

# Coordination of the Vernalization Response through a *VIN3* and *FLC* Gene Family Regulatory Network in *Arabidopsis*<sup>CIW</sup>

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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. Vernalization triggers the recruitment of chromatin-modifying complexes to a clade of flowering repressors that are epigenetically silenced via chromatin modifications. In *Arabidopsis thaliana*, VERNALIZATION INSENSITIVE3 (*VIN3*) and its related plant homeodomain finger proteins act together with Polycomb Repressive Complex 2 to increase repressive histone marks at floral repressor loci, including *FLOWERING LOCUS C* (*FLC*) and its related genes, by vernalization. Here, we show that *VIN3* family of proteins nonredundantly functions to repress different subsets of the *FLC* gene family during the course of vernalization. Each *VIN3* family protein binds to modified histone peptides *in vitro* and directly associates with specific sets of *FLC* gene family chromatin *in vivo* to mediate epigenetic silencing. In addition, members of the *FLC* gene family are also differentially regulated during the course of vernalization to mediate proper vernalization response. Our results show that these two gene families cooperated during the course of evolution to ensure proper vernalization response through epigenetic changes.**

## INTRODUCTION

The life cycle of plants is under the influence of changing environments. One important environmental cue is seasonal change. Periodic fluctuations of photoperiod and temperature serve as cues for plants to recognize changing seasons. Plants flower at a particular season during their life cycle as a result of adaptation. Proper timing of transition from vegetative to reproductive development ensures the survival of plant species. In some plant species, the prolonged cold of winter provides a crucial clue for plants to monitor seasonal changes. In biennial and winter-annual plants, exposure to prolonged period of winter cold renders plants responsive to inductive photoperiods, thus resulting in rapid flowering in the following spring. This process is known as vernalization (Sung and Amasino, 2005; Dennis and Peacock, 2007).

In the model plant *Arabidopsis thaliana*, the requirement for the vernalization response is mainly a result of *FLOWERING LOCUS C* (*FLC*) expression (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). Vernalization results in the epigenetic silencing of *FLC*. *FLC* encodes a MADS box DNA binding protein that functions to repress genes encoding the floral integrators, including *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* by directly binding to regulatory regions of *FT* and *SOC1* (Searle et al., 2006). *FLC* exists as a small gene family that includes *FLOWERING LOCUS M* (*FLM*)/*MADS AFFECTING FLOWERING1* (*MAF1*) and *MAF2*–*MAF5* (Ratcliffe et al., 2001, 2003; Scortecci et al., 2001; Helliwell et al., 2006; Searle et al., 2006). Like *FLC*, other *FLC* clade members act as floral repressors when they are ectopically expressed (Ratcliffe et al., 2003), and some of them have been reported to be repressed by vernalization (Ratcliffe et al., 2003; Gu et al., 2009; Sheldon et al., 2009).

Mechanisms underlying vernalization-mediated gene silencing are well documented for the *FLC* repression (Schmitz and Amasino, 2007; Kim et al., 2009). Vernalization triggers a series of chromatin remodeling complexes that affect histone modifications at *FLC* chromatin (Bastow et al., 2004; Sung and Amasino, 2004). *FLC* mRNA expression is repressed during the cold exposure, and several repressive histone marks accumulate at *FLC* chromatin, including methylations at Histone H3 Lys 9 and Histone H3 Lys 27 (H3K27). The accumulation of these histone modifications at *FLC* chromatin depends on the activity of chromatin-remodeling complexes, including Polycomb Repression Complex 2 (PRC2). PRC2 is an evolutionarily conserved H3K27 methyltransferase. Increased enrichment of PRC2 at *FLC* chromatin by vernalization is critical for the stable repression of *FLC* (Wood et al., 2006; De Lucia et al., 2008).

PRC2 biochemically copurifies with members of the *VERNALIZATION INSENSITIVE3* (*VIN3*) family of proteins, including *VIN3*, *VIN3-LIKE1* (*MIL1*)/*VERNALIZATION5* (*VRN5*) and *VIL2*/*VERNALIZATION5/VIN3-LIKE1* (*VEL1*) (Wood et al., 2006; De Lucia et al., 2008). The *VIN3* family of proteins contain a plant homeodomain (PHD) finger, and the association of PHD finger

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proteins with PRC2 has been proposed as a part of evolutionarily conserved mechanisms in Polycomb group (PcG)-mediated gene repression (De Lucia et al., 2008). Besides *FLC*, repressions of some other *FLC* clade members by vernalization, including *FLM/MAF1*, *MAF2*, and *MAF3*, also depend on the presence of *VIN3* and other PRC2 components (Sung et al., 2006; Sheldon et al., 2009), raising the possibility of coordinated regulation of *FLC* clade through chromatin modifications by vernalization.

Targets for PcG-mediated gene repression are also targets for Trithorax group (TrxG)-mediated gene activation (Schuettengruber et al., 2007). PcG and TrxG antagonistically act on common targets, and their activities determine fates of transcriptional activities of their target genes during various developmental processes. TrxG activity mediates active histone marks, including methylations at Histone H3 Lys 4 (H3K4) and H3 Lys 36 (H3K36) (Kim et al., 2005; Jiang et al., 2009; Tamada et al., 2009; Ko et al., 2010). Multiple members of a Trithorax homolog family and a Trithorax-related family (i.e., *ARABIDOPSIS HOMOLOG OF TRITHORAX1* [*ATX1*], *ATX2*, *ARABIDOPSIS TRITHORAX-RELATED7* [*ATXR7*], and *ATXR4*) are redundantly required for the activation of *FLC* and other members of *FLC* family (Jiang et al., 2009; Tamada et al., 2009; Yun et al., 2012). In addition, *EARLY FLOWERING IN SHORT DAYS* (*EFS*) is required for methylations both at H3K4 and H3K36 (Kim et al., 2005; Ko et al., 2010). Some members of the *FLC* family are also regulated by these activating chromatin-remodeling complex components for their active transcription (Jiang et al., 2009; Tamada et al., 2009; Yun et al., 2012). Despite the clear involvement of TrxG-related components in the activation of *FLC* and its clade, their fates during vernalization-mediated repressions of *FLC* and its family member genes have not been addressed.

In this study, we show that all members of *VIN3* family of proteins function to collectively repress different sets of members of *FLC* gene family during the course of vernalization. Contributions of *VIN3* family of proteins to vernalization-mediated repression of *FLC* gene family members differ in their target specificities and in their timing to function during the course of vernalization. Members of the *FLC* gene family are also differentially expressed during the course of vernalization due to differential associations of PcG and TrxG on their chromatin and function to ensure a proper vernalization response. In addition, we show that *VIN3* family proteins, PRC2, and Trithorax-like complexes are coordinated to mediate the vernalization response through coordinated repressions of *FLC* gene family members.

## RESULTS

### Expression Patterns of *VIN3* Family of Proteins and *FLC* Gene Family during the Course of Vernalization

Previously, mRNA expression patterns of *VIN3* gene family were determined using PCR during the course of vernalization (Sung et al., 2006; Greb et al., 2007). Using quantitative RT-PCR, we confirmed differential expression patterns among the *VIN3*

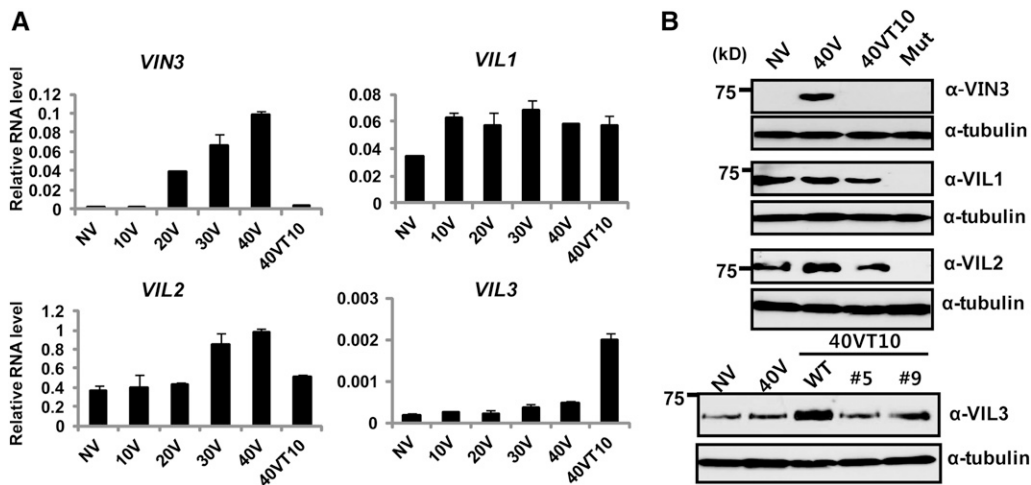
family (Figure 1A). *VIN3* mRNA is expressed only during the vernalizing cold. *VIL1/VRN5* mRNA is rather constitutively expressed, although there is a small increase by 10 d of cold. We also observed slight but significant upregulation of *VIL2/VEL1* mRNA during vernalizing cold (30 and 40 d of cold). Interestingly, *VIL3/VEL2* mRNA is distinctively increased after the cold exposure, suggesting its possible role in the vernalization after the cold exposure (Figure 1A). To explore roles of *VIN3* family members in vernalization, we obtained antibodies against all members of *VIN3* family of proteins (Figure 1B). Specificities of each antibody were tested using both recombinant proteins (see Supplemental Figure 1 online) and insertional mutants (for *VIN3*, *VIL1/VRN5*, and *VIL2/VEL1*) or knockdown lines (for *VIL3/VEL2*) (Figure 1B). Expression patterns of *VIN3* family of proteins are largely correlated with their transcripts (Figure 1B), demonstrating that regulation of *VIN3* family members mainly occurs at the transcription level.

To date, *FLC*, *FLM*, MADS AFFECTING FLOWERING 2 (*MAF2*) and MADS AFFECTING FLOWERING 3 (*MAF3*) are considered to be repressed as a result of vernalization. However, there have been conflicting observations on their expression changes by vernalization (Ratcliffe et al., 2001, 2003; Sheldon et al., 2009). Furthermore, MADS AFFECTING FLOWERING 4 (*MAF4*) and MADS AFFECTING FLOWERING 5 (*MAF5*) are not considered to be repressed by vernalization because no significant change in their mRNA expressions by vernalization was observed in previous studies (Ratcliffe et al., 2001, 2003; Sheldon et al., 2009). The lack of significant change at their mRNA levels by vernalization also suggested that they are dispensable for vernalization-mediated accelerated flowering response. However, previous studies mainly determined the mRNA levels of the *FLC* gene family before and after vernalization but not with various durations of cold exposure.

In our analysis, we determined the mRNA expression levels of the *FLC* gene family following various durations of vernalization treatment (Figures 2A to 2F). As reported previously, *FLC*, *FLM*, *MAF2*, and *MAF3* are repressed as a result of vernalization (Figure 2A to 2D). Interestingly, *MAF4* and *MAF5* show rather dynamic changes in their mRNA expression during the course of vernalization (Figures 2E and 2F). *MAF4* and *MAF5* transcripts are dramatically increased during early periods of the cold exposure and their expressions peak at 20 d of cold exposure (Figures 2E and 2F). Increased levels of *MAF4* and *MAF5* mRNA are eventually reduced to the levels comparable to those before cold exposure (Figures 2E and 2F), indicating that they may have a role during the early period of cold exposure.

### Precocious Vernalization Response in *maf4* and *maf5* Mutants

Our expression analysis indicates that *MAF4* and *MAF5* may contribute to vernalization response, especially during the early stage of vernalization (Figures 2E and 2F). To address the function of *MAF4* and *MAF5*, we obtained insertional mutants for *maf4* and *maf5* (Figure 3A; see Supplemental Figure 2 online). We first focused on flowering times of plants with 20 d of cold treatment, when significant upregulation of *MAF4* and *MAF5* was observed (Figure 3A; see Supplemental Figure 2A online).



**Figure 1.** Expression Analysis of *VIN3* Gene Family Members during the Course of Vernalization.

**(A)** mRNA expression analysis of *VIN3* gene family members during the course of vernalization using real-time RT-PCR. NV, nonvernalized; 10V, 10 d of vernalization; 20V, 20 d of vernalization; 30V, 30 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. Relative fold change was determined by normalization with the levels of *PP2A* gene. Real-time PCR experiments were performed with three biological replicates, and error bars indicate *sd*. Primer sequence information used for real-time RT-PCR analysis is shown in Supplemental Table 1 online.

**(B)** Immunoblot analysis of *VIN3* family of proteins during the course of vernalization. NV, nonvernalized; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. Corresponding insertional mutants were used as negative controls for *VIN3*, *VIL1/VRN5*, and *VIL2/VEL1* immunoblots. Two independent RNAi lines (#5 and #9) were used as negative controls for *VIL3/VEL2* immunoblots. WT, the wild type.

With 20 d of cold treatment, *maf4* and *maf5* mutants flower earlier than the wild-type plants (Figures 3A and 3B; see Supplemental Figure 2A online). Earlier flowering of *maf4* and *maf5* mutants after 20 d of cold suggests that *MAF4* and *MAF5* may play a role in preventing precocious vernalization response.

To test whether the earlier flowering observed in *maf4* and *maf5* mutants is unique among *FLC* gene family mutants, we determined flowering times with different durations of cold exposure in all *FLC* family mutants (Figures 3C and 3D). In the wild type, significant acceleration of flowering occurs only after 30 d of cold (Figures 3C and 3D). Prior to vernalization, *maf4* and *maf5* mutants do not show any significant difference in their flowering times compared with the wild type. Differences in flowering times of *maf4* and *maf5* mutants are apparent with 20 d of cold exposure, when the wild-type plants still do not show a significant vernalization response. After 40 d of vernalization, flowering times of *maf4* and *maf5* mutants are comparable to those of the wild-type plants, suggesting that the role of *MAF4* and *MAF5* is rather specific to a short period of cold exposure (i.e., 20 d of cold).

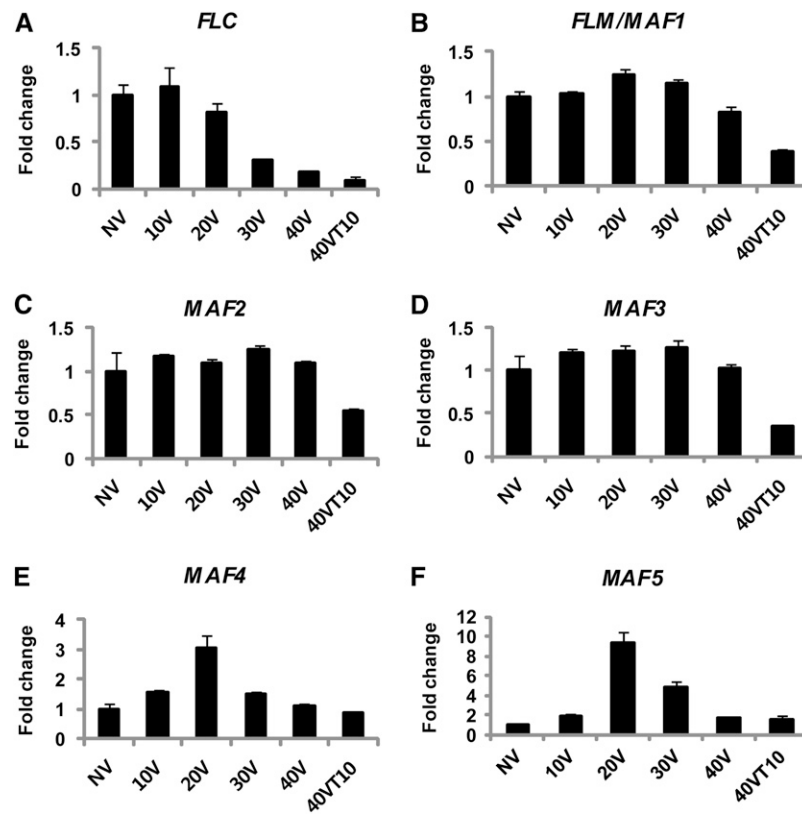
A previous study suggested that *MAF2* acts to prevent earlier flowering by a suboptimal duration of the cold exposure, based on earlier flowering observed in *maf2* mutants after a short period of cold exposure (~2 weeks; Ratcliffe et al., 2003). Indeed, both *maf1/flm* and *maf2* mutants also flower significantly earlier than the wild type after 20 d of cold exposure (Figures 3C and 3D; see Supplemental Figure 3 online). However, they flower significantly early even without vernalization and their flowering times are not significantly promoted by 20 d of cold exposure compared with nonvernalized controls (Figures 3C and 3D; see

Supplemental Figure 3 online). Taken together, our results show that *MAF4* and *MAF5* specifically function to prevent a precocious vernalization response by suboptimal duration of cold exposure.

#### Divergent Contributions of *VIN3* Gene Family to the Regulation of *FLC* Gene Family by Vernalization

*VIN3* and *VIL1/VRN5* were previously identified as essential components for vernalization-mediated *FLC* and *FLM/MAF1* repression (Sung et al., 2006; Greb et al., 2007). A mutation in *VIN3* also compromises expression of some members of the *FLC* gene family (Sheldon et al., 2009). *VIL2/VEL1* also biochemically copurifies with *VIN3* and *VIL1/VRN5* (De Lucia et al., 2008), but a mutation in *VIL2/VEL1* does not have a significant effect on the vernalization response (Kim and Sung, 2010). Nonetheless, *VIL2/VEL1* is required for the repression of *MAF5* to regulate flowering time under short-day conditions (Kim and Sung, 2010). In addition, we observed dynamic expression patterns of both the *VIN3* and *FLC* gene family during the course of vernalization (Figure 1). Their differential expressions during the course of vernalization indicate that the *VIN3* family of proteins may act on various combinations of *FLC* gene family members. To address the involvement of the *VIN3* gene family members in the vernalization-mediated regulation of the *FLC* gene family, we examined mRNA expression patterns of the *FLC* gene family members in all *VIN3* family mutants during the course of vernalization (Figure 4).

In *vin3* mutants, all members of the *FLC* gene family are derepressed throughout the course of vernalization (Figure 4A).



**Figure 2.** Expression Analysis of *FLC* Gene Family Members during the Course of Vernalization.

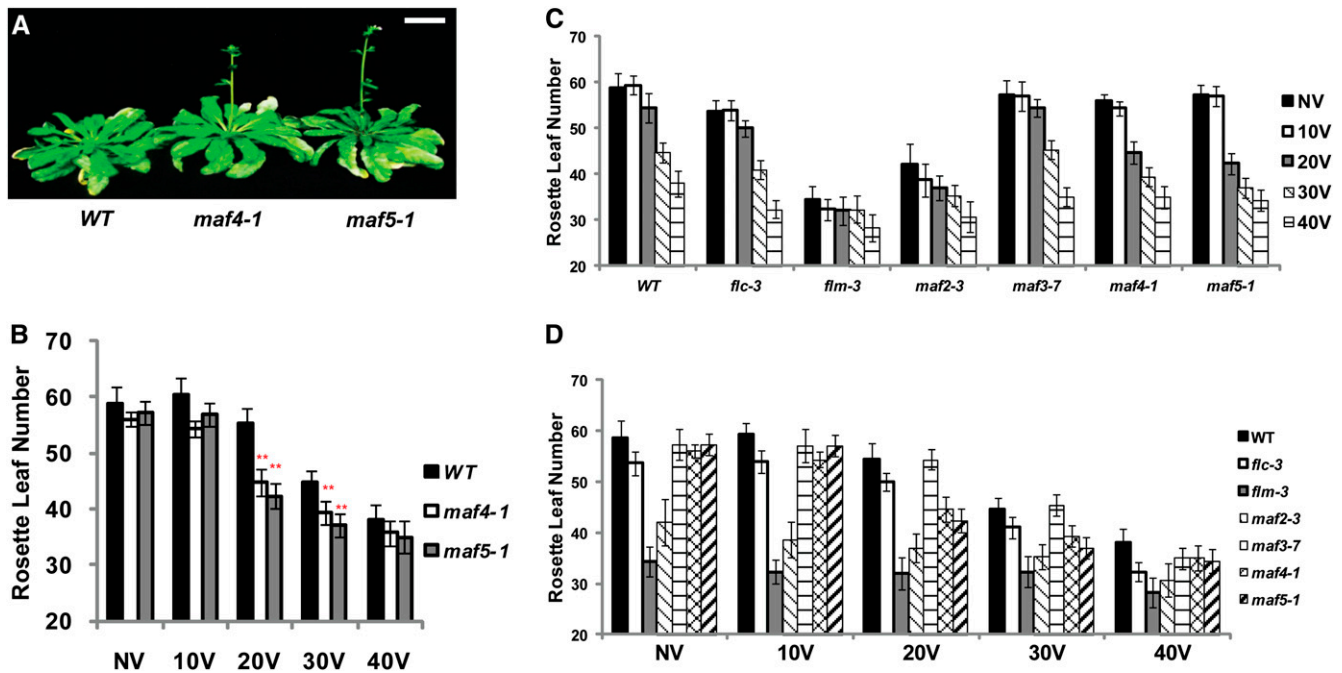
mRNA expression analysis of *FLC* gene family members during the course of vernalization using real-time RT-PCR. NV, nonvernalized; 10V, 10 d of vernalization; 20V, 20 d of vernalization; 30V, 30 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. Relative fold change was determined by normalization with the levels of the *PP2A* gene. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sd. Primer sequence information used for real-time RT-PCR analysis is shown in Supplemental Table 1 online.

No significant repression of *FLC* gene family was observed both during the cold exposure and after vernalization (Figure 4A). Thus, *VIN3* is required for the vernalization-mediated repression of all *FLC* gene family members.

Consistent with a previous report (Sung et al., 2006), both *FLC* and *FLM* are derepressed in *vil1/vrn5* mutants compared with the wild type after vernalization (Figure 4B). Unlike in *vin3* mutants, however, *FLC* and *FLM* are initially repressed during the cold exposure but are derepressed when plants return to warm growth temperature in *vil1/vrn5* mutants (Figure 4B). Other members of the *FLC* gene family are not affected in *vil1/vrn5* mutants, suggesting that *VIL1/VRN5* specifically acts to maintain the repressed states of *FLC* and *FLM* after cold exposure.

In *vil2/vel1* mutant plants, repression of *FLC* and *FLM* by vernalization still occurs (Figure 4C). During early periods of cold treatment, mRNA expression of *MAF4* and *MAF5* increase both in the wild type and in *vil2/vel1* mutants. However, *MAF4* and *MAF5* are not repressed by longer periods of cold treatment in *vil2/vel1* mutants (Figure 4C). Therefore, *VIL2/VEL1* is required for the vernalization-mediated repressions of *MAF4* and *MAF5* during the cold.

As indicated earlier, expression of *VIL3/VEL2* mRNA notably increase after vernalization (Figure 1), suggesting its role in the maintenance of vernalization response after the cold treatment. To address the role of *VIL3/VEL2* in vernalization, we created RNA interference (RNAi) lines for *VIL3/VEL2* (no T-DNA insertional mutant is available). We established multiple stable RNAi lines in which *VIL3/VEL2* expression is reduced (see Supplemental Figure 4A online; Figure 1B). *VIL3/VEL2* RNAi lines show reduced expression of *VIL3/VEL2* mRNA without compromising expression of other members of the *VIN3* gene family (see Supplemental Figure 5 online). *VIL3/VEL2* RNAi plants do not exhibit a significant difference in flowering time under both long days and short days compared with the wild type prior to cold treatment (see Supplemental Figure 4B online). However, *VIL3/VEL2* RNAi lines flower much later after the vernalization treatment (see Supplemental Figures 4C and 4D online). We selected two representative stable *VIL3/VEL2* RNAi lines and determined mRNA expression levels of *FLC* gene family members (Figure 4D). *VIL3/VEL2* RNAi lines showed significantly upregulated levels of *MAF4* and *MAF5* and to a lesser extent, *MAF2* and *MAF3*, but not those of *FLC* and *FLM/MAF1*. Derepression of *MAF2*~*MAF5* is more



**Figure 3.** *MAF4* and *MAF5* Function to Prevent Precocious Vernalization Response.

(A) *maf4* and *maf5* mutants flower earlier than the wild-type (WT) plants in response to 20 d of cold exposure. Plants were grown under short-day conditions (8 h light and 16 h dark) after vernalization. Bar = 2 cm.

(B) Flowering times of *maf4* and *maf5* mutants compared with the wild type after various durations of cold exposure. For flowering time measurements, the number of rosette leaves was counted when bolts were ~3 to 5 cm long. At least nine plants were used for each flowering time measurement.

(C) and (D) Flowering times of *FLC* gene family mutants compared with the wild type after various durations of cold exposure. NV, nonvernalized; 10V, 10 d of vernalization; 20V, 20 d of vernalization; 30V, 30 d of vernalization; 40V, 40 d of vernalization. All mutants are in the Columbia background, and flowering times were determined under short-day conditions (8 h light and 16 h dark) after the cold exposure. For flowering time measurements, the number of rosette leaves was counted when bolts were ~3 to 5 cm long. At least nine plants were used for each flowering time measurement. [See online article for color version of this figure.]

evident after the cold (Figure 4D; see Supplemental Figure 4E online), indicating that *VIL3/VEL2* is required for the maintenance of repressions of *MAF2~MAF5*.

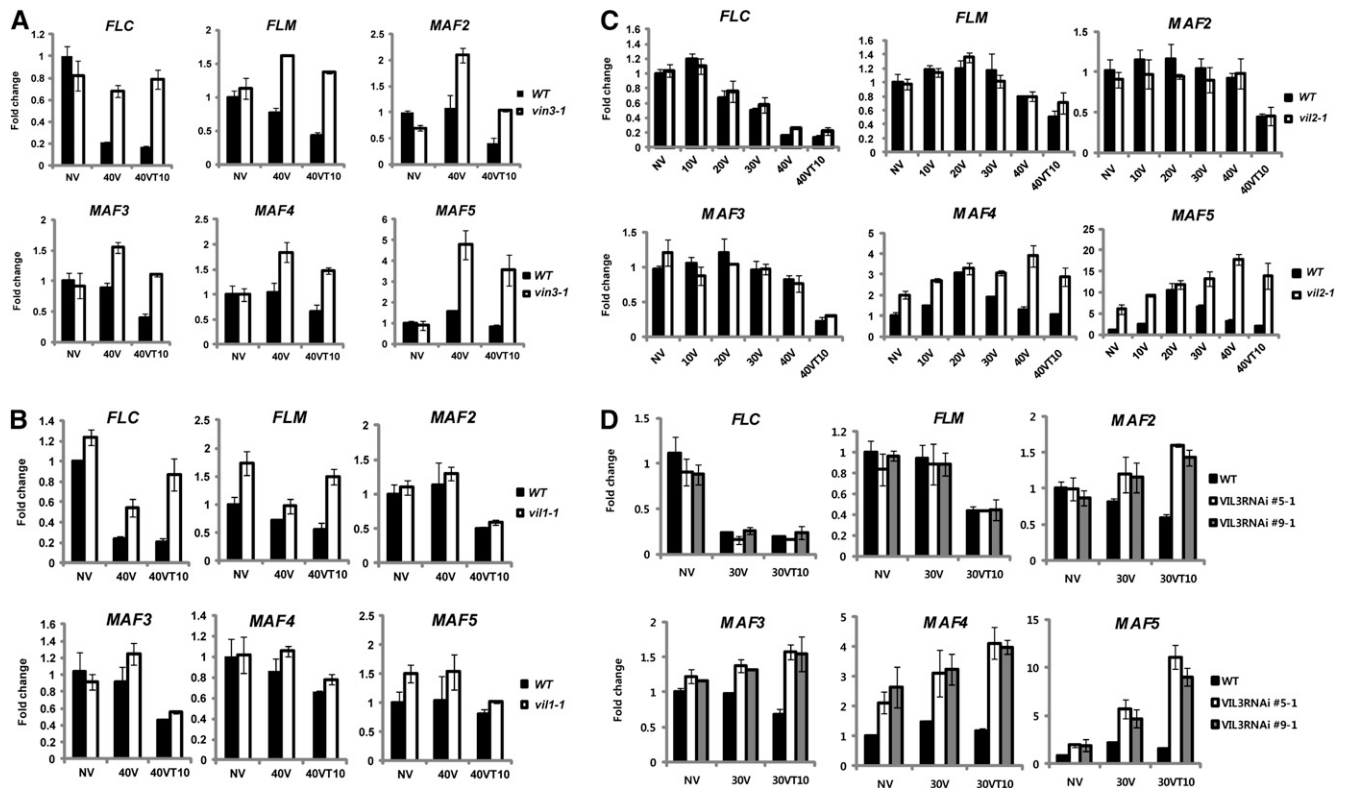
#### Direct Association of VIN3 Family of Proteins with Chromatin of *FLC* Gene Family Members during the Course of Vernalization

mRNA expression profiles of the *FLC* gene family members in *VIN3* family mutants indicate that there are divergent contributions by *VIN3* family proteins to the repression of the *FLC* gene family during the course of vernalization (Figure 4). To determine whether the effects of mutations in *VIN3* family genes on the *FLC* gene family are due to their direct associations with their target chromatin, we performed chromatin immunoprecipitation (ChIP) using antibodies against the *VIN3* family of proteins (Figure 5). *VIN3* protein is transiently associated with all members of *FLC* gene family chromatin during the cold, when *VIN3* is expressed (Figure 5B). Enrichments of *VIN3* protein at *FLC* gene family chromatin increased only during the cold.

Unlike *VIN3*, *VIL1/VRN5* enrichment is observed during the cold, and the increased enrichment persists even after the cold at *FLC* and *FLM/MAF1* chromatin (Figure 5C). We could not

observe significant enrichment of *VIL1/VRN5* at *MAF2~MAF5* chromatin (Figure 5C), consistent with the lack of derepression of *MAF2~MAF5* in *vil1/vrn5* mutants (Figure 4B). Consistent with the observation that *MAF4* and *MAF5* are derepressed in *vil2/vel1* mutants, enrichment of *VIL2/VEL1* protein at *MAF4* and *MAF5* chromatin is increased especially during the cold (Figure 5D), suggesting its role in establishing repression of *MAF4* and *MAF5* during the exposure to vernalizing cold.

mRNA expression analysis of *FLC* gene family in *VIL3/VEL2* RNAi lines indicated that *VIL3/VEL2* functions to maintain repressed states of *MAF4~MAF5* and to a lesser degree *MAF2~MAF3* (Figure 4D). To determine whether *VIL3/VEL2* directly binds to these *FLC* family chromatin, we performed ChIP using antibody against *VIL3/VEL2* (Figure 5E). Consistent with expression data, *VIL3/VEL2* accumulates at *MAF2*, *MAF3*, *MAF4*, and *MAF5* chromatin but not at *FLC* and *FLM/MAF1* chromatin. Enrichment of *VIL3/VEL2* at its target chromatin is persistent even after vernalizing cold, suggesting its role in maintaining the repression of *MAF2~MAF5* after the cold (Figure 5E). Taken together, all members of *VIN3* family protein directly associate with specific subsets of *FLC* gene family chromatin at certain times during the course of vernalization to coordinate repression of *FLC* gene family members.



**Figure 4.** Misregulations of *FLC* Gene Family Members in *VIN3* Gene Family Mutants during the Course of Vernalization.

(A) mRNA expression of *FLC* gene family members in *vin3* mutants during the course of vernalization. WT, the wild type.

(B) mRNA expressions of *FLC* gene family members in *vil1/vm5* mutants during the course of vernalization.

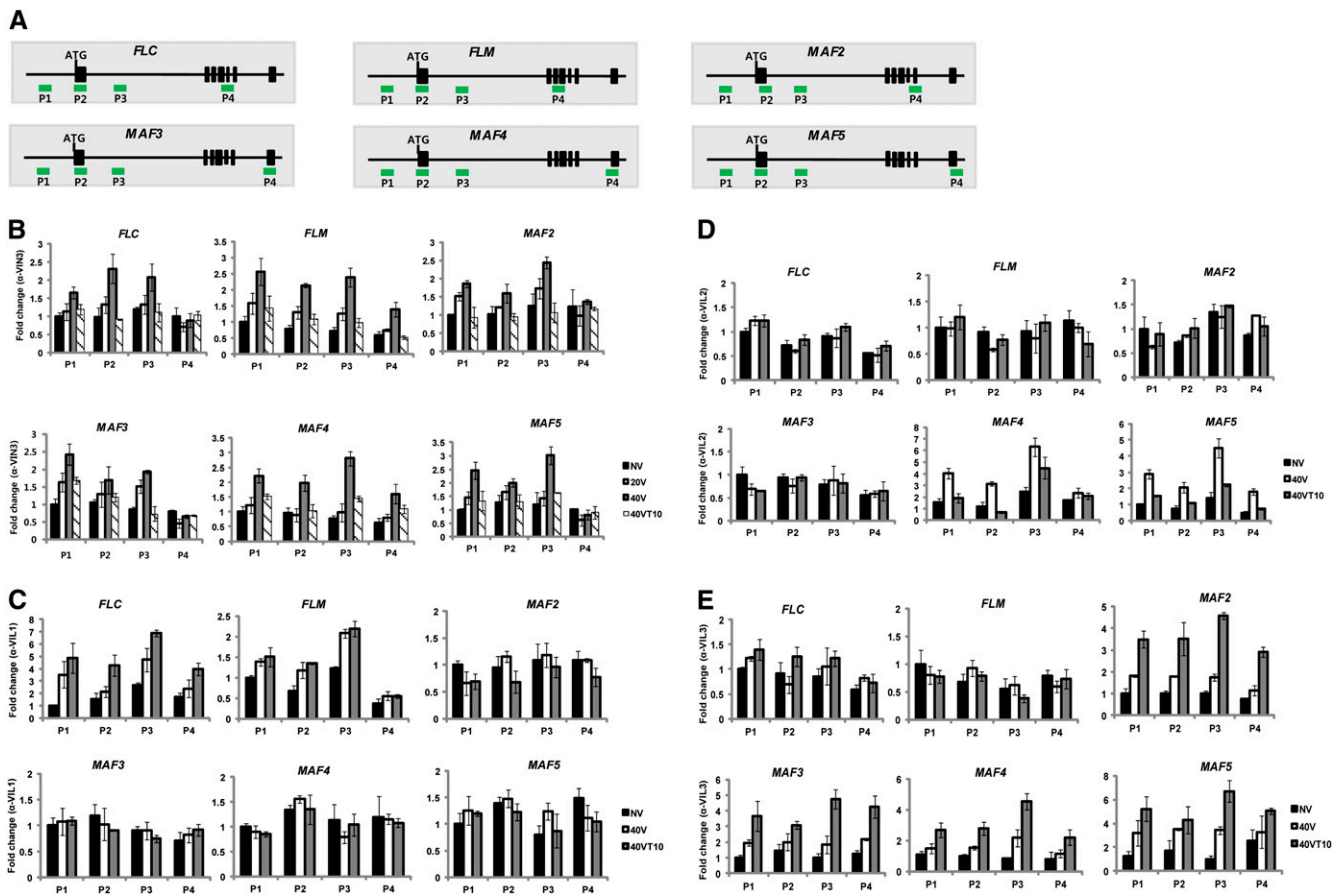
(C) mRNA expressions of *FLC* gene family members in *vil2/vel1* mutants during the course of vernalization.

(D) mRNA expressions of *FLC* gene family members in *VIL3/VEL2 RNAi* lines (#5 and #9) during the course of vernalization. NV, nonvernalized; 10V, 10 d of vernalization; 20V, 20 d of vernalization; 30V, 30 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. To show relative fold change, the value at nonvernalization of the wild type was converted to 1 and relative fold changes during the course of vernalization were presented. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sd.

### **VIN3 Family Proteins Preferentially Bind to H3K9me2**

*VIN3* family of proteins commonly contains a PHD finger motif (Figures 6A and 6B). The PHD motif can bind to various histone tails, ranging from nonmethylated histone H3 (Lan et al., 2007) to methylated histone H3 Lys 9 (Karagianni et al., 2008) and methylated histone H3 Lys 36 (Shi et al., 2007). In addition, we reported that *VIL2/VEL1* preferentially binds to the H3K9me2 histone peptide (Kim and Sung, 2010). To identify histone modifications to which the *VIN3* family of proteins bind, we performed *in vitro* histone peptide binding assays (Shi et al., 2006; Wysocka et al., 2006) using a series of synthesized histone peptides with various modifications (Figure 6C). All of the *VIN3* family of proteins preferentially bind to Histone H3 Lys 9 dimethylated (H3K9me2) peptides, although they also exhibit significant binding to dimethylated Histone H3 Lys 4 (H3K4me2). H3K9me2 is a modified histone mark that is enriched in repressed chromatin, including *FLC* after vernalization (Bastow et al., 2004; Sung and Amasino, 2004).

To address the specificity of the binding in detail, the putative tertiary structure of *VIN3* PHD finger domain was simulated using the SWISS-MODEL program (Figure 6B; see Supplemental Figure 5 online). The predicted structure of *VIN3* PHD domain contained two antiparallel  $\beta$ -sheets, commonly found in the PHD domain structure (Sanchez and Zhou, 2011). A hydrophobic aromatic ring residue is required to produce specific  $\pi$ -interaction with the polar methyl charge of the Lys (K) histone residue in PHD finger domain (Sanchez and Zhou, 2011). Based on a predicted structure of *VIN3* PHD motif, Phe-157, Trp-166, and Ser-175 residues of *VIN3* appear to form an aromatic cage that could serve as a docking site for a modified histone (Figure 6B; see Supplemental Figure 6 online). These residues are conserved in all members of the *VIN3* family of proteins (Figure 6A). To test if these cage-forming residues are important for modified histone binding, we introduce mutations to one of essential residues of this cage (Trp to Ala) in the PHD domain of *VIN3*, *VIL1/VRN5*, and *VIL3/VEL2*. These mutations completely abolished the histone peptide binding of the PHD



**Figure 5.** VIN3 Family of Proteins Are Enriched at Their Specific Target Chromatins of *FLC* Gene Family Members during the Course of Vernalization.

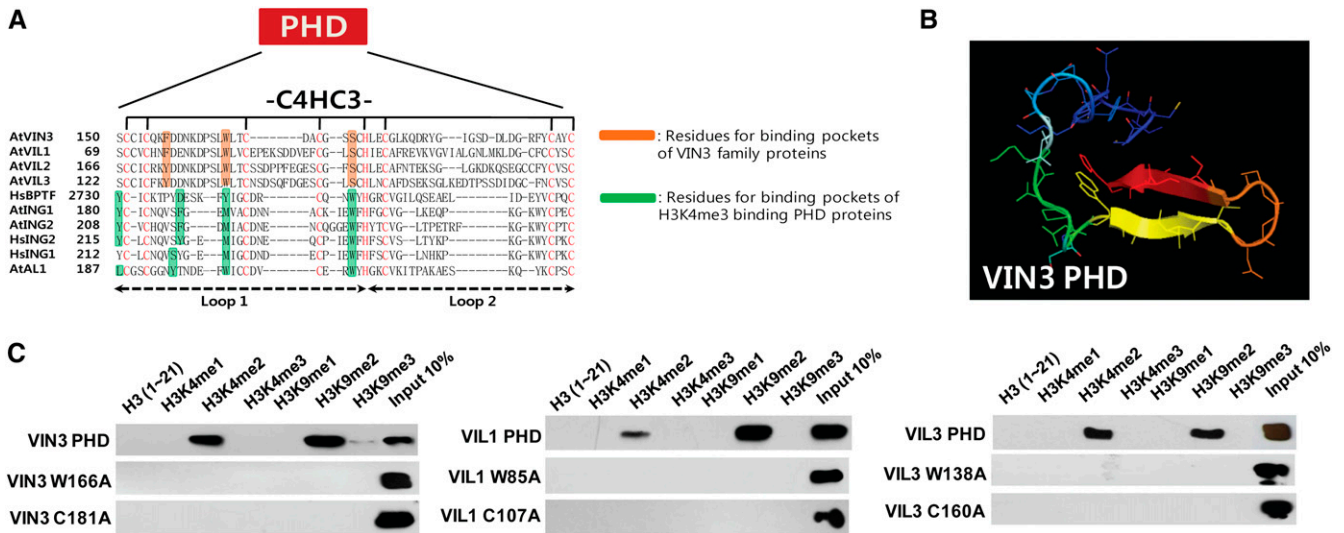
(A) Schematic representation of *FLC* gene family genomic regions (not scaled) and relative position of primers used in ChIP assays shown (P1 to P4 for each gene).  
 (B) VIN3 is enriched at all *FLC* gene family chromatins during the cold exposure.  
 (C) VIL1/VRN5 enriches at *FLC* and *FLM* chromatins, predominantly after the cold exposure.  
 (D) VIL2/VEL1 is enriched at *MAF4* and *MAF5* chromatins, predominantly during the cold exposure.  
 (E) VIL3/VEL2 is enriched at *MAF2*, *MAF3*, *MAF4*, and *MAF5* chromatins, predominantly after the cold exposure. NV, nonvernalized; 20V, 20 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sd. [See online article for color version of this figure.]

fingers of the VIN3 family of proteins (Figure 6C). Not surprisingly, disruption of the zinc fingers itself of VIN3 family proteins by the substitution of one of critical Cys residue with Ala residues completely abolished binding of VIN3 family proteins (Figure 6C). Thus, we concluded that the VIN3 family of proteins have a common binding preference for H3K9me2 histone mark and require a binding pocket structure consisting of two hydrophobic aromatic residues (Phe/Tyr and Trp) and one polar residue (Ser) in the PHD finger for H3K9me2 histone mark.

#### PRC2-Mediated Repression of *FLC* Clade Chromatin

Members of the VIN3 protein family copurify with components of PRC2 (Wood et al., 2006; De Lucia et al., 2008). PRC2 enriches at *FLC* chromatin by vernalization and mediates the increase in

levels of H3K27me3 at *FLC* chromatin, which signifies the stable silencing of *FLC* (Schubert et al., 2006; Wood et al., 2006; De Lucia et al., 2008; Jiang et al., 2008). As discussed earlier, all members of the *FLC* gene family undergo vernalization-mediated repression. Thus, we first determined levels of Histone H3 Lys27 trimethylation (H3K27me3) at chromatin of *FLC* gene family members during the course of vernalization (Figure 7). The H3K27me3 enrichment increases not only at *FLC* chromatin but also at all other members of *FLC* gene family chromatins as a result of vernalization (Figure 7). Furthermore, as is the case for the previously known roles of VIN3 and VIL1/VRN5 (Figures 8A and 8B), other VIN3 family proteins are also required for the H3K27me3 enrichment at their respective target *FLC* gene family member chromatin (Figures 8C and 8D), suggesting that all VIN3 family of proteins are required for the enrichment of H3K27me3.

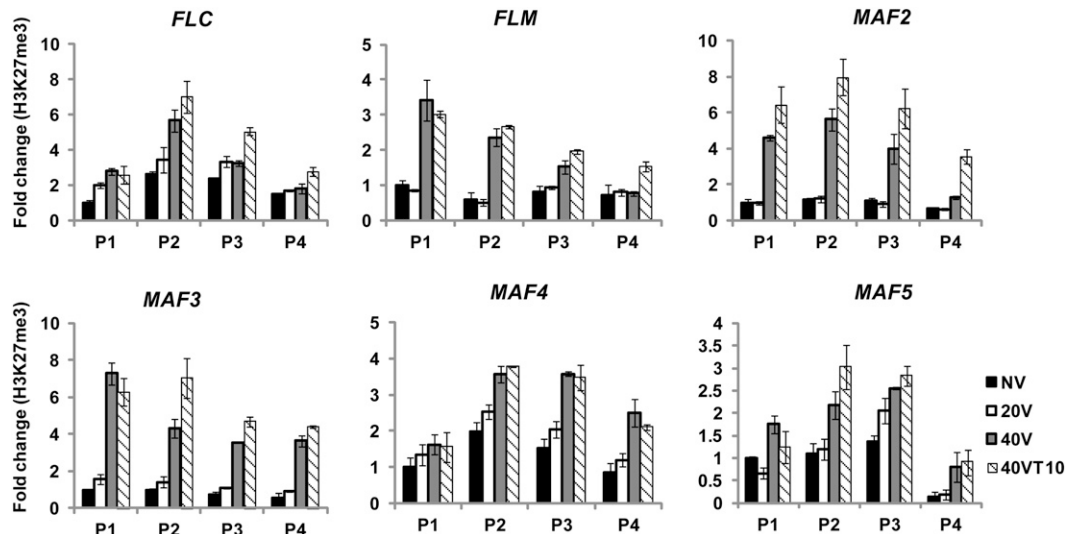


**Figure 6.** VIN3 Family of Proteins Preferentially Binds to H3K9me2 Peptides in Vitro.

(A) Multiple alignments of PHD fingers with known three-dimensional structure compared with the PHD fingers of VIN3 family of proteins. (B) Predicted three-dimensional structure of the PHD finger of VIN3. (C) In vitro histone peptides pull-down assay using recombinant VIN3 family of proteins. Full list of synthesized histone peptides used in this study are described in Supplemental Table 2 online.

To test whether PRC2 directly associates with *FLC* gene family chromatin, we used green fluorescent protein (GFP)-CLF transgenic plants to trace PRC2 enrichment at *FLC* gene family member chromatin (Figure 9). Consistent with the increase in H3K27me<sub>3</sub>, all *FLC* gene family chromatin showed significant

accumulation of GFP-CLF during the course of vernalization. It is interesting to note that PRC2 is enriched above the background level at *FLC*, *MAF4*, and *MAF5* chromatin prior to vernalization (compared with nontransgenic lines), and the PRC2 enrichment is further increased by vernalization. On the

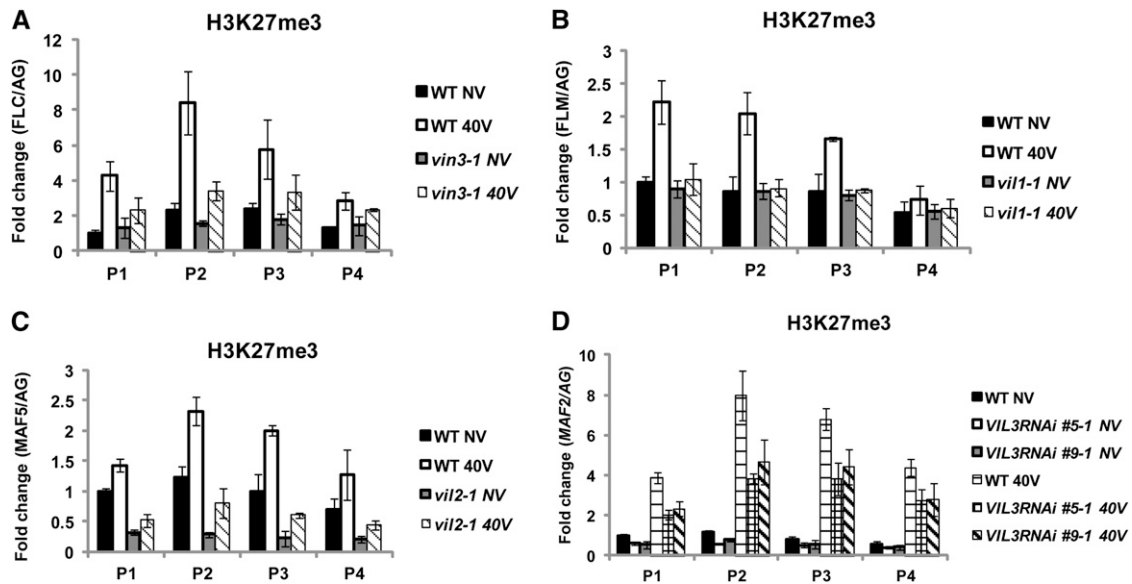


**Figure 7.** The Enrichment of H3K27me<sub>3</sub> at Chromatin of *FLC* Gene Family Members as a Result of Vernalization.

The level of H3K27me<sub>3</sub> enrichment at chromatin of *FLC* gene family members during the course of vernalization was determined by ChIP assays. Relative positions of primers used for each chromatin are depicted in Figure 5A. NV, nonvernalized; 20V, 20 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. To show relative fold change, the value at nonvernalization of the wild type was converted to 1 and relative fold changes during the course of vernalization were presented. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sp.

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**Figure 8.** Increased H3K27me3 Enrichments at Chromatins of *FLC* Gene Family Members by Vernalization Depends on *VIN3* Gene Family Members.

(A) The level of H3K27me3 enrichment at *FLC* chromatin between the wild type (WT) and *vin3* mutants as a result of vernalization. (B) The level of H3K27me3 enrichment at *FLM* chromatin between the wild type and *vil1/vrn5* mutants as a result of vernalization. (C) The level of H3K27me3 enrichment at *MAF5* chromatin between the wild type and *vil2/vel1* mutants as a result of vernalization. (D) The level of H3K27me3 enrichment at *MAF2* chromatin between the wild type and *VIL3/VEL2 RNAi* lines (#5 and #9) as a result of vernalization. Relative positions of primers used for each chromatin are depicted in Figure 5A. NV, nonvernalized; 40V, 40 d of vernalization. *AGAMOUS* (*AG*) was used as a control for normalization. To show relative fold change, the value at nonvernalization of the wild type was converted to 1 and relative fold changes during the course of vernalization were presented. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sd.

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other hand, *FLM/MAF1*, *MAF2*, and *MAF3* chromatins are enriched with PRC2 only by vernalization (Figure 9). This observation is also consistent with a genome-wide H3K27me3 enrichment study (Zhang et al., 2007). Taken together, our results show that all *FLC* gene family chromatins undergo PRC2-mediated H3K27me3 accumulation in a *VIN3* family protein-dependent manner by vernalization.

### Changes in Histone Modification at *FLC* Gene Family Chromatin

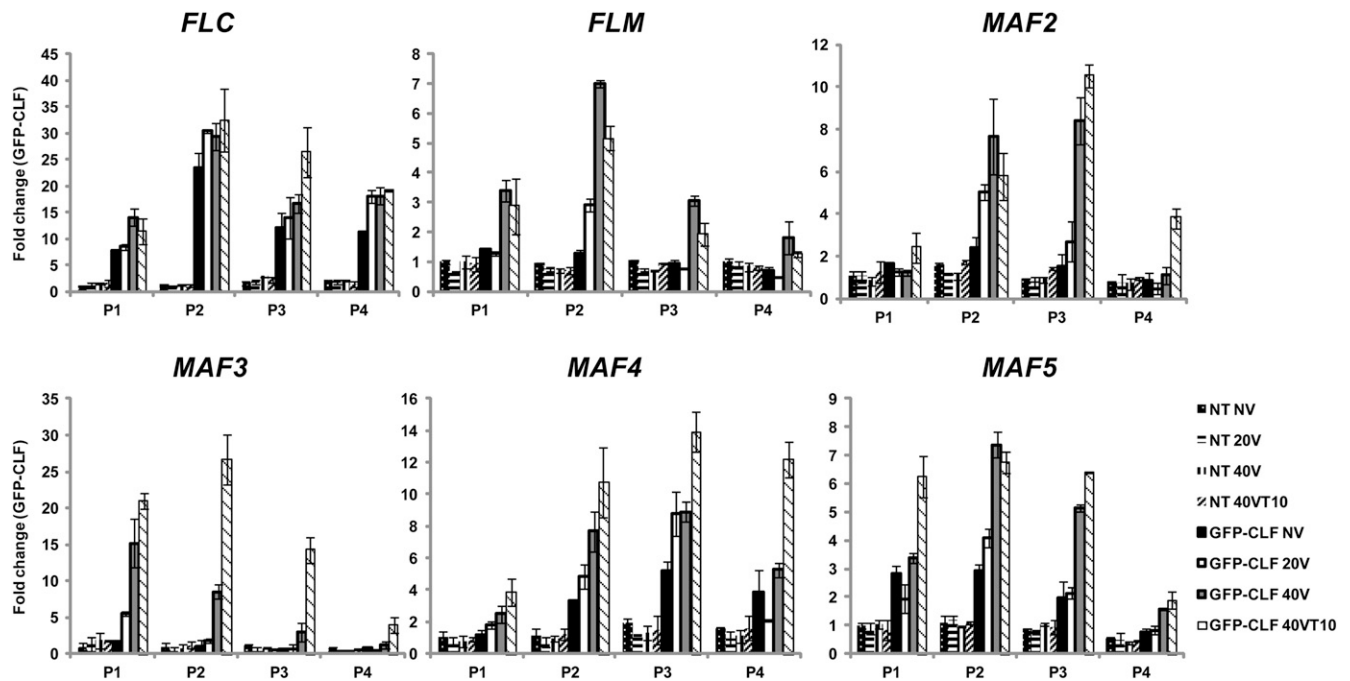
To address the *in vivo* implications of the *in vitro* histone binding preference by *VIN3* family proteins (Figure 6), we examined histone modifications at *FLC* gene family chromatin during the course of vernalization in the wild type and *VIN3* family mutants. Although *VIN3* family proteins are required for the enrichment of H3K27me3 at their target chromatin (Figure 7), *VIN3* family proteins do not exhibit binding to methylated H3K27 peptides (see Supplemental Figure 7 online). We examined the level of enrichment of H3K4me2 (a modification that *VIN3* family of protein binds *in vitro*) at chromatin of *FLC* gene family members during the course of vernalization. We did not observe a significant difference at the level of enrichment of H3K4me2 by vernalization at chromatin of *FLC* gene family members (see Supplemental Figure 8 online). Furthermore, no significant difference was observed in the level of enrichment of H3K4me2

between the wild type and *vin3* family mutants (see Supplemental Figure 8 online), suggesting that the level of H3K4me2 does not play a significant role in vernalization-mediated *FLC* gene family repression.

On the other hand, the level of enrichment of H3K9me2 (another modification that *VIN3* family of protein binds *in vitro*) increases as a result of vernalization at *FLC* gene family chromatin (Figures 10A to 10D). Furthermore, levels of H3K9me2 showed significant differences between the wild type and *vin3* family mutants at their representative target chromatin by vernalization (Figures 10A to 10D). Enrichment of H3K9me2 at *FLC* gene family chromatin is rather unique among PRC2 target loci. For example, another well-known H3K27me3-enriched *AGAMOUS* chromatin (Schubert et al., 2006) does not show significant enrichment of H3K9me2 at its chromatin (Figure 10). Thus, our results indicate that *VIN3* family proteins are required for the vernalization-mediated enrichment of H3K9me2 at *FLC* gene family chromatin through their affinity to H3K9me2.

### Behavior of Activating Chromatin-Modifying Complexes during the Course of Vernalization

One of the characteristic changes at *FLC* chromatin upon vernalization is the loss of Histone Lys 4 trimethylation (H3K4me3) enrichment (Bastow et al., 2004; Sung and Amasino, 2004; Sung et al., 2006; Greb et al., 2007; De Lucia et al., 2008). Multiple



**Figure 9.** Vernalization-Mediated Enrichments of PRC2 at Chromatins of *FLC* Gene Family Members.

ChIP assay using *GFP-CLF* transgenic plants to address the enrichment of CLF at chromatins of *FLC* gene family members during the course of vernalization. Nontransgenic plants (NT) were compared for the specificity of precipitation. Relative positions of primers used for each chromatin are depicted in Figure 5A. NV, nonvernalized; 20V, 20 d of vernalization; 40V, 40 d of vernalization; 40VT10, 40 d of vernalization followed by 10 d of normal growth temperature. Real-time PCR experiments were performed with three biological replicates and error bars indicate SD. AG was used as a control. First, relative enrichment of each fragment was calculated based on comparison to quantitative PCR using INPUT material. Second, relative enrichment was calculated by comparison to the AG proximal promoter control (Schubert et al., 2006). To show relative fold change, the value of nonvernalization time point in nontransgenic plants was converted to 1 and relative fold changes during the course of vernalization were presented.

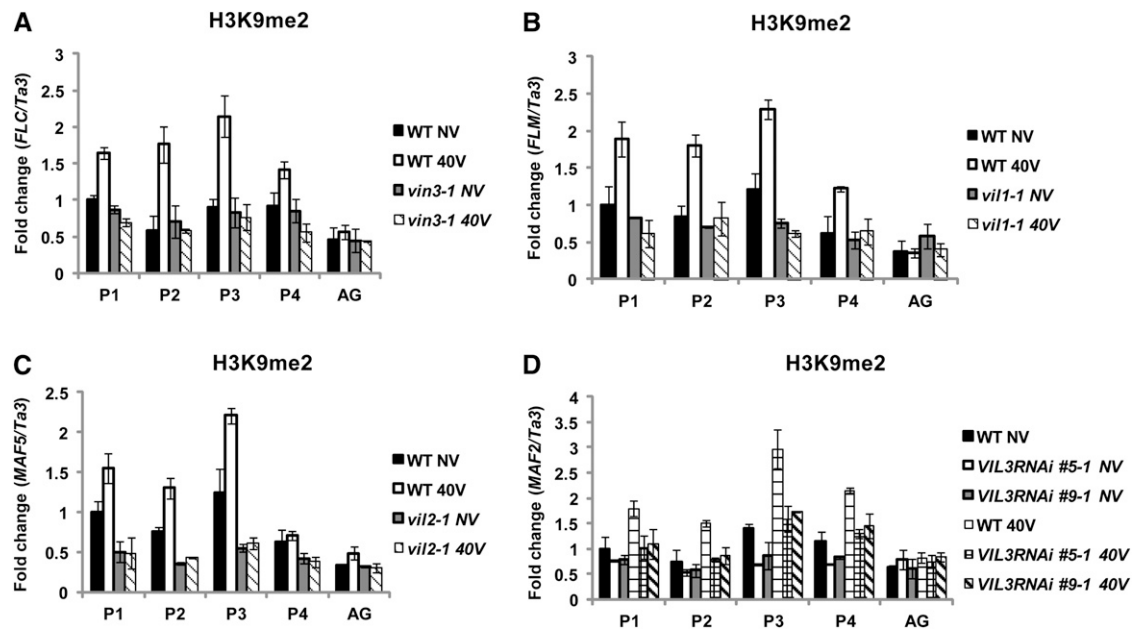
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activating chromatin modifying complexes have been identified to be responsible for H3K4me3 enrichment at *FLC* chromatin and, thus, the active state of *FLC* prior to vernalization (Kim et al., 2005; Jiang et al., 2009; Tamada et al., 2009; Ko et al., 2010). Although the resulting decrease in levels of H3K4me3 enrichment at *FLC* chromatin by vernalization is well known (Bastow et al., 2004; Sung and Amasino, 2004; Sung et al., 2006; Greb et al., 2007; De Lucia et al., 2008), the fate of activating complexes during the course of vernalization has not been addressed.

We tested subsets of known activating chromatin modifying complex components affecting *FLC* activation for their roles in the regulation of *FLC* gene family during the course of vernalization. First, we measured expression levels of *FLC* gene family between the wild type and mutants for known components of activating chromatin modifying complexes, including *efs*, *atx1-1*, *atxr7-1*, and *atx1-1 atxr7-1*, during the course of vernalization (see Supplemental Figure 9 online). *EFS* encodes a SET domain protein that mediates both H3K4me3 and H3K36me2 at *FLC* chromatin prior to the cold (Ko et al., 2010) and is also required for activation of some other members of *FLC* gene family (Kim et al., 2005; see Supplemental Figure 9 online). *ATX1* and *ATXR7* are Trithorax-type SET domain proteins, which mediate H3K4

methylation in *Arabidopsis*. They are redundantly required for the activation of *FLC* and some other members of the *FLC* gene family (see Supplemental Figure 9 online). It is also likely that additional members of Trithorax-type SET domain proteins are redundantly involved in the activation of *FLC* and other members of *FLC* gene family (Yun et al., 2012). Most members of *FLC* gene family are downregulated in *efs* mutants or in *atx1 atxr7* double mutants during the course of vernalization, although there are variations in degrees of severity (see Supplemental Figure 9 online). Interestingly, upregulations of *MAF4* and *MAF5* with 20 d of cold treatment are significantly reduced in *efs* and *atx1 atxr7* mutants, suggesting that *EFS*, *ATX1*, and *ATXR7* play roles in the transient activation of *MAF4* and *MAF5* during the early periods of cold exposure.

Although it has been documented that *EFS* and some Trithorax-type methyltransferases directly bind to *FLC* chromatin to mediate H3K4me3 and to activate *FLC* (Tamada et al., 2009; Ko et al., 2010), it has not been determined whether these components are removed from *FLC* chromatin by vernalization (when *FLC* is silenced). Thus, we used epitope-tagged transgenic lines for *EFS* and *ATXR7* to monitor these activating chromatin-modifying complexes at *FLC* gene family chromatin during the course of vernalization (Figure 11; see Supplemental



**Figure 10.** Increased H3K9me2 Enrichments at Chromatins of *FLC* Gene Family Members by Vernalization Depends on *VIN3* Gene Family Members.

AG was used as a negative control. Ta3 was also used as a control for normalization

(A) The level of H3K9me2 enrichment at *FLC* chromatin between the wild type (WT) and *vin3* mutants as a result of vernalization.

(B) The level of H3K9me2 enrichment at *FLM* chromatin between the wild type and *vil1/vrn5* mutants as a result of vernalization.

(C) The level of H3K9me2 enrichment at *MAF5* chromatin between the wild type and *vil2/vel1* mutants as a result of vernalization.

(D) The level of H3K9me2 enrichment at *MAF2* chromatin between the wild type and *VIL3/VEL2 RNAi* lines (#5 and #9) as a result of vernalization. Relative positions of primers used for each chromatin are depicted in Figure 5A. NV, nonvernalized; 40V, 40 d of vernalization. To show relative fold change, the value at nonvernalization of the wild type was converted to 1 and relative fold changes during the course of vernalization were presented. Real-time PCR experiments were performed with three biological replicates, and error bars indicate sd.

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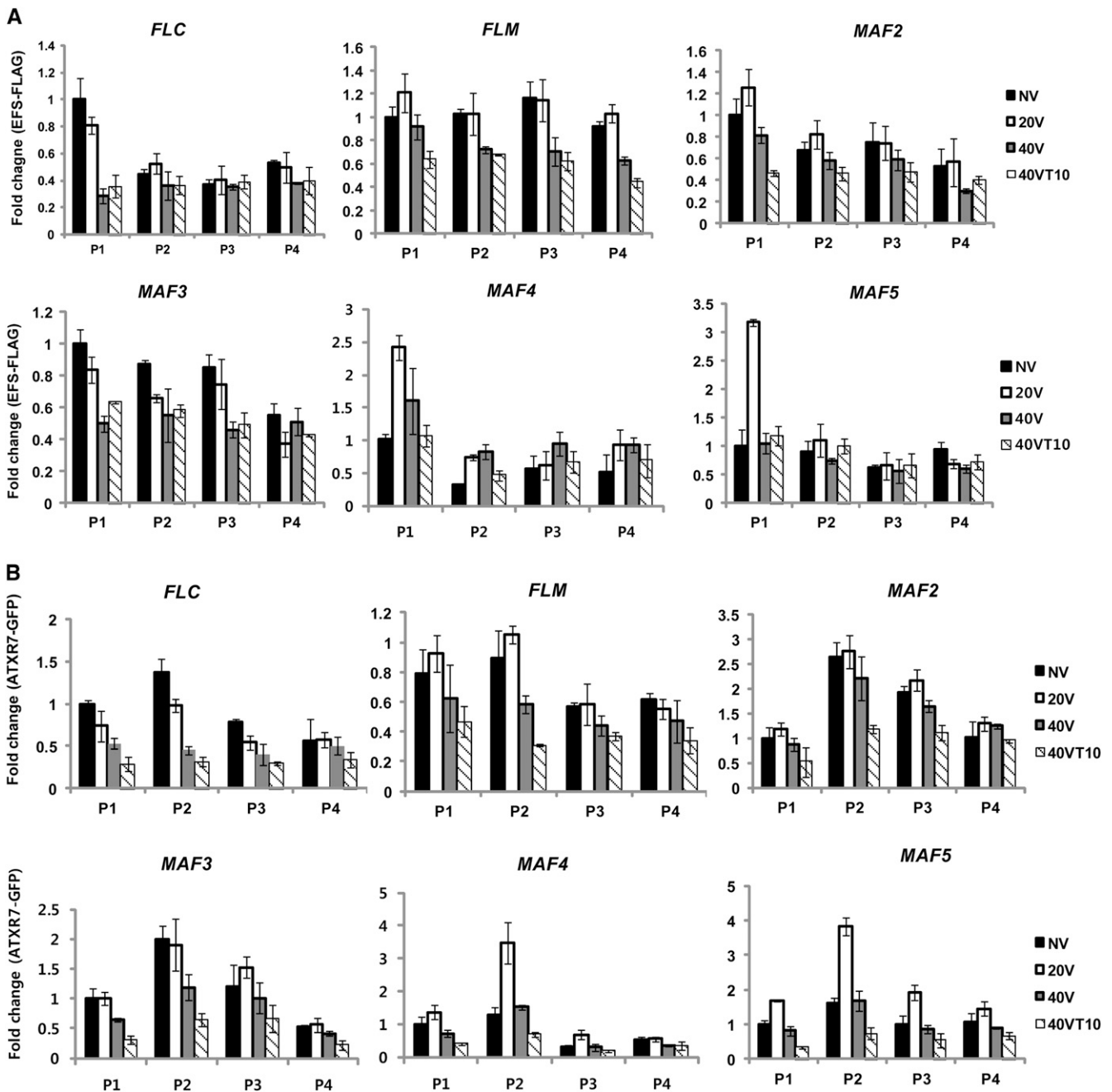
Figures 10 and 11 online). Before vernalization, both EFS and ATXR7 showed enrichment at chromatin of all *FLC* gene family members, especially around their respective transcriptional start site regions, which is consistent with previous reports (Tamada et al., 2009; Ko et al., 2010). By vernalization, enrichments of EFS and ATXR7 at *FLC* gene family chromatins are reduced (Figures 11A and 11B), suggesting that activating chromatin-modifying complexes are replaced by repressive chromatin complexes (i.e., PRC2) at chromatin of *FLC* gene family members as a result of vernalization (Figure 9). It is interesting to note that enrichments of EFS and ATXR7 at *MAF4* and *MAF5* chromatin are transiently increased by 20 d of cold when their expressions peak (Figure 4). Therefore, *MAF4* and *MAF5* chromatin becomes enriched with activating chromatin-modifying complexes by short-term cold, but the increased enrichment of EFS and ATXR7 during early period of cold exposure is eventually replaced by increased enrichments of PRC2 (Figure 9).

## DISCUSSION

Our study shows that all members of *VIN3* and *FLC* gene families function to mediate the vernalization response in *Arabidopsis*. Among members of *VIN3* gene family, *VIN3* appears to be a master regulator in vernalization, since it is required for

repression of all members of the *FLC* gene family (Figure 4A). Consistent with its expression profile, *VIN3* only functions during the cold exposure (i.e., *VIN3* protein associates with chromatin of the *FLC* gene family only during cold exposure) and appears to be required for initial repression of *FLC* gene family during the cold (Figure 5B). *VIL2/VEL1* acts redundantly in part with *VIN3*, as it is required for initial repression of *MAF4* and *MAF5* (Figure 4C), and *VIL2/VEL1* protein is transiently enriched at *MAF4* and *MAF5* chromatin during the cold exposure (Figure 5D). Unlike *VIN3* and *VIL2/VEL1*, *VIL1/VRN5* and *VIL3/VEL2* function even after the cold exposure. *VIL1/VRN5* and *VIL3/VEL2* appear to contribute to H3K27me3 and H3K9me2 enrichments both during and after cold (Figures 5C, 5E, and 12). Thus, it is likely that there is functional divergence among *VIN3* family of proteins in their temporal involvement in vernalization. Furthermore, there are clear differences in their target specificities (Figure 12).

Although *VIN3* is enriched at all *FLC* gene family chromatin, other members of the *VIN3* gene family are involved in the repressions of only specific members of the *FLC* gene family. Despite differences in their temporal and target specificities, they are all required for repression of their respective target *FLC* gene family. PRC2 biochemically copurifies with members of *VIN3* family of proteins, suggesting they act together to mediate epigenetic silencing of the *FLC* gene family. Consistent with this

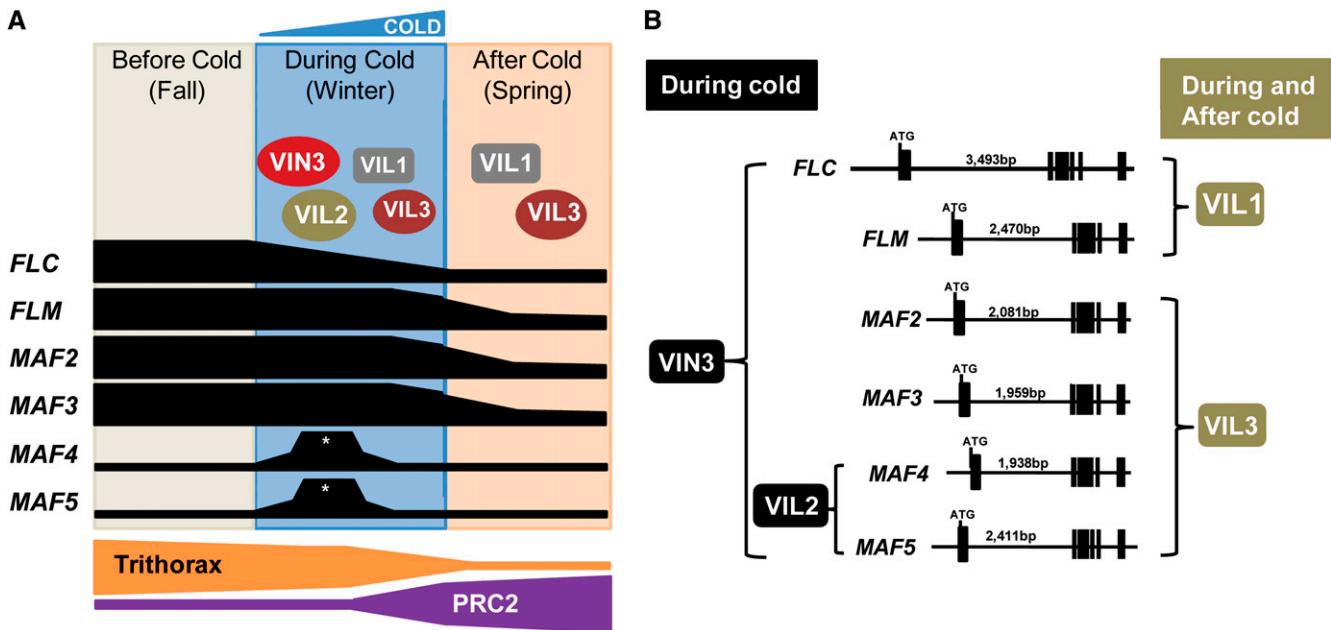


**Figure 11.** Dynamic Changes in Enrichments of EFS and ATXR7 at Chromatins of *FLC* Gene Family Members during the Course of Vernalization.

ChIP assay using *EFS-FLAG* and *ATXR7-GFP* transgenic plants to address the enrichments of EFS and ATXR7 at chromatins of *FLC* gene family members during the course of vernalization. Real-time PCR experiments were performed with three biological replicates, and error bars indicate s.e. [See online article for color version of this figure.]

notion, all *FLC* gene family chromatin is eventually enriched with PRC2 (Figure 9), and PRC2 is shown to mediate H3K27me3 enrichment. It should be noted that we could not observe significant binding of *VIN3* family of proteins to H3K27me3 peptides *in vitro* (see Supplemental Figure 7 online), although all members of the *VIN3* gene family are required for enrichment of

H3K27me3 at their respective target chromatin (Figure 7). Instead, all of the *VIN3* family of proteins binds to H3K9me2, another histone modification that is enriched at chromatin of the *FLC* gene family by vernalization. Furthermore, the H3K9me2 enrichment by vernalization depends on the presence of *VIN3* gene family (Figure 10). Thus, the *VIN3* family of proteins likely



**Figure 12.** Schematic Representations of Regulatory Networks between *VIN3* and *FLC* Gene Families.

(A) *FLC*, *FLM*, *MAF2*, and *MAF3* are repressed by vernalization through coordinated activities of *VIN3* family of proteins and PRC2. *MAF4* and *MAF5* are transiently activated during an early period of cold exposure (asterisks) but eventually repressed by *VIN3* family of proteins and PRC2.

(B) Target specificities of *VIN3* family of proteins.

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provides positive feedback between two repressive histone modifications at their target chromatin in part through the PRC2 activity. In addition, *VIN3* family proteins bind to an H3K4me2 in *in vitro* binding assay. It has been reported that unlike H3K4me3, H3K4me2 is not coupled with gene activation in *Arabidopsis* (Zhang et al., 2009). Consistent with this, we did not observe any significant change at the level of H3K4me2 at chromatin of *FLC* gene family. Although levels of H3K4me2 enrichment are not changed by the extended cold exposure, it is possible that H3K4me2 binding preference by *VIN3* family of proteins may play a role in facilitating *FLC* repression upon vernalization.

As the *VIN3* gene family shows functional divergences among its members, the *FLC* gene family also exhibits functional divergence among its members. In particular, *MAF4* and *MAF5* function to prevent a precocious vernalization response. *maf4* and *maf5* mutant plants respond to a suboptimal, short period of cold exposure (Figure 3A; see Supplemental Figure 2A online). One characteristic of vernalization response is that it is a response to a prolonged period of cold exposure. It is not known how plants measure the duration of cold to trigger vernalization response. Our results indicate that *MAF4* and *MAF5* function, at least in part, to ensure plants to respond to a long period of cold (i.e., winter) to promote flowering. A previous report showed that *MAF2* may function to prevent a precocious vernalization response (Ratcliffe et al., 2003). In our analysis, however, *maf2* mutants flower much earlier even prior to vernalization and they do not significantly respond to short-term cold (Figure 3B).

Furthermore, transient increases in levels of mRNA expression of *MAF4* and *MAF5* by a short-term cold (Figures 2E and 2F) suggest that *MAF4* and *MAF5* are specifically responsive to short-term cold to prevent a precocious vernalization response.

Although there is no net reduction in *MAF4* and *MAF5* transcripts after vernalization, *MAF4* and *MAF5* chromatin undergo dramatic changes during the course of vernalization. Initial increases in their transcription by short-term cold are correlated with increased enrichments of activating chromatin remodeling complexes. Further cold exposure eventually triggers enrichments of *VIL2/VEL1*, *VIL3/VEL2*, and PRC2 at *MAF4* and *MAF5* chromatin and, thus, enrichments of repressive histone marks. Furthermore, these changes at chromatin depend on two *VIN3*-related proteins, *VIL2/VEL1* and *VIL3/VEL2*. Mutations in *VIL2/VEL1* and *VIL3/VEL2* result in derepressions of *MAF4* and *MAF5* after vernalization (Figures 5D and 5E). Therefore, eventual repressions of *MAF4* and *MAF5* by vernalization should be considered as part of vernalization-mediated repression.

Dynamic expression profiles of the *FLC* gene family during the course of vernalization coincide with differential enrichments of chromatin-modifying complexes, including *VIN3* family proteins, PRC2, and Trithorax-type activating complexes. In a recent study, the PRC2 enrichment at *FLC* chromatin by vernalization is mediated by a long noncoding RNA, COLDAIR (Heo and Sung, 2011). Here, we describe diverse recruitment of the *VIN3* family of proteins, which are associated with PRC2. How *VIN3* family proteins achieve their target specificity remains to be addressed. Our study presents a detailed analysis of the dynamics of

repressive and activating chromatin remodeling complexes at multiple environmentally regulated loci. Related but yet distinct functions among members of gene families show that plants have evolved sophisticated regulatory networks among members of gene families to interpret an environmental input (i.e., winter cold) to developmental transitions (i.e., flowering). Our thorough analysis will also be helpful to use this regulatory network between *VIN3* and *FLC* gene families as a model to address mechanistic details of epigenetic regulation of developmental genes.

## METHODS

### Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* were germinated on agar plates for 10 d in short-day conditions and vernalized at 4°C under short days. Seedling plants were transplanted to soil or harvested for total RNA isolation. Vernalization treatments were as previously described (Kim et al., 2010). In short, nonvernalized seedlings were grown for 11 d after germination under short-day conditions. For 40VT0 and 40VT10 plants, seedlings were grown for 7 and 4 d, respectively, after seed germination and then seedlings were subjected to cold treatment. Samples were harvested for experiments right after the cold treatment (40VT0) or grown for 10 d more (40VT10) under short days. Plants were grown at 22°C in different photoperiods for measurement of flowering time as described in each experiment. All of the *Arabidopsis* plants used in his study were in the Columbia background except the *GFP-CLF* transgenic line (CaMV35S: *GFP:CLF* in *clf*; *FRI* in Wassilewskija background). Information on mutants used in this study is shown in Supplemental Table 3 online.

### Development of Transgenic Lines

For *GFP:MAF4* and *MAF5:GFP* transgenic lines, cDNA of each *MAF4* and *MAF5* was amplified and cloned into pENTR-D TOPO cloning vector (Invitrogen) followed by LR reaction into the plant expression Gateway vector pB7FWG2,0 using LR enzyme (Invitrogen). Each construct was transformed into *maf4-1* (SALK\_028506) (Gu et al., 2009) and *maf5-1* (SALK\_095092) mutants. For *VIL3/VEL2* RNA knockdown lines, 317 bp (+1798 to 2114) of 3' region of *VIL3/VEL2* was amplified and digested with *AscI-SwaI* and *BamHI-SpeI* restriction enzymes, respectively, and cloned into pFGC1008 RNAi vector to produce double-stranded RNA structure. Construct was transformed into Columbia-0 wild-type plants. All transgenic lines were selected on selective antibiotic medium and were confirmed by PCR. Stable homozygous T3 transgenic plants were used for experiments. The cauliflower mosaic virus 35 promoter-driven *GFP-CLF* construct (Schubert et al., 2006) was previously described and used for this study. *ATXR7-GFP* (Tamada et al., 2009) and *EF5-FLAG* (Ko et al., 2010) constructs including the native promoter regions were previously described and used for ChIP assay.

### Production of Polyclonal Antibodies

Amino acids sequences of *VIN3* family proteins were analyzed with the OptimumAntigen design tool (GenScript) to identify good antigen sequences. Selected synthetic peptide antigens of *VIN3* family proteins were injected into rabbit for production of polyclonal antibodies (GenScript). The synthetic peptide sequences used as antigens are as follows: *VIN3* (493-KRDIYKKGKQGNGK), *VIL1/VRN5* (464-DDAVSNGRRKNNN), *VIL2/VEL1* (473-PKKPSSKNEDNNSP), and *VIL3/VEL2* (75-EIIPSPKRQRDLV). Synthesized peptides were injected into rabbit, and resulting antiserum were further purified by affinity purification (GenScript).

### Real-Time Quantitative RT-PCR Analyses

Total RNA was isolated using a Plant RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Before reverse transcription reaction, DNase I (Invitrogen) treatment was performed for 30 min at 37°C to eliminate contaminated genomic DNA from total RNAs. First-strand cDNA synthesis was performed on 2 µg of RNA using the M-MLV System (Promega). Real-time quantitative RT-PCR was performed using SYBR green dye reaction mixture (Thermo Scientific) on an ABI prism 7900HT sequence detection system (Life Technologies). Each sample was quantified in triplicate, and the relative transcript level of each gene was determined by normalization of the resulting expression levels versus that of *PP2A*, as described (Czechowski et al., 2005). Error bars indicate standard deviation from triplicate samples. For primer sequences used in real time RT-PCR analyses, see Supplemental Table 1 online.

### ChIP-Quantitative PCR Analyses

Whole seedling plants were harvested for grinding. ChIP assays were performed as previously described (Kim and Sung, 2010). For primer sequences, see Supplemental Table 1. Quantitative PCR reaction was done using SYBR green dye reaction mixture (Thermo Scientific) according to the manufacturer's instructions. Relative enrichment of each fragment was calculated based on comparison to quantitative PCR using INPUT material. Second, relative enrichment was calculated by comparison to each listed control region. All ChIP assays were performed at least three times from at least two biological replicates and produced similar results. Error bars indicate SD from triplicate samples. Quantitative PCR reaction was performed on 7900HT Fast Real-Time PCR system (Life Technologies).

### In Vitro Histone Peptide Pull-Down Assay

cDNAs of full-length and PHD-only *VIN3* family genes were amplified by RT-PCR and cloned into the pVP13 (Thao et al., 2004) expression vector. Recombinant full-length and PHD-only *VIN3* family proteins were purified using a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen). Biotinylated histone peptides were purchased from Millipore (see Supplemental Table 2 online). After pull down, proteins were transferred using a mini trans-blot electrophoresis transfer cell apparatus (Bio-Rad). His-tag antibody (Santa Cruz Biotech) was used for immunoblot analysis. Commercial antibodies used in this study are listed in Supplemental Table 4 online.

### Prediction of Putative Tertiary Structure of the *VIN3* PHD Domain

Tertiary structure prediction for the PHD finger domain of *VIN3* was performed using the Swiss-Model website (<http://swissmodel.expasy.org/>) that simulates tertiary structure based on the reported protein structure of a sequence with similarity to it (Arnold et al., 2006). The acquired PDB file was graphically visualized using Pymol version 1.4.0 program ([www.pymol.com](http://www.pymol.com)). Briefly, a template sequence of *VIN3* PHD domain (loop 1 region, CICQKFDNDKPSLWLTCDACGSSCHGRCV-GILQSEALIDEYV) was submitted into the template identification step, and then predicted structure was produced based on parent template PDB file (2ri7A) in automatic modeling mode of the Swiss-Model website.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *VIN3* (At5g57380), *VIL1/VRN5* (At3g24440), *VIL2/VEL1* (At4g30200), *VIL3/VEL2* (At2g18880), *FLC* (At5g10140), *FLM/MAF1* (At1g77080), *MAF2* (At5g65050), *MAF3* (At5g65060), *MAF4* (At5g65070), *MAF5* (At5g65080), *ATX1* (At2g31650), *ATXR7* (At5g42400), and *EF5*

(At1g77300). Germplasm used was *maf4-1* (SALK\_028506) and *maf5-1* (SALK\_095092).

#### Supplemental Data

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

**Supplemental Figure 1.** Immunoblots Using Recombinant VIN3 Family of Proteins.

**Supplemental Figure 2.** Flowering Times of *maf4* and *maf5* Mutants.

**Supplemental Figure 3.** Flowering Times of *maf2* Mutants by Various Durations of Cold Exposure.

**Supplemental Figure 4.** *VL3* Knockdown Lines Compromise Vernalization Response.

**Supplemental Figure 5.** Specificity of *VIN3* Family Mutants.

**Supplemental Figure 6.** Predicted Histone Binding Pocket of the *VIN3* PHD Finger Motif.

**Supplemental Figure 7.** In Vitro Histone Peptides Pull-Down Assay Using Recombinant *VIN3* Family Proteins.

**Supplemental Figure 8.** H3K4me2 Enrichment at Chromatin *FLC* Gene Family Chromatin.

**Supplemental Figure 9.** Expression Analysis of the *FLC* Gene Family during the Course of Vernalization in Activating Chromatin-Modifying Complex Components.

**Supplemental Figure 10.** Dynamic Changes in Enrichment of EFS at Chromatins of *FLC* Gene Family during the Course of Vernalization.

**Supplemental Figure 11.** Dynamic Changes in Enrichment of ATXR7 at Chromatins of *FLC* Gene Family during the Course of Vernalization.

**Supplemental Table 1.** Primer Sequences Used in This Study.

**Supplemental Table 2.** Histone Peptides Used for in Vitro Pull-Down Assay.

**Supplemental Table 3.** Information on Mutants Used in This Study.

**Supplemental Table 4.** Commercial Antibodies Used for This Study.

#### ACKNOWLEDGMENTS

We thank Richard Amasino for providing ATXR7-GFP transgenic line seeds and Yoo-Sun Noh for EFSFLAG transgenic line seeds. This work was supported by the National Science Foundation (IOS 0950785) and by the National Institutes of Health (R01 GM100108A1).

#### AUTHOR CONTRIBUTIONS

D.-H.K. and S.S. conceived and designed the experiments, analyzed the data, and wrote the article. D.-H.K. performed the experiments.

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