino, 2003; He et al., 2004; Noh et al., 2004; Oh et al., 2004; He and Amasino, 2005; Kim et al., 2005; Zhao et al., 2005; Choi et al., 2007; Saleh et al., 2008; Tamada et al., 2009). Some of these genes encode proteins with chromatin modification functions, including components of RNA Polymerase II-associated factor 1 (PAF1) complex (*VIP3, VIP4, ELF7,* and *ELF8*), a Histone H3 Lys-36 methyltransferase (*EFS*), a Histone H3 Lys-4 methyltransferase (ATX1, ATX2, and ATXR7), and a SWR1-related nucleosome remodeling factor (*PIE1*).

Mitotically stable repression of *FLC* by vernalization is also achieved by chromatin modifications (Michaels and Amasino, 1999; Bastow et al., 2004; Sung and Amasino, 2004). *FLC* mRNA expression is repressed during the course of cold exposure, and several repressive histone marks accumulate at *FLC* chromatin, including methylations at Histone H3 Lys-9 (H3K9) and Histone H3 Lys-27 (H3K27). The accumulation of histone modifications at *FLC* chromatin depends on the activity of chromatin remodeling complexes. During the course of cold exposure, POLYCOMB REPRESSION COMPLEX2 (PRC2), which has H3K27 methyltransferase activity, is enriched at *FLC* chromatin (Wood et al., 2006; De Lucia et al., 2008) and establishes the stable repression of *FLC* through H3K27 methylation. PRC2 biochemically copurifies with members of the VERNALIZATION INSENSITIVE3 (VIN3) family of proteins, including VIN3, VIN3-LIKE1 (VIL1)/ VERNALIZATION5 (VRN5), and VIL2/VERNALIZATION LIKE1 (VEL1; Wood et al., 2006; De Lucia et al., 2008).

The vernalization response involves two phases. The first is a cold perception that measures the cumulative time of exposure to cold. Vernalization requires cold exposure over the course of weeks rather than minutes or hours. The second phase is essentially the output of the cold perception. When a sufficient duration of cold has been perceived, a series of changes of gene expression ensue, ultimately leading to the epigenetic repression of *FLC*. VIN3, which is a repressive chromatin-remodeling component, is induced only after a sufficient duration of cold has been perceived. One of the early molecular events in the vernalization response is the induction of *VIN3* by prolonged cold exposure. Upstream of *VIN3*, there must be a biochemical mechanism to sense cold. However, nothing is known about the upstream event. The induction of *VIN3* by cold is unique in that *VIN3* induction takes several days of cold, unlike many cold-induced genes, which are induced within hours of cold exposure (Thomashow, 2001). Furthermore, *VIN3* mRNA expression is quickly rerepressed once plants are moved to warm temperature.

Interestingly, the induction of *VIN3* also involves changes in active histone marks at *VIN3* chromatin, including Histone H3 acetylation, Histone H4 acetyla-

tion, and Histone H3 Lys-4 trimethylation (H3K4me3; Finnegan et al., 2005; Bond et al., 2009). However, no chromatin remodeling complexes have been identified to have roles in those changes at *VIN3* chromatin.

Here, we show that *VIN3* is in a constitutively silenced state, which is mediated by the presence of a transposable element (TE)-derived sequence in its promoter region and by the components of repressive complexes, including PRC2 and LHP1. In addition, the full extent of *VIN3* induction by vernalization requires components of activating complexes, including PAF1 and EFS. Thus, *VIN3* expression is under the influence of chromatin level regulators. Furthermore, *VIN3* chromatin is in a transiently bivalent state when *VIN3* mRNA is induced, having both a repressive histone mark and an active histone mark at *VIN3* chromatin. Our results show that *VIN3* is under a constitutively repressed state, which is transiently relieved from repression only when sufficient cold is provided.

RESULTS

A Transposable Element Is Present in the *VIN3* Promoter Region, and H3K9 Dimethylation Is Enriched at *VIN3* Chromatin Prior to Vernalization

We surveyed possible regulatory sequences in 5' upstream regions of *VIN3*. Interestingly, we located a MuDR-type transposable element-derived sequence at -185 to -765 from the transcription start site (Fig. 1A). The presence of a transposable element-derived sequence at *VIN3* promoter region has been assigned as AT5TE83600 (www.arabidopsis.org). The sequence
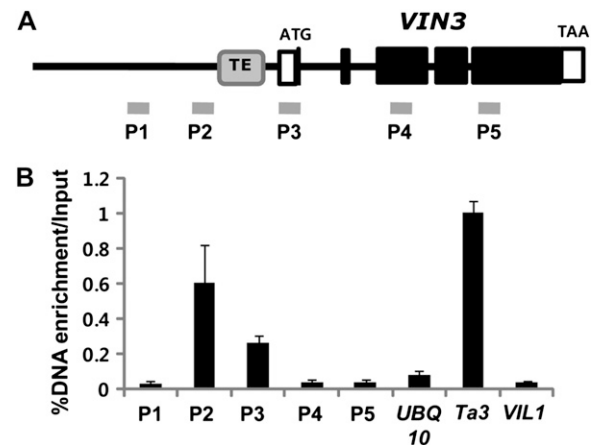

Figure 1. H3K9me2 enrichment at *VIN3* chromatin. A, Schematic diagram showing the gene structure of *VIN3*. The solid boxes indicate coding regions, and the open boxes indicate untranslated regions (UTRs). Gray box indicates the transposable element-derived sequence (TE). Solid bars (P1 to P5) indicate regions for primer pairs used for ChIP assays. B, ChIP-quantitative PCR to quantify the relative enrichment of H3K9me2. *UBQ10, Ta3,* and *VIL1* regions were used to compare the levels of H3K9me2 enrichments. Primer sequence information used for ChIP assay is shown in Supplemental Table S1.

showed 55% and 53% nucleotide sequence identities with transposable elements, AT3G29634 and AT5G28495, respectively (Supplemental Fig. S1). The presence of a transposable element-derived sequence raised the possibility that the *VIN3* upstream region contains a heterochromatin island by the insertion of a transposable element. It is not unprecedented that an insertion of a transposable element could create local heterochromatin. In fact, some allelic variants of *FLC* are due to insertions of transposable elements in its first intron (Michaels et al., 2003; Liu et al., 2004). In the case of *FLC*, two different classes of transposable elements create silenced *FLC* alleles due to extensive Histone H3 Lys 9 dimethylation (H3K9me2) that originated from the transposable element and spread throughout the entire *FLC* genomic region (Michaels et al., 2003; Liu et al., 2004). Similarly, we observed a localized enrichment of H3K9me2 around the transposable element into the *VIN3* genomic region in a nonvernalized condition (Fig. 1B). This suggests that *VIN3* chromatin is in a repressed state prior to the exposure to cold. Previous genomic studies to identify H3K9me2-enriched regions using genomic tiling arrays did not identify the *VIN3* promoter region as an H3K9me2-enriched region (Rehrauer et al., 2010; Zhou et al., 2010). It is possible that the level of enrichment at *VIN3* promoter is relatively lower than other regions and localized around the transposable element-derived sequence (Fig. 1B).

VIN3 Is Derepressed in *lhp1* and *clf;swn* Double Mutants

The presence of a transposon-derived sequence at *VIN3* promoter region prompted us to test a series of mutants that are involved in gene silencing for their effects on the *VIN3* expression. We tested a series of Arabidopsis mutants involved in (1) DNA methylation, *met1-7* and *drm1;drm2*; (2) RNA interference production, *rdr2-1*, *rdr6-14*, *dcl3-1*, *nrpd1a-4*, and *nrpd1b-11*; and (3) Histone methylation, *kyp-2* and *lhp1-4*. Among tested mutants, we observed a significantly elevated level of *VIN3* mRNA expression prior to the cold exposure in *lhp1* mutants (Fig. 2A).

VIN3 exists as a small gene family including *VIL1/VRN5*, *VIL2/VEL1*, and *VIL3/VEL2*. Some members of the gene family also show vernalization-mediated regulation at the transcriptional level (Sung et al., 2006b; Greb et al., 2007). A survey of promoter regions of other members of the family revealed no other members harbor a transposable element in their promoter regions, with an exception of *VIL3*, which has a Helitron-related transposable element 2 kb upstream of the transcription start site (Fig. 2B). Furthermore, only *VIN3* exhibits derepressed expression without the vernalization treatment in *lhp1* mutants (Fig. 2C). Taken together, the regulation by the insertion of a transposable element and by LHP1 is specific to *VIN3* among the gene family.

LHP1 shows a functional overlap with PRC2 in Arabidopsis (Hennig and Derkacheva, 2009).
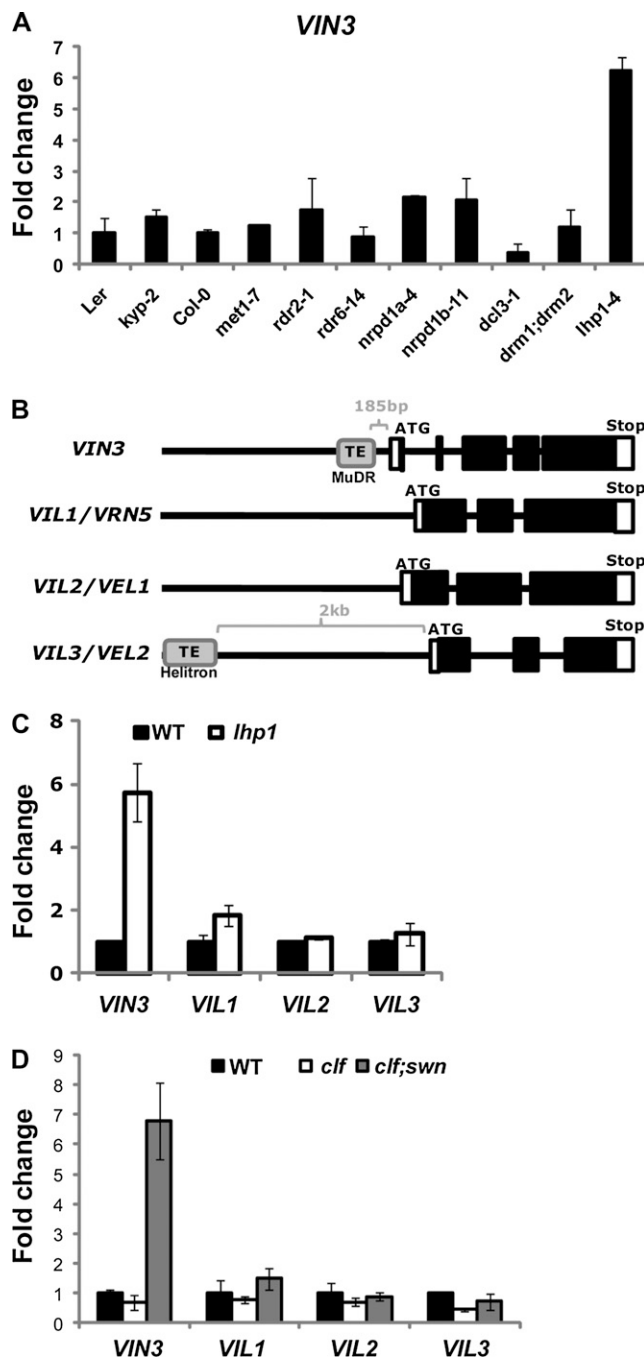

Figure 2. Involvement of LHP1 and PRC2 in the repression of *VIN3* gene in a nonvernalized condition. A, Real-time reverse transcription (RT)-PCR analysis on *VIN3* expression between wild types (Col-0 and L*er*) and a series of mutants. B, Schematic diagram showing the gene structures of *VIN3* family genes (*VIN3*, *VIL1/VRN5*, *VIL2/VEL1*, and *VIL3/VEL2*). The solid boxes indicate the coding regions, and the open boxes indicate UTRs. C, Real-time RT-PCR analysis on *VIN3* family genes between the wild type (WT; *FRI-Col*) and *lhp1-4* mutants in NV condition. D, Real-time RT-PCR analysis on *VIN3* expression in the wild type (Col), *clf-29*, and *clf-29/swn-2* mutants in NV condition. C and D, Relative fold change was determined by normalization with the levels of *PP2A* gene as previously reported (Czechowski et al., 2005). Primer sequence information used for real-time RT-PCR analysis is shown in Supplemental Table S1.

CURLY LEAF (CLF) and SWINGER (SWN) are two Arabidopsis homologs of E(z), which is a core component of PRC2. To address the functional overlap of PRC2 with LHP1 in the regulation of *VIN3*, we obtained *clf;swn* double mutants. Indeed, *clf;swn* double mutants do have an elevated level of *VIN3* mRNA before cold exposure similar to *lhp1* (Fig. 2D). *clf* single mutant alone does not cause the derepression of *VIN3*, suggesting the functional redundancy between *CLF* and *SWN*. Similar to the derepression of *VIN3* in *lhp1* mutants, mutations in *clf;swn* only affect *VIN3* and not other gene family members (Fig. 2D).

LHP1 and CLF Directly Associate with *VIN3* Chromatin But Do Not Dissociate from *VIN3* Chromatin When *VIN3* Is Induced by Vernalization

Because lesions in *LHP1* or *CLF/SWN* result in the derepression of *VIN3* in a nonvernalized condition, we address whether LHP1 and CLF/SWN directly regulate *VIN3* expression through physical association with *VIN3* chromatin using chromatin immunoprecipitation (ChIP) assays. Using GFP-tagged *LHP1* and GFP-tagged *CLF* transgenic lines, both of which fully rescue *lhp1* and *clf* mutants, respectively (Schubert et al., 2006; Sung et al., 2006a), we observed strong enrichments of LHP1 and CLF at *VIN3* chromatin

Figure 3. ChIP assay using *LHP1:GFP* and *GFP:CLF* transgenic plants. A, Schematic diagram showing the gene structure of *VIN3*. Solid boxes indicate the coding regions, and open boxes indicate UTR. Gray box indicates the transposable element-derived sequence (TE). Solid bars (P1 to P5) indicate regions for primer pairs used for ChIP assays. B, ChIP assay using *LHP1:GFP* transgenic lines. C, ChIP assay using *GFP:CLF* transgenic line. *AGAMOUS* (*AG*) and *UBQ10* were used as controls. As an input control, the preimmunoprecipitated DNA after sonication was used (B and C). D, ChIP assay using *LHP1:GFP* transgenic lines followed by real-time quantitative PCR. E, ChIP assay using *GFP:CLF* transgenic line followed by real-time quantitative PCR. For quantifications, the relative enrichments compared to input were calculated, and relative enrichments are shown compared to those of the control, *AG* (D and E). IP, Immunoprecipitation; NV, nonvernalized; 40V and 40VT0, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature; WT, wild type.
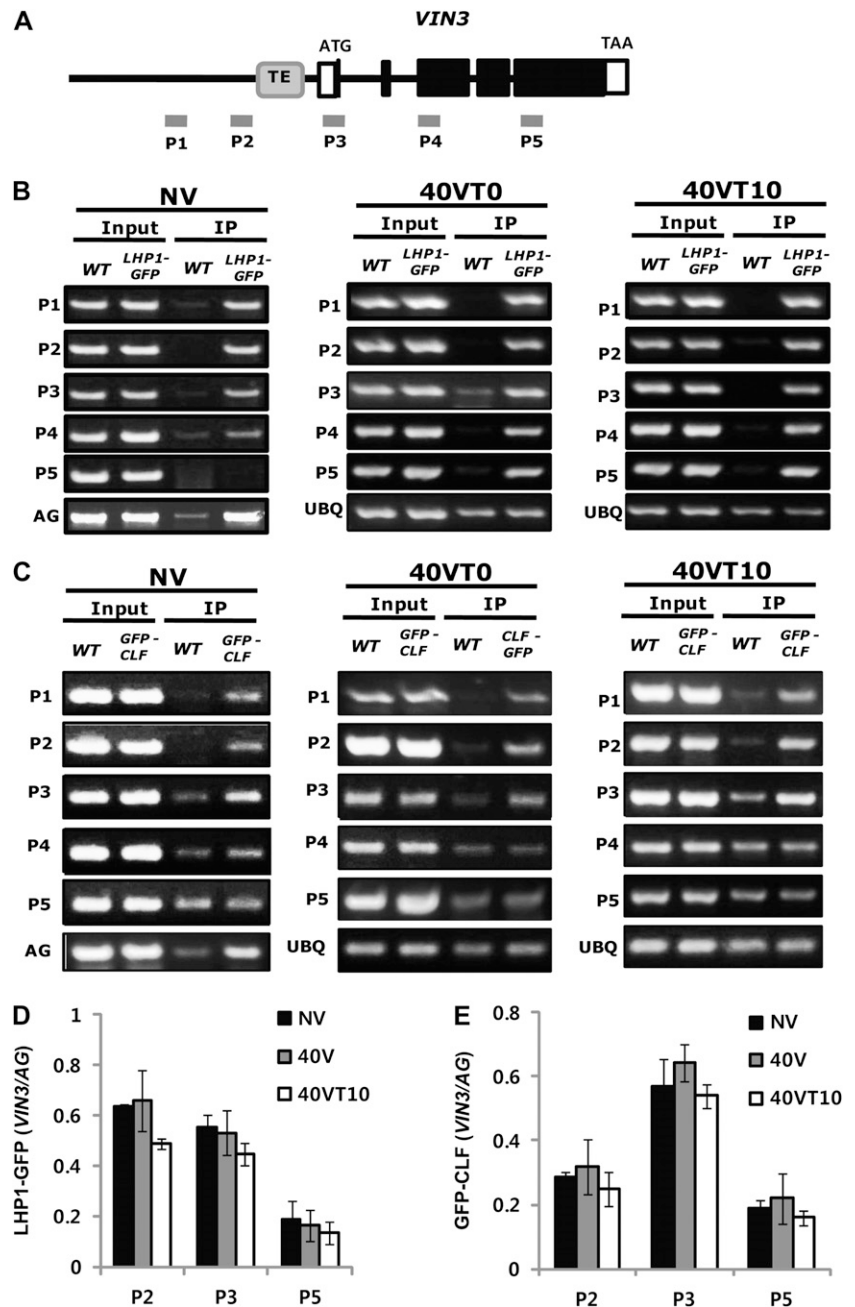
by ChIP (Fig. 3, B and C). The associations of LHP1 and CLF with *VIN3* chromatin appear to be robust throughout the course of vernalization (Fig. 3, B and C). To confirm constitutive associations of LHP1 and CLF with *VIN3* chromatin, we compared the relative enrichments of LHP1 and CLF at *VIN3* chromatin during the course of vernalization using ChIP followed by real-time quantitative PCR (Fig. 3, D and E). We did not detect significant difference in the enrichments of LHP1 and CLF at *VIN3* chromatin even when *VIN3* is highly expressed during the cold exposure (Fig. 3, D and E). This shows that LHP1 and CLF are constitutively present at *VIN3* chromatin regardless of the expression state of *VIN3*.

VIN3 Is Induced Faster by Cold in *lhp1* Mutants

Constitutive association of LHP1 with *VIN3* chromatin during the course of vernalization suggests that cold-induced expression of *VIN3* must overcomes the repressive activity of LHP1. Although *VIN3* is derepressed in *lhp1* mutants prior to the cold exposure, the vernalization treatment still significantly up-regulates *VIN3* to levels comparable to those of the wild type

(Fig. 4A). In addition, derepressed levels of *VIN3* in *lhp1* or *clf;swn* double mutants do not fully recapitulate the levels of vernalization-induced *VIN3* expression (Figs. 2, C and D, and 4A). Thus, the level of *VIN3* induction by vernalization cannot be achieved solely by the removal of LHP1 or CLF/SWN.

To address the contribution of *LHP1* in the *VIN3* induction kinetics, we examined the level of *VIN3* mRNA expression during the course of vernalization, including shorter periods of cold treatment in *lhp1* mutants (Fig. 4B). Interestingly, *VIN3* is induced by shorter period of cold exposure in the absence of *LHP1* (Fig. 4B). With 5 to 10 d of cold exposure, *VIN3* is significantly induced in *lhp1* mutants, while no significant induction of *VIN3* is observed in the wild type (Fig. 4B). This suggests that the slow kinetics of *VIN3* induction in the wild type is at least in part due to the repression by LHP1.

Histone Modification Landscapes Change at *VIN3* Chromatin during the Course of Vernalization

The presence of a transposable element at the promoter region of *VIN3* and the involvement of LHP1 and CLF/SWN in the repression of *VIN3* led us to examine chromatin modifications at *VIN3* chromatin during the course of vernalization (Fig. 5). Histone H3 Lys-27 trimethylation (H3K27me3) is often correlated with gene repression and is mediated by the PRC2 complex. Interestingly, we did not observe any significant change in the level of H3K27me3 at *VIN3* chromatin during the course of vernalization (Fig. 5B). This result is consistent with the constitutive association of CLF, which is an H3K27 methyltransferase, with *VIN3* chromatin. On the other hand, an active mark, H3K4me3, is enriched when *VIN3* is highly expressed during cold exposure (Fig. 5C). Consistent with the active transcription of *VIN3*, RNA polymerase II (Pol II) enrichment at *VIN3* chromatin increases during the cold exposure (Fig. 5D). Thus, during the cold exposure, *VIN3* chromatin contains both a repressive mark and an active mark, creating a bivalent state. We observed that H3K9me2 is enriched around *VIN3* coding regions that originated from a transposable element prior to the cold exposure (Fig. 1B). To evaluate the dynamics of H3K9me2 enrichment at *VIN3* by vernalization, we examined the relative enrichments of H3K9me2 during the course of vernalization. Interestingly, the level of enrichment of H3K9me2 is transiently reduced when *VIN3* is highly expressed during cold exposure (Fig. 5E), suggesting that the induction of *VIN3* by vernalization overcomes the effect of a transposable element at its promoter region.

Activating Components Are Involved in the Induction of *VIN3* by Vernalization

It is interesting to note that common repressive components (i.e. LHP1 and CLF/SWN) are involved in the repressions of both *VIN3* and its target, *FLC*.
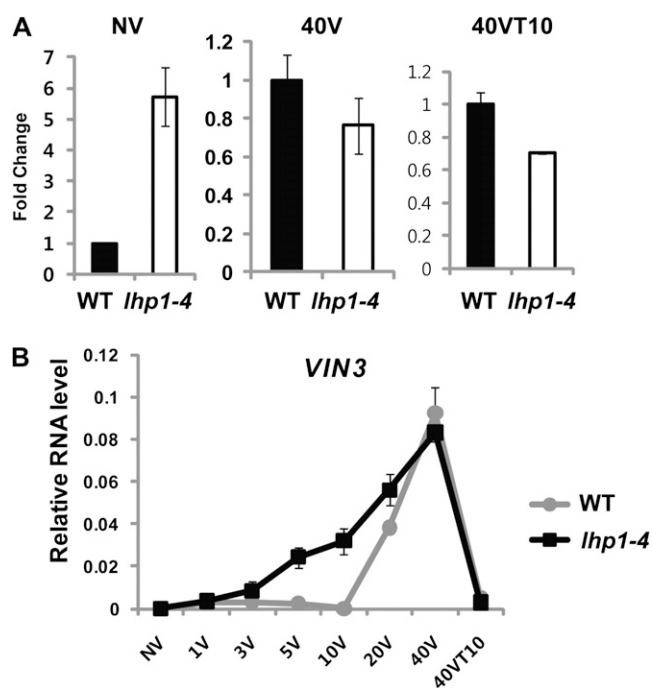

Figure 4. Real-time RT-PCR analysis on *VIN3* expression between the wild type (*FRI*-Col) and *lhp1-4* (in *FRI*-Col) mutants during the course of vernalization. A, Nonvernalized (NV); 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. WT, Wild type. B, Shorter periods of cold exposure. 1V, 1 d of cold; 3V, 3 d of cold; 5V, 5 d of cold; 10V, 10 d of cold; 20V, 20 d of cold. Relative fold change was determined by normalization with the levels of *PP2A* gene as previously reported (Czechowski et al., 2005). Primer sequence information used for real-time RT-PCR analysis is shown in Supplemental Table S1.
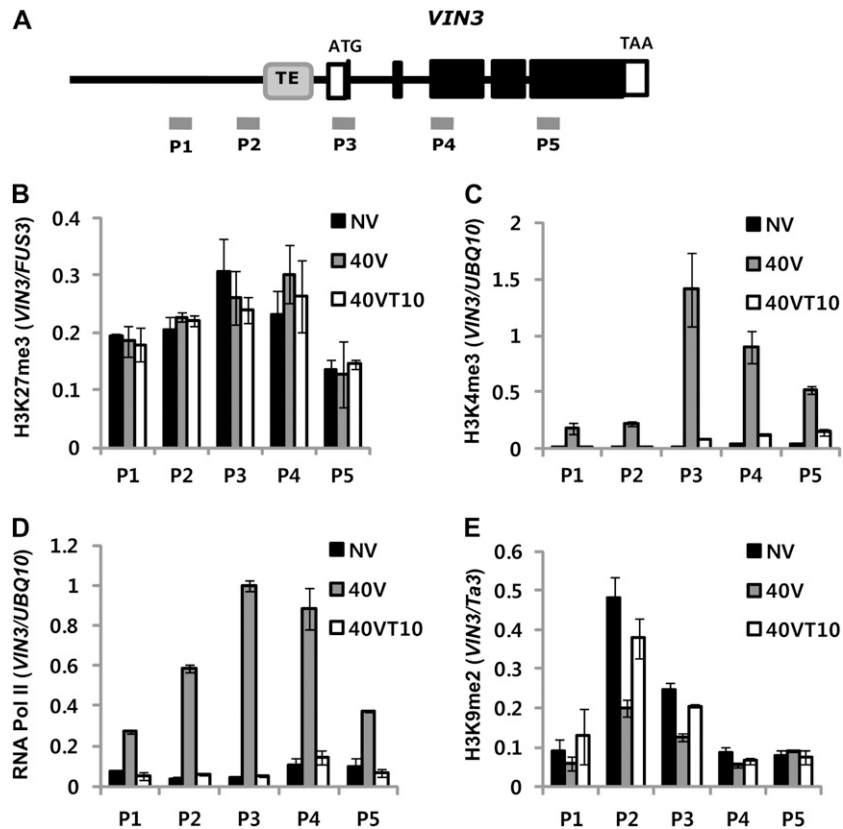
Figure 5. ChIP assays using anti-H3K27me3, H3K9me2, Pol II, and H3K4me3 antibody during the course of vernalization. A, Schematic diagram showing the gene structure of *VIN3*. Solid boxes indicate the coding regions, and open boxes indicate UTRs. Gray box indicates the transposable element-derived sequence (TE). Solid bars (P1 to P5) indicate regions for primer pairs used for ChIP assays. B, ChIP assays followed by real-time quantitative PCR using the anti-H3K27me3 antibody during the course of vernalization. For quantifications, the relative enrichments compared to input were calculated, and relative enrichments are shown compared to those of the control, *FUS3*. A *FUS3* region was used as a positive control as previously reported (Makarevich et al., 2006). NV, Nonvernalized. C, ChIP assay followed by real-time quantitative PCR using the anti-H3K4me3 antibody during the course of vernalization. For quantifications, the relative enrichments compared to input were calculated, and relative enrichments are shown compared to those of the control, *UBQ10*. D, ChIP assay followed by real-time quantitative PCR using the anti-Pol II antibody (8WG16) during the course of vernalization. The Pol II antibody (8WG16) detects both phosphorylated and nonphosphorylated forms of RNA Pol II. For quantifications, the relative enrichments compared to input were calculated, and relative enrichments are shown compared to those of the control, *UBQ10*. E, ChIP assay followed by real-time quantitative PCR using the anti-H3K9me2 antibody during the course of vernalization. For quantifications, the relative enrichments compared to input were calculated, and relative enrichments are shown compared to those of the control, *Ta3*. Primer sequence information used for ChIP assay is shown in Supplemental Table S1. NV, Nonvernalized; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature.

Furthermore, the contributions of PRC2 components, CLF and SWN, to the repressed state of *VIN3* suggest that other *FLC* regulators may also be involved in the regulation of *VIN3*. Particularly, increased enrichment of H3K4me3 at *VIN3* chromatin during cold exposure indicates that H3K4me3-mediated activating complexes are also involved in the regulation of *VIN3*. To test the involvement of such activating components, we examined the *VIN3* expression pattern in *elf7* mutants. *ELF7* encodes a homolog of PAF1, which commonly associates with Trithorax-mediated gene activations. Interestingly, the induced levels of *VIN3* by vernalization are significantly lower in *elf7* mutants than in the wild type (Fig. 6A). Similar reduction in the induced

level of *VIN3* is observed in *efs* mutants (Fig. 6B), another mutation in activating components for *FLC*. However, it should be noted that *VIN3* is still induced by 40 d of vernalizing cold treatment both in *elf7* and *efs* mutants (Fig. 6). Thus, activating components that are required for the *FLC* expression are also necessary for the full extent of *VIN3* induction by vernalization, in which *VIN3* expression must overcome the repressive effects of LHP1 and the PRC2 complex.

CONCLUSION

We identified multiple factors that contribute to the repressed state of *VIN3* prior to cold exposure. We
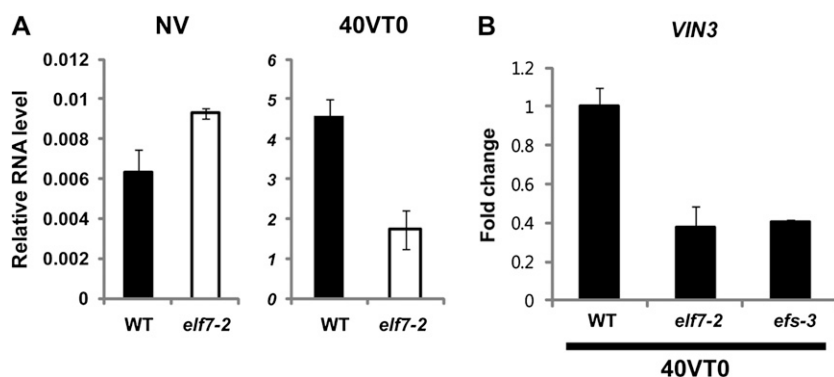
Figure 6. *ELF7* and *EFS* are required for the full induction of *VIN3* by vernalization. A, Real-time RT-PCR analysis on *VIN3* expression in the wild type (WT; Col-0) and *elf7-2* mutants in nonvernalized (NV) and 40 d of vernalization (40VT0) conditions. B, Real-time RT-PCR analysis on *VIN3* expression in the wild type, *elf7-2*, and *efs-3* mutants during vernalization. Relative fold change was determined by normalization with the levels of *PP2A* as previously reported (Czechowski et al., 2005).

observed derepression of *VIN3* in the absence of components of PRC2, CLF and SWN, and LHP1. In addition, a transposable element at the *VIN3* promoter region appears to contribute the repressed state of *VIN3* prior to cold.

Interestingly, there is no significant change in the level of H3K27me3, a repressive chromatin mark, on *VIN3* chromatin, even when *VIN3* is highly expressed (Fig. 4B). This suggests that the induction of *VIN3* can overcome the effect of H3K27me3, a representative repressive histone mark. On the other hand, an active histone mark, H3K4me3, is highly enriched at the 5′ end of *VIN3* chromatin during the cold exposure (Fig. 4C). Thus, when *VIN3* is induced during the cold exposure, *VIN3* chromatin appears to become a bivalent domain (Bernstein et al., 2006), containing both a repressive mark, H3K27me3, and an active mark,

H3K4me3. The bivalent domains are proposed to be characteristic of genes that are silent but poised to be activated or vice versa and thus provide flexibility of gene expression in undifferentiated cells (Azuara et al., 2006; Bernstein et al., 2006). Here, we observed a bivalent domain that is transiently activated by an environmental cue, winter cold.

PRC2 and LHP1 are constitutively associated with *VIN3* chromatin regardless of the expression state of *VIN3*. However, the lack of repressive components (CLF/SWN or LHP1) cannot recapitulate the induced level of *VIN3* expression without vernalization. It is possible that the induction of *VIN3* by cold is due to the combined contributions of both derepression of transposons and the removal of LHP1-PRC2 from *VIN3* chromatin by cold. However, examination of the LHP1 enrichment at *VIN3* chromatin indicates
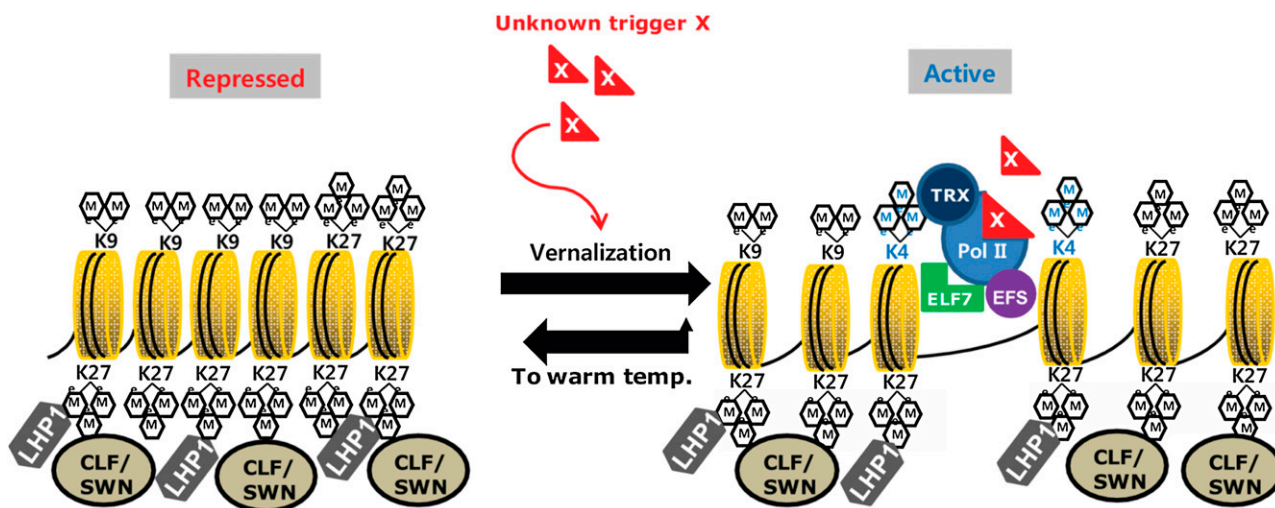

Figure 7. Schematic model on the regulation at *VIN3* chromatin by vernalization. In an NV condition, LHP1 and CLF-containing PRC2 complexes are necessary for the repressed state of *VIN3*. H3K9me2 and H3K27me3 are enriched at *VIN3* chromatin prior to vernalization. When plants are exposed to a prolonged cold (i.e. winter), unknown upstream components (X) induce *VIN3*, although LHP1 and PRC2 complexes are still enriched at *VIN3* chromatin. The induction of VIN3 causes the decrease in enrichments of H3K9me2 mark and the increase in enrichments of H3K4me3 mark at the transcription start site of *VIN3*. The increased enrichments of H3K4me3 are in part due to the activity of activating complexes (i.e. PAF1, EFS, and Trithorax-like proteins). Once plants return to warm growth temperature, H3K4me3 mark decreases and H3K9me2 mark increases again at *VIN3* chromatin in the absence of an unknown trigger (X). [See online article for color version of this figure.]

that LHP1 enrichment at *VIN3* chromatin is static throughout the course of vernalization (Fig. 3, B and D). Accordingly, a similar finding was observed for the CLF enrichment at *VIN3* chromatin (Fig. 3, C and E). This is consistent with the lack of changes at the level of enrichment of H3K27me3 at *VIN3* chromatin during the course of vernalization (Fig. 5B).

The derepressed *VIN3* in *lhp1* mutants does show ectopic induction by cold. This suggests that the slow kinetic of *VIN3* induction by vernalization is due to in part by the LHP1-mediated repression. Furthermore, activating chromatin remodeling complexes are also necessary for the full extent of *VIN3* induction by vernalization (Fig. 6). Thus, during the earlier cold exposure, activating complexes (i.e. PAF1 and EFS) need to overcome the repressive effect by LHP1 and PRC2. This may explain, at least in part, the slow kinetics of the *VIN3* induction by cold. However, it is worth noting that the full extent of *VIN3* induction in *lhp1* mutants still requires more than 20 d of cold. In addition, the rerepression of *VIN3* after the exposure to vernalizing cold still occurs in the absence of *LHP1* (Fig. 4B).

Activating complex components (i.e. PAF1 and EFS) are required for the full extent of *VIN3* induction by vernalization (Fig. 6). PAF1 often functionally associates with Trithorax-like components for the *FLC* activation (Tamada et al., 2009). Furthermore, the enrichment of H3K4me3 at *VIN3* chromatin is transiently increased when *VIN3* is induced. Thus, it is likely that Trithorax-like proteins are also involved in the induction of *VIN3* (Fig. 7). Although activating complex components are necessary for the full extent of *VIN3* induction by vernalization, the *VIN3* induction by vernalization still occurs in the absence of either PAF1 or EFS. Thus, it is probable that there are unidentified cold-induced activators and/or cold-repressed repressors involved in the induction of *VIN3* by vernalization, which could be enhanced by activating complex components (Fig. 7).

In conclusion, PRC2-LHP1 and the transposable element affects the repressed state of *VIN3* prior to cold and the effects of PRC2-LHP1 and that of the transposable element need to be overcome by unknown factors (X) along with the activity of activating chromatin remodeling complexes (Fig. 7).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) were used as the wild type in this study. *clf-29* (Salk_021003), *lhp1-4* (referred also as *tfl2-2*), *clf-29;swn-2*, *rdr2-1*, *rdr6-14*, *nrpd1a-4*, *nrpd1b-11*, *drm1; drm2*, *met1-7* (salk_076522), *kyp-2* (L*er* background), *elf7-2*, and *efs-3* were used in this study. Standard growth conditions were 22°C under illumination with white fluorescent light. The photoperiodic cycle was 16 h light/8 h dark (long day) or 8 h light/16 h dark (short day). For vernalization treatments, seeds were surface sterilized, placed on agar-solidified germination medium, and grown for 7 d at 22°C growth chamber and transferred to 4°C for 40 d under short-day photoperiods.

RNA Extraction and Quantitative Real-Time PCR Analysis

Seven-day-old seedling plants were harvested for RNA extraction. Total RNA was extracted using the Qiagen Plant RNeasy mini kit. Before reverse transcription, total RNA was treated for 30 min at 37°C with RNase-free DNase I (Invitrogen) to eliminate contaminated genomic DNA, and then total RNA (2 to 3 μg) was used for cDNA synthesis using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR reaction was done using SYBR green dye reaction mixture (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reaction was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems). PCR primers used for quantitative real-time PCR analysis are listed in Supplemental Table S1. The relative expression level of each gene was normalized using PP2A as described previously (Czechowski et al., 2005).

ChIP Assay

ChIP experiments were performed as previously reported (Johnson et al., 2002) with some modifications. Seven-day-old seedling plants were used for ChIP analysis. To elute DNA from immunoprecipitated complexes, the QIAquick PCR purification kit (Qiagen) was used. Antibodies recognizing H3K9me2 (Abcam catalog number ab1220), H3K27me3 (Abcam catalog number ab6002), H3K4me3 (Abcam catalog number ab8580), and Pol II (Abcam catalog number ab817) were purchased and used for ChIP assay with 10 μg per each reaction. All ChIP assays were done at least three times from at least two biological replicates and produced similar results. The quantitative real-time PCR reaction was done using SYBR green dye reaction mixture (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reaction was performed on a 7900HT Fast Real-Time PCR system. For primer sequences used in ChIP assay, refer to Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. TE sequence alignment.

Supplemental Table S1. Primer sequence information.

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