A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of *FLC* during vernalization

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Vernalization, the acceleration of flowering by winter, involves cold-induced epigenetic silencing of *Arabidopsis FLC*. This process has been shown to require conserved Polycomb Repressive Complex 2 (PRC2) components including the Su(z)12 homologue, VRN2, and two plant homeodomain (PHD) finger proteins, VRN5 and VIN3. However, the sequence of events leading to *FLC* repression was unclear. Here we show that, contrary to expectations, VRN2 associates throughout the *FLC* locus independently of cold. The vernalization-induced silencing is triggered by the cold-dependent association of the PHD finger protein VRN5 to a specific domain in *FLC* intron 1, and this association is dependent on the cold-induced PHD protein VIN3. In plants returned to warm conditions, VRN5 distribution changes, and it associates more broadly over *FLC*, coincident with significant increases in H3K27me3. Biochemical purification of a VRN5 complex showed that during prolonged cold a PHD-PRC2 complex forms composed of core PRC2 components (VRN2, SWINGER [an E(Z) HMTase homologue], FIE [an ESC homologue], MSI1 [p55 homologue]), and three related PHD finger proteins, VRN5, VIN3, and VEL1. The PHD-PRC2 activity increases H3K27me3 throughout the locus to levels sufficient for stable silencing. *Arabidopsis* PHD-PRC2 thus seems to act similarly to Pcl-PRC2 of *Drosophila* and PHF1-PRC2 of mammals. These data show *FLC* silencing involves changed composition and dynamic redistribution of Polycomb complexes at different stages of the vernalization process, a mechanism with greater parallels to Polycomb silencing of certain mammalian loci than the classic *Drosophila* Polycomb targets.

Arabidopsis | PHD protein | PRC2 | chromatin modifications

Polycomb-group (PcG) proteins were identified first in *Drosophila melanogaster* as factors necessary to maintain the repressed state of homeotic (*Hox*) genes (1). They also were identified as regulators of *Hox* genes in vertebrates and have been implicated in stem cell identity, cancer, imprinting, and chromosome X inactivation (1, 2). Recent genomic analysis has shown that up to 5% of genes in mice, humans, and *Drosophila* are PcG targets (3). Contrary to the original belief that PcG proteins maintain chromatin states, they now are thought to play a dynamic role in the transcriptional regulation of many genes (2).

PcG proteins function in multiprotein complexes. Polycomb Repressive Complex 2 (PRC2) consists of core components, E(Z) (a histone methyltransferase), extra sex combs (ESC), p55, and Su(z)12 (4), and is widely conserved from plants to humans (4, 5). PRC2 maintains repressed chromatin states through posttranslational modification of histone tails (specifically histone 3 lysine 27 trimethylation, H3K27me3). In flies, H3K27me3 is recognized by the chromodomain of Pc, one of the components of the PRC1 complex. However, components of PRC1 seem to be absent from plant genomes (4).

Polycomb silencing is involved in many aspects of plant development (5, 6) and was found to be the mechanistic basis of vernalization, the acceleration of flowering by prolonged cold. Vernalization, one of the best examples of environmentally induced epigenetic regulation, is an adaptation that has evolved to enable plants to overwinter in the vegetative state and flower in spring, when conditions are favorable (7). Prolonged cold causes the down-regulation of the gene encoding the *Arabidopsis thaliana* floral repressor *FLC* (8, 9), and this repression then is maintained epigenetically during subsequent development of the plant until expression is reset in the embryo (10). This epigenetic silencing requires a Polycomb mechanism involving VRN2, one of the three *Arabidopsis* Su(z)12 homologues (11), and histone H3K27 methylation (12–14). A role for other PRC2 components CURLY LEAF (CLF), SWINGER (SWN), and FIE has been suggested through RNAi knockdown experiments and co-immunoprecipitation analysis (15). Mutant analysis has shown that stable silencing also requires VRN1, a DNA-binding protein with two plant-specific B3 domains (16); LHP1, the *Arabidopsis* HP1 homologue (17, 18); and two PHD finger proteins, VIN3, which is induced by prolonged cold (18), and VRN5 (19), also reported as VIL1 (20). VIN3 and VRN5 are two components of a five-member VEL gene family in the *Arabidopsis* genome (21); two other members, VEL1/VIL2 and VEL2/VIL3, also contain a PHD finger and a fibronectin III domain (19–21). All VEL proteins contain a conserved C-terminal region required for dimerization (19).

Current models of how these factors mediate vernalization have been based on comparison with other Polycomb systems and pairwise interaction analysis. Here, we characterize the association of the *trans* factors with *FLC* at different phases of vernalization and report the biochemical purification of the vernalization-specific Polycomb complex. We find both a constitutive association of VRN2 with *FLC* and a cold-induced association of the PHD finger protein VRN5 with a specific intronic domain in *FLC*. Purification of a VRN5 complex from vernalized seedlings showed formation of a vernalization-specific PHD complex, consisting of core PRC2 components (VRN2, SWINGER [an E(Z) HMTase homologue], FIE [ESC homologue], MSI1 [p55 homologue]), and three PHD finger proteins, VRN5, VIN3, and VEL1. Post-cold, VRN5 associates more broadly over *FLC* coincident with increased H3K27me3. Vernalization-induced epigenetic silencing of *FLC* therefore involves differential association and changed composition of distinct Polycomb complexes, a mechanism that shows many parallels with Polycomb silencing in mammals.

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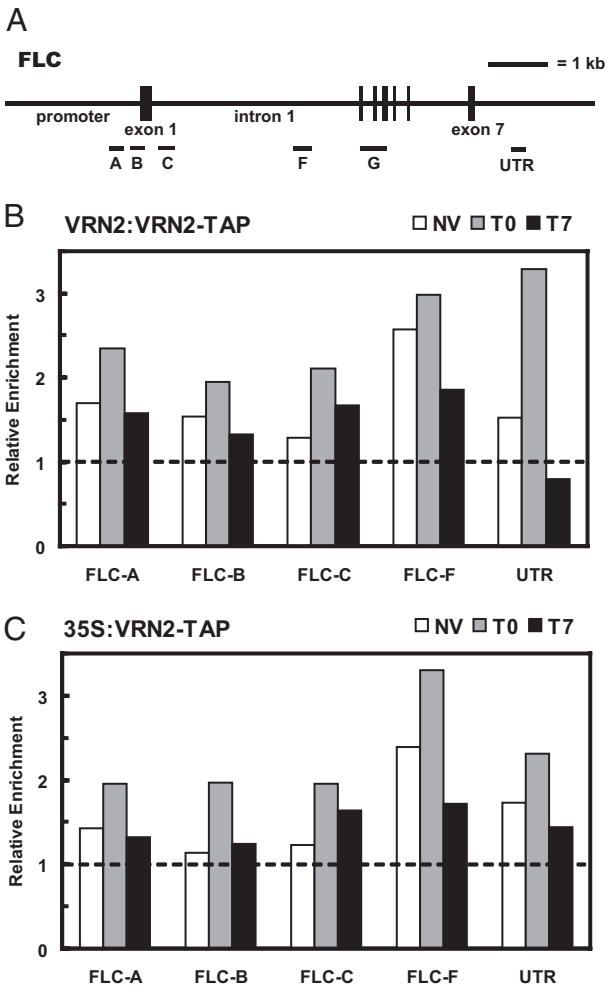


Fig. 1. VRN2 is constitutively associated with *FLC*. (*A*) Schematic representation of the *FLC* locus. (*B* and *C*) ChIP showing association of a VRN2-TAP protein fusion expressed under the control of the *VRN2* or the *35S* promoter with different *FLC* regions. VRN2-TAP is a carboxyl-terminal fusion of the tandem affinity purification (TAP) tag (38) to the VRN2 protein. The construct was transformed into the *vrn2–1 fca-1* mutant; both fusions rescue the *vrn2* phenotype. Q-PCR results are normalized relative to an *fca-1* control (not containing a TAP-tagged protein), represented as a dashed line. Each histogram shows the results from each fusion protein for one complete experiment; replicate experiments (Fig. S1) all showed enrichment relative to the control over the entire locus.

Results

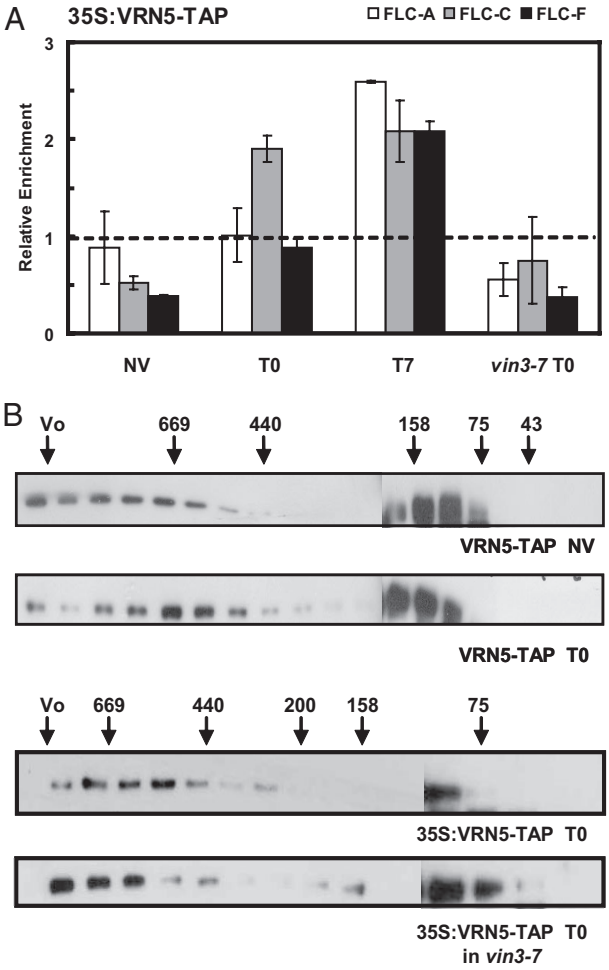
VRN2 Associates Constitutively Along the Whole *FLC* Locus, Whereas VRN5 Shows a Dynamic Association. We explored PRC2 complex localization at *FLC* by analyzing the association of VRN2, the Su(z)12 homologue required for vernalization, with the target *FLC* locus. Stable *Arabidopsis* lines were generated carrying complementing VRN2-tandem affinity purification (TAP) translational fusions, either as a fusion with its own promoter or with the strong viral 35S promoter. Chromatin immunoprecipitation (ChIP) assays on these lines showed VRN2 associates with all regions of the *FLC* locus analyzed [Fig. 1 and supporting information (SI) Fig. S1]. VRN2 association also was found at all phases of the vernalization process, with clear enrichment detected before cold (NV), immediately at the end of the cold (T0), and after subsequent growth in the warmth for 7 days (T7). A constitutive association of VRN2 is supported by several observations: increased *FLC* expression in nonvernalized *vrn2* seedlings (11, 19), H3K27me3 at *FLC* in nonvernalized plants (see Fig. 3*B* and ref. 22), and reduced H3K27me3 at *FLC* in nonvernalized *vrn2* seedlings (see Fig. 3*C*). Nevertheless, VRN2 (PRC2) association alone clearly is not sufficient to mediate the vernalization-induced repression of *FLC*, suggesting that other factors are involved.

In contrast to VRN2, the binding of the PHD finger VRN5 to *FLC* was much more specific. Using ChIP assays and nonvernalized *Arabidopsis* lines carrying a complementing 35S-VRN5-TAP fusion, we did not find VRN5 associated with *FLC* (Fig. 2*A*). In plants harvested immediately after prolonged cold (T0), VRN5 was found to localize to one specific *FLC* region near the 5′ end of intron 1 (Fig. 2*A*, region C). No association was detected with the *FLC* promoter, transcription start site, or central region of the gene. Specific VRN5 association with region FLC-C after prolonged cold was confirmed using independent complementing *Arabidopsis* lines carrying either a VRN5-TAP fusion fused to VRN5 promoter sequences (VRN5-VRN5-TAP) or a VRN5 fusion to EYFP rather than TAP (VRN5-VRN5-EYFP) (Fig. S2). After subsequent growth in the warmth following prolonged cold (T7), VRN5 was detected in all regions of *FLC* tested (Fig. 2*A*, regions A, C, and F). In all our ChIP experiments the enrichment of VRN2-TAP and VRN5-TAP lines at *FLC* was limited to 1.5- to 3-fold as compared with background from a control line not carrying the TAP-tag fusion. This low but reproducible enrichment may be caused by *FLC* being expressed in relatively few cells in the meristems and vasculature, whereas the chromatin was extracted from whole seedlings.

VRN5 has been shown to heterodimerize with the cold-inducible homologous PHD finger protein VIN3 (19). We therefore analyzed whether VRN5 association with *FLC* was dependent on VIN3 and found this to be the case (Fig. 2*A*). VIN3 has been reported to localize to the *FLC* promoter and intron 1 in plants equivalent to the T0 plants described here (18). However, we were not able to detect any significant association of VRN5 with *FLC* outside region C in T0 seedlings (Fig. 2*A*).

VRN5, VEL1, and VIN3 Are Components of a Vernalization-Specific PHD-PRC2 Complex. To investigate how VRN5 association with *FLC* related to VRN2 function and changed histone modification at *FLC*, we explored the *in vivo* protein partners of VRN5. Gel filtration analysis showed that in vernalized plants VRN5 forms a complex of ~ 650 kD (or complexes in the same size range) (Fig. 2*B Upper*). We therefore performed biochemical purification of the VRN5-VRN5-TAP complex from vernalized seedlings. *In vivo* partners of VRN5 were found to include core PRC2 components, the E(Z) homologue, SWINGER (23), the ESC homologue FIE (24), the p55 homologue MSI1 (25), and the Su(z)12 homologue VRN2, plus two other PHD finger proteins, VIN3 and VEL1 (Fig. 2*C* and Dataset S1). The higher abundance of VEL1 compared with VIN3 (Fig. 2*C*) might reflect specific degradation or release of VIN3 during complex isolation. Alternatively, VRN5 may be able to associate with VEL1 or VIN3. Yeast analysis showed that both VIN3 and VEL1 can heterodimerize with VRN5, but, unlike the other two, VRN5 did not homodimerize (19).

Gel filtration analysis of VRN5 complexes showed that VRN5 is present in a larger complex in nonvernalized seedlings than in vernalized seedlings (Fig. 2*B Upper*). It also showed that redistribution of VRN5 into the smaller MW complex is dependent on VIN3 (Fig. 2*B Lower*). This result is consistent with VRN5 incorporation into the vernalization-specific PRC2 complex at *FLC* occurring only after VIN3 induction. We interpret all these data as suggesting that prolonged cold results in Polycomb machinery assembling into a novel complex, which we call "PHD-PRC2," that localizes specifically to the 5′ end of intron 1. Subsequently, VRN5 distributes over a wider domain of *FLC*.



C

VRN5-TAP associated proteins

At Number	Name	Mass(KDa)	Peptides	Prot.Score	Drosophila
At3g24440	VRN5	66.8	21	593	Pcl (?)
At4g02020	SWN	95.3	16	433	E(z)
At4g30200	VEL1	75.8	17	290	Pcl (?)
At5g58230	MSI1	48.1	6	182	p55
At3g20740	FIE	41.2	5	127	Esc
At4g16845	VRN2	51.1	5	83	Su(z)12
At5g57380	VIN3	67.1	3	38	Pcl (?)

Fig. 2. VRN5 association with *FLC* and PHD-PRC2 complex composition. (*A*) ChIP showing association of 35S-VRN5-TAP protein with different *FLC* regions. 35S-VRN5-TAP is a carboxyl-terminal fusion to the TAP-tag that rescues a *vrn5–1 fca-1* mutant phenotype. Q-PCR results are normalized relative to the *fca-1* control (not containing a TAP-tagged protein), represented as a dashed line. The graph shows the average enrichment ± SE obtained from three independent ChIP experiments. Semiquantitative PCR analysis revealed no association of VRN5 in *FLC* regions B, G, and UTR in T0 plants (data not shown). (*B*) Size exclusion chromatography analysis of VRN5-TAP protein. VRN5-TAP protein eluted as part of a multiprotein complex and free protein of ≈ 88 kDa. Protein extracts were prepared from seedlings before vernalization (NV) or after cold treatment (T0) and separated through Superdex 200. Size markers (kDa) are indicated. Vo, void volume. (*C*) VRN5-TAP–associated proteins. Plants carrying a fully functional VRN5-TAP fusion expressed under the control of the endogenous promoter were harvested after 8 weeks cold and used in TAP-tag affinity purification. Protein components were identified using LC-MS/MS (Dataset S1). The list includes the proteins previously reported to be involved in vernalization and Polycomb function by genetic or molecular analysis. A protein was judged to be associated with the VRN5-TAP protein when it was absent from the vernalized *fca-1* control material taken through the same purification procedure. Full details on the peptides and scores from the TAP purification and control sample are included in *SI Text* available on line as an Excel spreadsheet.

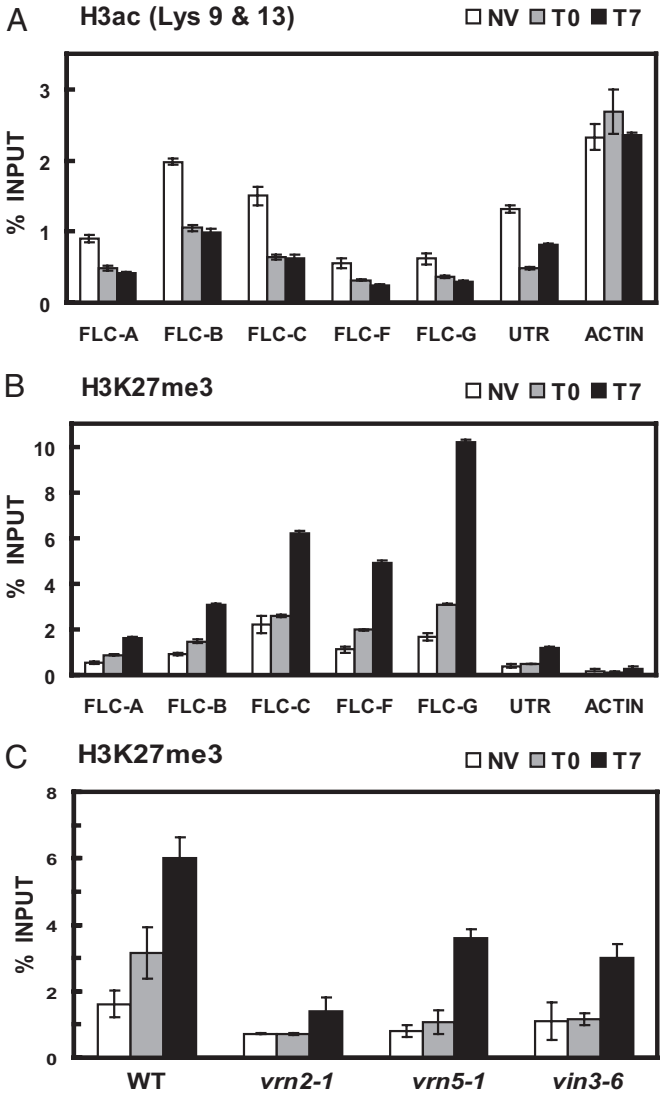


Fig. 3. Histone modifications at the *FLC* locus. Immunoprecipitated DNA was analyzed by real-time qPCR, and enrichment was represented as percentage of INPUT (% INPUT). All ChIP were normalized for histone H3 occupancy. Data in the graphs are the average of four qPCR assays from two independent ChIP experiments; the bars represent standard error. (*A*) ChIP analysis of histone H3 acetylation. (*B*) ChIP analysis of histone H3K27 trimethylation. (*C*) qPCR results of H3K27me3 ChIP for region FLC-C in plants carrying a *vrn2*, *vrn5*, or *vin3* mutation. Region C is shown, but the same profile was also observed for all other *FLC* regions.

VRN5 and VIN3, Like *Drosophila* Pcl and Human PHF1, Are Required for High Levels of H3K27 Trimethylation. To understand how PHD-PRC2 association at the 5′ end of intron 1 (region C) might affect *FLC* chromatin, we explored the profile of a number of histone modifications. Consistent with an important role in *FLC* regulation, histone H3 acetylation (H3Ac) levels were highest around the 5′ end of the gene in regions B and C in nonvernalized plants and decreased significantly in plants exposed to prolonged cold, when PHD-PRC2 is associated (Fig. 3*A*). H3K27me3 at *FLC* mirrored VRN2 distribution (Fig. 1) and covered the whole locus in both nonvernalized and vernalized plants (Fig. 3*B*). After the cold, and coincident with redistribution of VRN5 along the *FLC* gene, H3K27me3 levels increased across the entire locus (Fig. 3*B*) (14).

It is tempting to speculate that the *Arabidopsis* PHD-PRC2 complex is analogous to the Pcl-PRC2 complex in flies (26) and

the PHF1-PRC2 complex in humans (27, 28), both of which lead to substantial increases in H3K27me3 and both of which have PHD proteins associated with core PRC2 machinery. This functional analogy is supported by the quantitative analysis of H3K27me3 levels in *vrn2*, *vrn5*, and *vin3* mutants (Fig. 3*C*). Loss of VRN2 led to a greater reduction in H3K27me3 at *FLC* after vernalization (Fig. 3*C*) than seen with the loss of either VRN5 or VIN3. Therefore, high levels of H3K27me3 seem to be required for stable silencing, and the PHD proteins play a critical role in enhancing PRC2 activity to achieve these levels. This situation is similar to the one described for *Drosophila* and mammals in which *su(z)12* mutations abolish lysine 27 methylation, whereas *Drosophila* Pcl, or its human homologue PHF1, is required to generate high levels of H3K27me3 (26–28). However, *Drosophila* Pcl seems to be dispensable for histone 3 lysine 27 dimethylation (H3K27me2) (26), but reports concerning mammalian PHF1 are contradictory (27, 28). H3K27m2 increases after vernalization over the whole *FLC* locus, and this increase is VRN5/VIN3 dependent (Fig. S3*A* and *B*) (18, 20).

Discussion

We have investigated the mechanism by which prolonged cold induces Polycomb silencing of the *Arabidopsis FLC* locus during different phases of vernalization. We found a constitutive association of the core PRC2 component, VRN2, across the whole *FLC* locus (Fig. 1). This widespread distribution of VRN2 over *FLC* is unlike the localized Su(z)12 at Polycomb Response Elements in *Drosophila Hox* genes (29, 30) and is more similar to the "blanket type" binding of SUZ12 at the *Hox* loci in proliferating human embryonic fibroblasts (31) and at genes encoding developmental regulators in human ES cells (32, 33). This pattern of PRC2 complex distribution throughout the locus also has been described for CLF distribution at the *Arabidopsis AGAMOUS* target (13). Surprisingly, the association of VRN2 is not dependent on the transcriptional state of the target locus (Fig. 1). Instead, we found that vernalization-induced *FLC* repression is triggered by the association of the PHD finger protein VRN5 to a specific domain in *FLC* intron 1 during cold, in a process that depends on the cold-induced PHD protein VIN3. This intronic domain is included in a region defined as required for repression by vernalization in a characterization of *FLC cis* elements (34), but further analyses will be required to show whether this specific region of *FLC* intron 1 could act as a Polycomb Repressive Element on its own. It will be interesting to investigate whether VRN5 binding to this intronic region is dependent on VRN2. However, this analysis could be complicated by redundancy with other *Arabidopsis* Su(z)12 homologues, such as EMBRYONIC FLOWER 2 (EMF2) (23). Genetic analysis has shown that *vrn2* does not block the vernalization response completely (19), and double mutant analysis has revealed that VRN2 and EMF2 have some overlapping functions (6). In plants returned to warmth, VRN5 distribution changes, and it associates more broadly over *FLC*, coincident with significant increases in H3K27me3, maintaining the silencing of *FLC* throughout the rest of the life cycle (Fig. 2*A*).

Genetic analyses have defined a number of PcG factors in *Arabidopsis* (5, 6), and several groups have used different approaches in attempting to address the issue of the composition of the vernalization-specific PRC2 complex (15, 23). To date, however, no PRC2 complex has been isolated from plants biochemically. Biochemical purification of VRN5-TAP–tagged protein showed that the PHD protein VRN5 forms a protein complex with its homologues VIN3 and VEL1 and with the PRC2 core components VRN2 (Su(z)12 homologue), SWINGER (EZ HMTase homologue), FIE (ESC homologue), and MSI1 (p55 homologue) (Fig. 2*C* and Dataset S1). This result is consistent with a previous report of the presence of VRN2 in a complex containing VIN3, FIE, CLF, and SWN shown by a candidate pulldown approach (15). However, probably because of redundancy, the genetic analysis had been unable to confirm a role for these components in the vernalization response: (*i*) *clf* or *swn* mutants had no defect in flowering time after vernalization (J. Goodrich, personal communication); (*ii*) MSI1 has been reported to regulate seed development but to have no role in *FLC* regulation (35); and (*iii*) RNAi knockdown analysis of VEL1 did not reveal a function for this PHD finger protein in vernalization (19). The direct *in vivo* identification of the components of the vernalization-specific PRC2 complex removes these ambiguities. It shows which of the *Arabidopsis* E(z) homologues, SWN, CLF, or MEDEA, plays the predominant role in cold-induced repression of *FLC* (15), unambiguously identifies MSI1 as a member of the vernalization complex, and allows the identification of VEL1 as a new vernalization component. Further experiments are underway to address the role of VEL1 in vernalization, to see if VEL1 also distributes more widely along *FLC* chromatin post-cold, and to assess whether the triple VEL-PHD mutant *vin3vrn5vel1* is completely insensitive to vernalization. The VEL family is conserved between *Arabidopsis* and cereal species (Ref. 36 and J. Cockram, D. Laurie, and C.D., unpublished result), so it will be interesting to see if this family of proteins has a conserved function in vernalization across a wide range of species.

The PHD-PRC2 complex shows interesting similarities with the recently characterized Pcl-PRC2 complex in *Drosophila* and PHF-PRC2 in mammals (26–28). Absence of the PHD proteins in each case led to reduced H3K27me3 levels, suggesting that PHD proteins are required for high levels of H3K27 trimethylation which in turn are required for stable epigenetic repression of the target gene. *In vitro* activity analysis of Pcl-PRC2 showed Pcl increases methyltransferase activity of the PRC2 complex (26). In plants the three VEL proteins, each with a single PHD, might serve the same function as one protein with two PHDs, but how VRN5/VIN3/VEL1 increases *Arabidopsis* PRC2 function and the degree of functional redundancy of the VEL proteins remains to be established. No clear sequence homology is found between the *Arabidopsis* VEL family members and *Drosophila* Pcl or mammalian PHF1, possibly suggesting independent evolution of a similar mechanism.

In summary, we have defined a sequence of events required for the vernalization-induced repression of *FLC* by a Polycomb mechanism (Fig. 4). Despite the functional analogy of the players of the Polycomb system in *Drosophila* and *Arabidopsis*, we find the patterns of PRC2 localization and H3K27me3 differ. For the *Drosophila Hox* loci PcG protein complexes constitutively assemble in a highly localized fashion at the Polycomb Repressive Element, often located many kilobases from the target, and changes in H3K27me3 distribution define the transcriptional state of the target gene (29, 30). For *Arabidopsis FLC* silencing, both the composition of the PRC2 complex and its distribution along the *FLC* locus change during vernalization, leading to increased but not changed distribution of H3K27me3 levels. In our view, this process is more similar to what happens at certain targets in human nondifferentiated cells (31) in which specific developmental signals can trigger PcG-induced repression via Polycomb proteins already associated with the target locus. This mechanism therefore may extend to other plant targets and be relevant to Polycomb silencing in other organisms.

Materials and Methods

Plant Material and Growth Conditions. For nonvernalizing plants, seeds were surface sterilized and sown on MS media plates (no glucose), stratified at 4°C for 2 d, and grown for 10 d in long-day conditions (16 h light at 20°C, 8 h darkness at 16°C). For vernalized plants, seeds were surface sterilized and sown on MS media plates (no glucose), pregrown for 7 days, and then transferred to 4°C under short-day conditions (8 h light, 16 h darkness) before being returned to long-day conditions (16 h light at 20°C, 8 h darkness at 16°C).

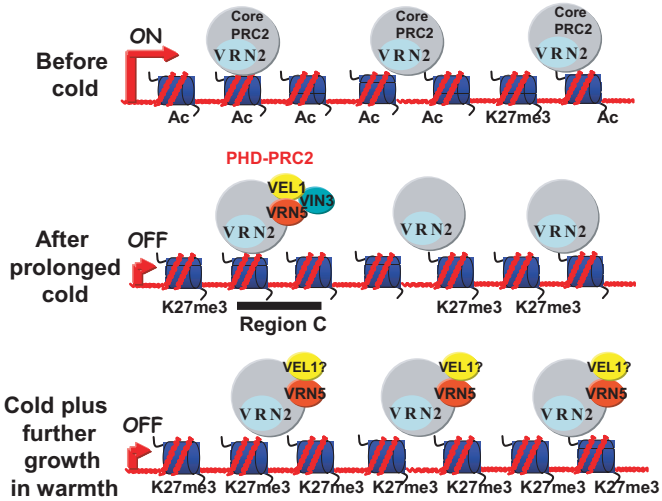


Fig. 4. Possible sequence of events in the assembly and association of specific Polycomb complexes at different stages of epigenetic silencing of *FLC* during vernalization. In nonvernalized plants the PRC2 core complex is associated with the whole *FLC* locus. Prolonged cold leads to VRN5 localization specifically at region C as part of the PHD-PRC2 complex with VIN3 and VEL1 and to a decrease in histone acetylation. In plants returned to warm conditions, VIN3 is no longer expressed, and VRN5 associates more widely throughout the locus, concomitant with a significant increase in H3K27 trimethylation.

Plants were vernalized for 8 weeks for the VRN2-TAP ChIP experiments, analysis of histone modifications, and TAP-tag purification and were vernalized for 6 weeks for the VRN5-TAP ChIP experiments and gel filtration analysis. T0 seedlings were harvested immediately after prolonged cold, whereas T7 seedlings were grown for a further 7 days in the warmth after transfer from cold. All genotypes were in a Landsberg *erecta* background; an active *FRIGIDA* had been introduced by transformation to generate vernalization-responsive lines (11, 19).

Chromatin Immunoprecipitation and Real-Time Quantitative PCR Analysis. ChIP assays were performed as previously described (37). For the TAP-tag ChIP the same protocol was followed with minor modifications: chromatin preparations were precleared for 1 h at 4°C with 100 μl of Sepharose 6 Fast Flow beads (GE Healthcare Bio-Sciences) and then were immunoprecipitated overnight at 4°C with 100 μl of IgG Sepharose 6 Fast Flow beads. All ChIP experiments were quantified by RT qPCR. For the TAP-tag ChIP, data are represented as the ratio of ChIP (FLC/ACTIN)/INPUT (FLC/ACTIN) of the different samples normalized to *fca-1* (a line not carrying the TAP-tag). For the histone modifications, we estimated the absolute fraction of DNA recovered from the INPUT (%INPUT) by comparing the reaction threshold cycle (CT) of the ChIP sample to a dilution of its own INPUT. Specific histone modifications were analyzed using anti-acetyl-histone H3 lysine 9 and 14 from Upstate Biotechnology (catalog no. 06–599), or anti-trimethyl-histone H3 lysine 27 from Thomas Jenuwein (rabbit 6523, 5 bleed). Histone H3 levels were assayed using anti-H3 core antibody from Abcam, and VRN5−:VRN5–EYFP was detected using an anti-GFP antibody from Invitrogen.

Oligonucleotides. 5′Af (aggcgagtggttctttgtttt)3′, Ar (ctttgctacttttgcattgcc); Bf (ttcaagtcgccggagatact), Br (cgtggcaatcttgtcttcaa); Cf (acctgggttttcatttgttcc), Cr (tcactcaacaacatcgagcac); Ff (tgaactcatgaaagaggcgtt), Fr (gactgcttccaattcatttgc); Gf (ttgatcgatatgggaaacagc), Gr (caaggtgttcctccagttgaa); UTR-f (caaaaggttgaggaactttgtacc), UTR-r (ccttcatggatgacggaacta); Actin-f (cgttcgctttccttagtgttagct), Actin-r (agcgaacggatctagagactcaccttg).

TAP-Tag Purification. The TAP purification protocol described in Ref. 38 was used with modifications. Seedlings (≈ 65 g fresh weight), grown on MS media plates (no glucose) for 1 week under long-day conditions and then vernalized for 8 weeks, were harvested in liquid nitrogen and ground to a powder using dry ice and a coffee blender. The powder was thawed in two volumes of extraction buffer (39) containing 1% Igepal, 10 mM β-mercaptoethanol, 2 mM benzamidine, 1 mM PMSF, 10 μM 3,4-dichloroisocoumarin, and ''complete'' protease inhibitor mixture (Roche). Proteins were extracted on a rotating platform for 1 h at 4°C, centrifuged twice at 14000 *g* for 30 min at 4°C, and filtered through a single layer of Miracloth. Centrifuged extracts were incubated with IgG Sepharose 6 ''Fast Flow'' beads (500 μl of beads for every 35 ml of protein extract) overnight at 4°C and were washed five times with 10 ml of extraction buffer with 0.1% Nonidet P-40, without inhibitors, and once with 10 ml of TEV cleavage buffer (50 mM Tris-HCl, pH 8,0, 150 mM NaCl, 0,1% IGEPAL, 0,5 mM EDTA, 1 mM dithiothreitol [DTT]). Elution from the IgG beads was performed by incubation for 4 h at 4°C with 200 U of TEV protease in cleavage buffer containing 1 μM E-64. Pooled eluates were incubated overnight at 4°C with 1.4 ml of calmodulin resin (50% slurry) in calmodulin-binding (CB) buffer (10 mM Tris-HCl, pH 8,0, 150 mM NaCl, 0,1% IGEPAL, 10 mM β-mercaptoethanol, 1 mM Mg acetate, 1 mM imidazole pH 8.0, 2 mM $CaCl_2$) containing 1 μM of E-64. Unbound material was eluted by gravity flow. Beads were washed with 10 ml of CB buffer and then with 40 ml CB buffer without Igepal and E64. Elution from the beads was done with 5 ml of calmodulin-elution buffer (10 mM Tris-HCl, pH 8,0, 150 mM NaCl, 10 mM β-mercaptoethanol, 1 mM Mg acetate, 1 mM imidazole pH 8.0, 10 mM EGTA). The eluate was trichloroacetic acid precipitated and washed twice with chilled acetone.

Liquid Chromatography-Mass Spectroscopy/Mass Spectrometry Methods. Protein pellets were resuspended in 100 mM ammonium bicarbonate with 0.1% Rapigest (Waters). Samples were heated to 95°C for 5 min and sonicated for a further 5 min before reduction with DTT and alkylation with iodoacetamide. Proteins were digested with trypsin (Promega) at 37°C overnight. Rapigest detergent was removed by the addition of 0.1% TFA, incubation at 37°C for 30 min, and centrifugation. Samples were lyophilized, and peptides were dissolved in 1% acetonitrile, 0.1% formic acid for MS analysis. Liquid chromatography (LC)-MS/MS analysis was performed using a LTQ Orbitrap mass spectrometer (Thermo Electron Corp.) and a nanoflow-HPLC system (Surveyor, Thermo Electron). Peptides were applied to a precolumn (C18 pepmap100, LC Packings) connected to a self-packed C18 8-cm analytical column (BioBasic resin, ThermoElectron; Picotip 75 μm i.d., 15 μm tip, New Objective). Peptides were eluted by a gradient of 2% to 30% acetonitrile, in 0.1% formic acid, over 40 min at a flow rate of ≈ 250 nL min^{-1}. MS1 were acquired in the Orbitrap at resolution 60,000. Up to four data-dependent MS/MS acquisitions (the four most abundant ions in each cycle) were acquired in the LTQ mass spectrometer for the range of 300:1700 *m/z*; a minimum signal of 1000 ion counts were required to trigger MS/MS; a collision energy 35% was used; and dynamic exclusion was applied after 1 repeat hit, for 60 sec. In all cases the mass spectrometer was operated in positive ion mode with a nano-spray source and a capillary temperature of 175°C. No sheath gas was used, and the source voltage and focusing voltages were optimized for the transmission of the peptide MRFA. Peak lists (as .dta) files were extracted by extract_msn (ThermoFisher Corp.), the dta generation parameters were: range 300.00–3500.00; MW; an absolute minimum intensity threshold of 1000 ion counts; MS/MS scan were combined for the same precursor ion if they were within the range of 20 scans, there was no minimum requirement to combine scans; the individual minimum ion count was 10 ions, charge state was assigned automatically using ZSA processing (default values); MS level was determined automatically. Dta files were merged with merge.pl (Matrix Science) and searches with Mascot version 2.2 (Matrix Science) against the *Arabidopsis* genome supplemented with common contaminants (TAIR7 plus trypsin, keratins; sequences collated by Thermo Electron Corp. resulting in 32721 sequences; 13342617 residues) with the fixed modification being carbamidomethyl Cys and the variable modification being oxidized Met. Mass values: monoisotopic protein mass, unrestricted; peptide mass tolerance, ± 5 ppm; fragment mass tolerance, ± 0.6 Da. Two missed cleavages were allowed with trypsin. A second Mascot search was performed allowing error-tolerant modification of all robustly identified proteins from the previous round. Identified peptides were validated further with the use of Scaffold software (Proteotype).

Gel Filtration Analysis. Protein complexes were extracted from 0.4 g of *Arabidopsis* seedlings (grown as described previously). Seedlings were harvested into liquid nitrogen, ground with a chilled drill bit, and thawed in TAP extraction buffer (39) (1:2 w:v) without Igepal and containing 10 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, 10 μM 3,4-dichloroisocoumarin, and ''complete'' protease inhibitor mixture (Roche). Proteins were extracted on a rotating platform for 1 h at 4°C and centrifuged twice at 20,000 *g* for 10 min at 4°C. Extracts were concentrated by using Centricon columns (Microcon YM-10) and quantified. Size standards (HMW Gel filtration calibration kit, Amersham) or equal amounts of extracts (≈ 500 μg) were loaded onto a Superdex 200 column (Amersham) attached to an Akta FPLC system (Amersham) using extraction buffer without protease inhibitors as running buffer. Fractions of 0.5 ml were collected on elution from the column and concentrated by addition of 10% (vol/vol) of Strataclean resin (Stratagene). The resin was

resuspended in 30 μl SDS/PAGE loading buffer, vortexed, and boiled for 5 min. After centrifugation, the supernatant was electrophoresed on 10% SDS/PAGE and blotted using PAP antibody (Sigma).

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