The *Arabidopsis thaliana* vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3

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In *Arabidopsis thaliana*, the promotion of flowering by cold temperatures, vernalization, is regulated via a floral-repressive MADS box transcription factor, FLOWERING LOCUS C (FLC). Vernalization leads to the epigenetic repression of *FLC* expression, a process that requires the polycomb group (PcG) protein VERNALIZATION 2 (VRN2) and the plant homeodomain protein VERNALIZATION INSENSITIVE 3 (VIN3). We demonstrate that the repression of *FLC* by vernalization requires homologues of other Polycomb Repressive Complex 2 proteins and VRN2. We show *in planta* that VRN2 and VIN3 are part of a large protein complex that can include the PcG proteins FERTILIZATION INDEPENDENT ENDOSPERM, CURLY LEAF, and SWINGER. These findings suggest a single protein complex is responsible for histone deacetylation at *FLC* and histone methylation at *FLC* in vernalized plants. The abundance of the complex increases during vernalization and declines after plants are returned to higher temperatures, consistent with the complex having a role in establishing *FLC* repression.

epigenetics | polycomb group proteins

Many plants require an extended period of cold to initiate the transition from vegetative to reproductive growth, a process known as vernalization. Vernalization of the apical meristem may precede, sometimes by many months, the transition of the meristem from vegetative to floral growth (1). In *Arabidopsis*, vernalization down-regulates the expression of *FLOWERING LOCUS C* (*FLC*), a repressor of the floral transition (2, 3). The length of the vernalization period correlates quantitatively with the degree of repression of *FLC* (4). In aerial tissues, the repressed level of *FLC* expression is maintained throughout subsequent growth (5). Regardless of the length of vernalization treatment and the degree of reduction in *FLC* activity, *FLC* expression is reset to normal levels in the next generation. These characteristics of *FLC* expression suggest that vernalization regulates *FLC* via an epigenetic mechanism.

The vernalization response in *Arabidopsis* requires the proteins VERNALIZATION 2 (VRN2; ref. 6) and VERNALIZATION INSENSITIVE 3 (VIN3; ref. 7). Loss of function of either protein leads to a loss of the vernalization response and a failure to down-regulate *FLC* after vernalization.

VRN2 is homologous to the *Drosophila melanogaster* polycomb group protein Suppressor of Zeste 12 [Su(Z)12; ref. 8]. Su(Z)12 is a component of Polycomb Repressive Complex (PRC) 2, which regulates genes in a number of developmental pathways including those involving homeotic genes (9–11). PRC2 complexes have four core protein components: Su(Z)12, Extra Sex Combs (ESC), Enhancer of Zeste [E(Z)] and P55 (12–15). The core PRC2 complex is ≈600 kDa, and it can be associated with additional proteins including Polycomb-like (PCL) and the histone deacetylase RPD3 (16) in a complex of 1,000 kDa. The E(Z) protein has histone 3 lysine 27 (H3K27) methyltransferase activity; addition of this mark of inactive chromatin is thought to be the basis of the repression of gene expression by PRC2 (17). *Arabidopsis* has homologues of PRC2 proteins that are required for the regulation of various developmental pathways (18). The best characterized is the FIS repression complex, a 650-kDa complex involved in seed development that includes FERTILIZATION INDEPENDENT ENDOSPERM (FIE), MEDEA (MEA), MULTICOPY SUPPRESSOR OF IRA1 (MSI1), and FERTILIZATION INDEPENDENT SEED 2 (FIS2), homologues of ESC, E(Z), P55, and Su(Z)12, respectively (19, 20).

VIN3 is a member of a plant-specific protein family with plant homeodomain (PHD) and fibronectin 3 (FNIII) domains (7). VIN3 protein binds to regions of the promoter and first intron of *FLC*. Unlike the constitutively expressed VRN2 mRNA, the VIN3 mRNA is present at very low abundance during growth at warm temperatures, with expression increasing progressively during a vernalization treatment and returning to prevernalized levels when the plant is returned to normal temperatures (7). This cold-driven accumulation of VIN3 mRNA may be part of a mechanism to time the duration of vernalization and ensure that short cold periods do not promote flowering.

The repression of *FLC* expression after vernalization is accompanied by modifications to histones associated with the *FLC* locus. In nonvernalized plants, FLC chromatin has high histone H3 acetylation (H3Ac) and H3K4 trimethylation (me3), marks of active chromatin but low levels of the inactive marks H3K9me2 and H3K27me2. After vernalization, H3Ac and H3K4me3 are reduced and H3K9me2 and H3K27me2 are increased (7, 21, 22). These changes suggest that the formation of a repressed chromatin state at *FLC* after vernalization is the basis of the epigenetic regulation of *FLC*. Loss of VIN3 function prevents loss of H3Ac and methylation of H3K9 and H3K27 in vernalized plants. In *vrn2* mutants, vernalization gives a transient loss of H3Ac but there is no methylation of H3K9 or H3K27 after vernalization (7). These data suggest that VIN3 may recruit a histone deacetylase to *FLC* and that VRN2 acts as part of a PRC2-like complex to methylate histone H3 to epigenetically repress *FLC* expression in vernalized plants.

In this paper, we use epitope-tagged proteins to show that VRN2 is associated with the polycomb group protein homologues FIE, SWINGER (SWN; also known as EZA1; ref. 23), and CURLY LEAF (CLF) in a PRC2-like complex and that these proteins are required for the repression of FLC by vernalization. We also show that VRN2 and VIN3 can be part of the same protein complex, suggesting a physical link between these two components of the vernalization response mechanism.

Results

VRN2 Protein Abundance Increases During Vernalization. The *vrn2-1* mutant in the late flowering *fca-1* background does not flower

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Abbreviations: LD, long day; PRC, polycomb repressive complex.

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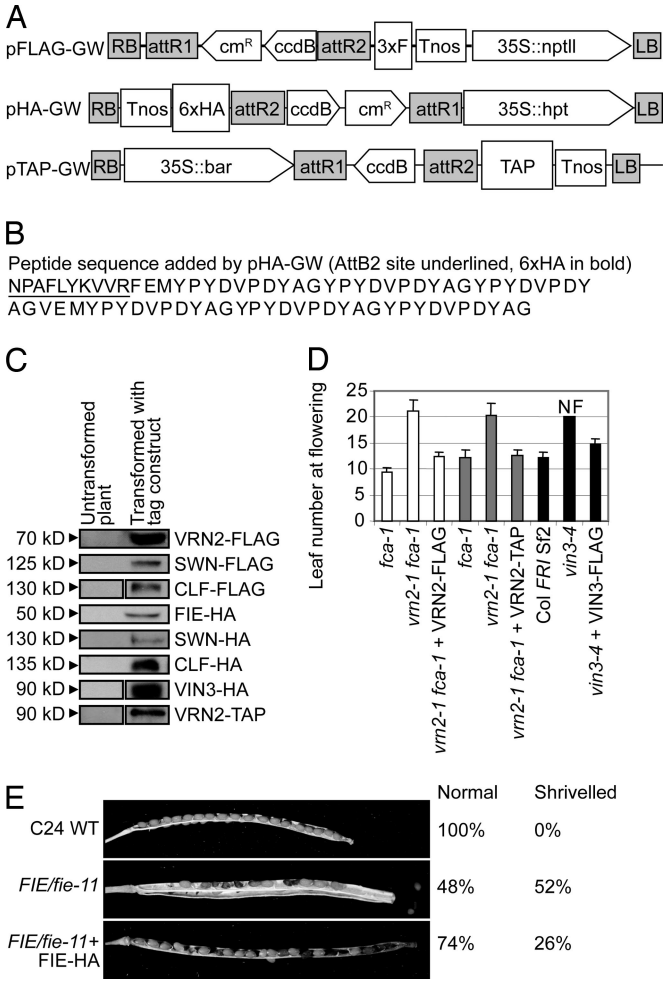

Fig. 1. Epitope tagging constructs, protein expression, and complementation of mutant phenotypes. (*A*) Outline of the T-DNA region of pFLAG-GW (pCH252; ref. 24), pHA-GW, and pTAP-GW. RB, T-DNA right border; 35S, CaMV 35S promoter; *npt* II, neomycin phosphotransferase; hpt, hygromycin phosphotransferase; Tnos, nopaline synthase terminator; cm^R, chloramphenicol-resistance gene; ccdB, conditional cell death B gene; 3X F, 3X FLAG epitope; LB, T-DNA left border; attR1 and attR2, Gateway recombination sites; 6xHA, 6x HA epitope; bar, phosphinothricin-resistance gene; TAP, tandem affinity purification epitope. (*B*) Additional peptide sequence added to proteins expressed from pHA-GW. (*C*) Western blot detection of VRN2-FLAG, SWN-FLAG, CLF-FLAG, FIE-HA, SWN-HA, CLF-HA, VIN3-HA, and VRN2-TAP in transformed *Arabidopsis* plants. Estimates of fusion protein size are indicated based on migration relative to protein standard markers. (*D*) Leaf number at bolting of *fca-1*, *vrn2-1 fca-1*, *vrn2-1 fca-1* + VRN2-FLAG (T2 plants), *vrn2-1 fca-1* + VRN2-TAP (T3 plants), Col^{FRISf2}, *vin3-4*, and *vin3-4* + VIN3-FLAG (T2 plants). Seed from transgenic lines and control lines were vernalized on plates for 4 weeks then transferred to LD. Values are the average rosette leaf number at flowering, error bar is SD. Experiments with VRN2-FLAG (open columns), VRN2-TAP (gray columns), and VIN3-FLAG (black columns) were conducted separately (NF; not flowered). (*E*) The FIE-HA construct was used to transform heterozygous *fie-11* (25), and the phenotypes of seeds in the siliques of the resulting T1 plants scored as normal or shriveled. Seed from heterozygous *FIE/fie-11* plants are 50% shriveled (26) due to the maternal embryo lethality of *fie*. *FIE/fie-11* plants carrying the FIE-HA transgene are 74% wild type, showing that the *fie-11* mutation is complemented (see *Supporting Text*, which is published as supporting information on the PNAS web site).

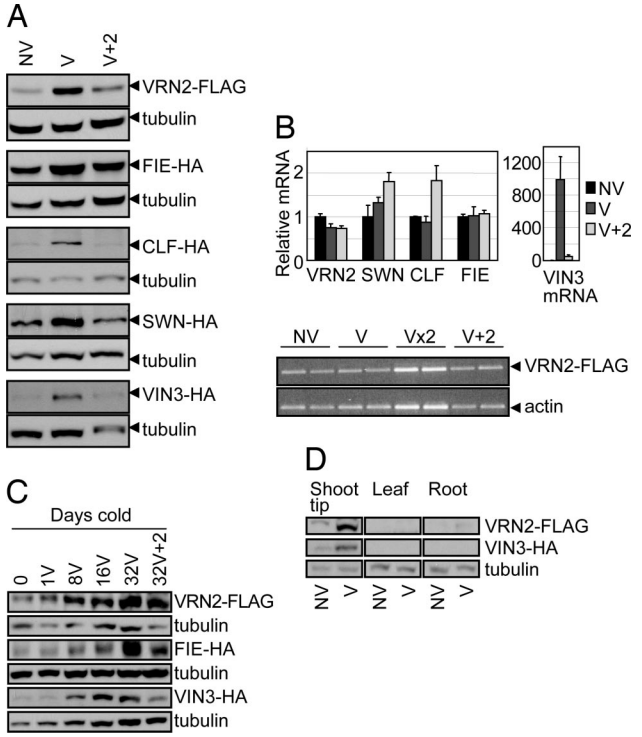

Fig. 2. Expression of VRN2-FLAG, SWN-HA, CLF-HA, FIE-HA, and VIN3-HA proteins. (*A*) Western blot analysis of total protein extracts from plants expressing VRN2-FLAG, SWN-HA, CLF-HA, FIE-HA, or VIN3-HA grown for 14 LD (NV), 12 LD + 4-week vernalization (V), and 12 LD + 4-week vernalization + 2 LD, (V + 2). Blots were reprobed with anti-tubulin antibody to confirm uniform loading. (*B*) Quantitative RT-PCR analysis of expression of endogenous VRN2, SWN, CLF, FIE, and VIN3 mRNAs in wild-type C24 plants (error bars are SD) and RT-PCR analysis of VRN2-FLAG mRNA, (Vx2, a PCR with twice the amount of cDNA added to demonstrate a 2-fold difference in mRNA content could be detected). (*C*) Time course of VRN2-FLAG, FIE-HA, and VIN3-HA protein expression. Plants were grown for 12 LD then transferred to 4°C, samples were taken after 0, 1, 8, 16, and 32 days at 4°C and 32 days at 4°C followed by 2 LD. (*D*) Organ specificity of VRN2-FLAG and VIN3-HA protein expression. Proteins extracted from shoot tips, leaves (cotyledons and first leaf pair), and roots of plants grown for 14 LD (NV) or 12 LD followed by 32 days at 4°C (V).

early in response to vernalization (6). A genomic VRN2-FLAG fusion construct (Fig. 1) complemented the *vrn2-1* mutant (Fig. 1*D*) so it is likely that the fusion protein is correctly expressed and has normal VRN2 function. The VRN2-FLAG protein is present in nonvernalized plants, shows a marked increase in abundance after 4 weeks of vernalization, and returns to approximately prevernalized levels when plants are moved back to higher temperatures after vernalization (Fig. 2*A*). VRN2-FLAG (and endogenous VRN2) mRNA abundance does not change in vernalized plants (Fig. 2*B*), so the change in VRN2-FLAG protein abundance is likely to be due to altered translation rate or protein stability.

The E(Z) homologue MEA is expressed predominantly in the female gametophyte and developing seed but not in mature seeds and seedlings (27), so it is unlikely to be involved in the vernalization response. The two other E(Z) homologues, CLF and SWN, are mainly expressed in the shoot tip (28, 29); both interact with the VEFS domain of VRN2 in a yeast two-hybrid assay (29) and, so together with the single ESC homologue, FIE, they are potential members of a complex with VRN2. Western blot analysis of the expression of HA-tagged versions of the FIE, CLF, and SWN genes (Fig. 1) showed a similar increase in all three proteins at the end of vernalization, with decreased expression after plants were returned to the higher temperature regime (Fig. 2*A*). Because the endogenous mRNAs encoding these proteins did not show a corresponding change (Fig. 2*B*), we concluded that these changes are a posttranscript effect as observed for VRN2-FLAG.

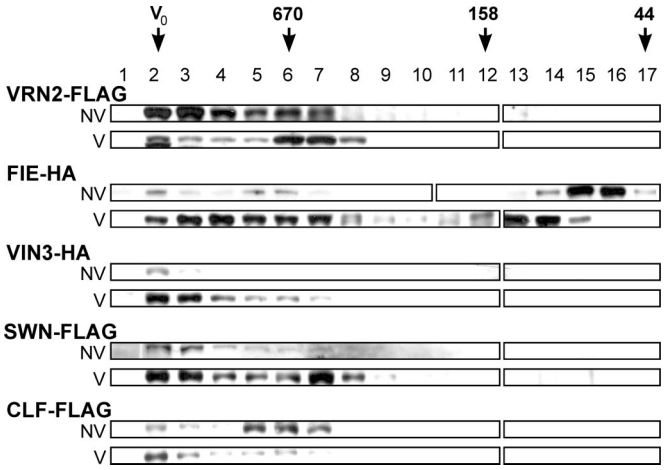

Fig. 3. Size exclusion chromatography analysis of VRN2, FIE, SWN, CLF, and VIN3 epitope-tagged proteins in native extracts before and after vernalization. Protein extracts from plants before vernalization (NV) or after 4-week cold treatment (V) and separated through Superdex S200. Size markers (kilodaltons) are indicated along the top (V_o, void volume).

The mRNA-encoding VIN3 is expressed at a low level in normal temperatures (Fig. 2*B*) and accumulates during vernalization, proportional to the duration of the cold treatment. It rapidly declines after a return to normal conditions after vernalization (ref. 7; Fig. 2*B*). When VIN3-HA (Fig. 1) is expressed in *Arabidopsis thaliana*, the protein abundance at the end of vernalization reflects that of VIN3 mRNA (Fig. 2 *A* and *B*). To test the relationship between the accumulation of PRC2 homologues and that of VIN3 during vernalization, VRN2-FLAG, FIE-HA, and VIN3-HA plants were sampled after different lengths of cold treatment (Fig. 2*C*). All of the proteins showed progressive increases in abundance with increasing time in the cold and reduced abundance after plants were returned to higher temperature conditions. The parallel changes in expression of these proteins during vernalization suggest a common regulation. The expression of VIN3, CLF, and SWN in the shoot tip (7, 28, 29), similar to VRN2 (Fig. 2*D*), is consistent with data showing that vernalization perception and response is located in the shoot apical region (30).

VRN2 and VIN3 Are Components of High Molecular Mass Protein Complexes. If VRN2 is acting as part of a PRC2-like complex, it should be in a high molecular mass fraction with other PRC2 components in native protein extracts. In the inflorescence, the PRC2 components FIE, MEA, and MSI1 are present in a 650-kDa complex (19); FIE also occurs as a monomeric protein. Proteins were separated from plants expressing VRN2-FLAG, FIE-HA, SWN-FLAG, CLF-FLAG, and VIN3-HA by size exclusion chromatography by using a Superdex S200 column, which separated components from 40 kDa to ≈1,000 kDa. FIE-HA was present in low molecular mass fractions and in fractions between 600 and 1,000 kDa (Fig. 3). VRN2-FLAG, SWN-FLAG, and CLF-FLAG were detected only in the 600- to 1,000-kDa fractions (Fig. 3*A*), similar to the reported sizes of PRC2 complexes in *Drosophila* (16), but extending to higher molecular masses than the 650-kDa FIE–MEA–MSI1 complex. The wide size range of the protein complexes suggests that these proteins may be present in complexes with different compositions. The VIN3-HA protein mostly eluted at ≈1,000 kDa in extracts from vernalized plants. Separation of VRN2-FLAG, FIE-HA and VIN3-HA proteins on a Sephacryl S500 column showed that the proteins present in the void volume of the S200 column migrate at ≈1,000 kDa (data not shown). There was no apparent difference in the sizes of complexes between nonvernalized and vernalized plants (Fig. 3).

VRN2 Is Part of a Complex with the Polycomb-Like Proteins FIE, CLF, and SWN. To determine whether VRN2 is in a complex with any of the PRC2 proteins, a *vrn2-1 fca-1* + VRN2-FLAG line was retransformed with FIE-HA, CLF-HA, or SWN-HA. All three HA-tagged proteins were detected in FLAG immunoprecipi-

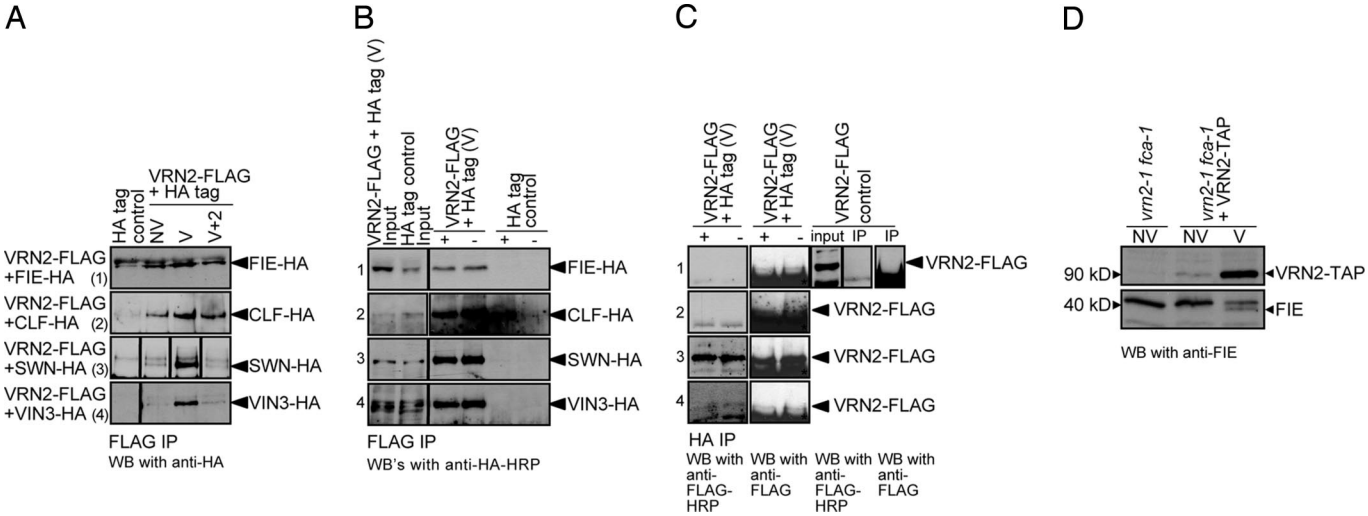

Fig. 4. Coimmunoprecipitation of proteins associated with VRN2. (*A*) FLAG immunoprecipitates from *vrn2-1 fca-1* + VRN2-FLAG lines retransformed with FIE-HA (1), CLF-HA (2), SWN-HA (3), and VIN3-HA (4) probed with anti-HA antibody. Plants grown for 12 LD (NV), 12 LD + 4-week vernalization (V), and 12 LD + 4-week vernalization + 2 LD (V + 2). HA tag control is a line expressing the relevant HA-tagged protein but not the VRN2-FLAG protein. Western blots (WB) are probed with anti-HA antibody. (*B*) Input protein extracts and FLAG immunoprecipitates treated with micrococcal nuclease (+) or untreated (−) probed with anti-HA antibody (this experiment was carried out by using independent transgenic lines to those used in *A*), blots are probed with anti-HA-HRP antibody-enzyme conjugate. (*C*) HA immunoprecipitates treated with micrococcal nuclease (+) or untreated (−) probed with anti-FLAG-HRP antibody-enzyme conjugate (by using the same plant lines as in *B*). (*D*) Calmodulin-agarose purified proteins from *vrn2-1 fca-1* + VRN2-TAP plants probed with anti-FIE antibodies (also detects protein A epitope in VRN2-TAP). A single box separated by black lines are from a single image of a Western blot. *, IgG detected by anti-mouse secondary antibody is visible in blots probed with anti-FLAG.

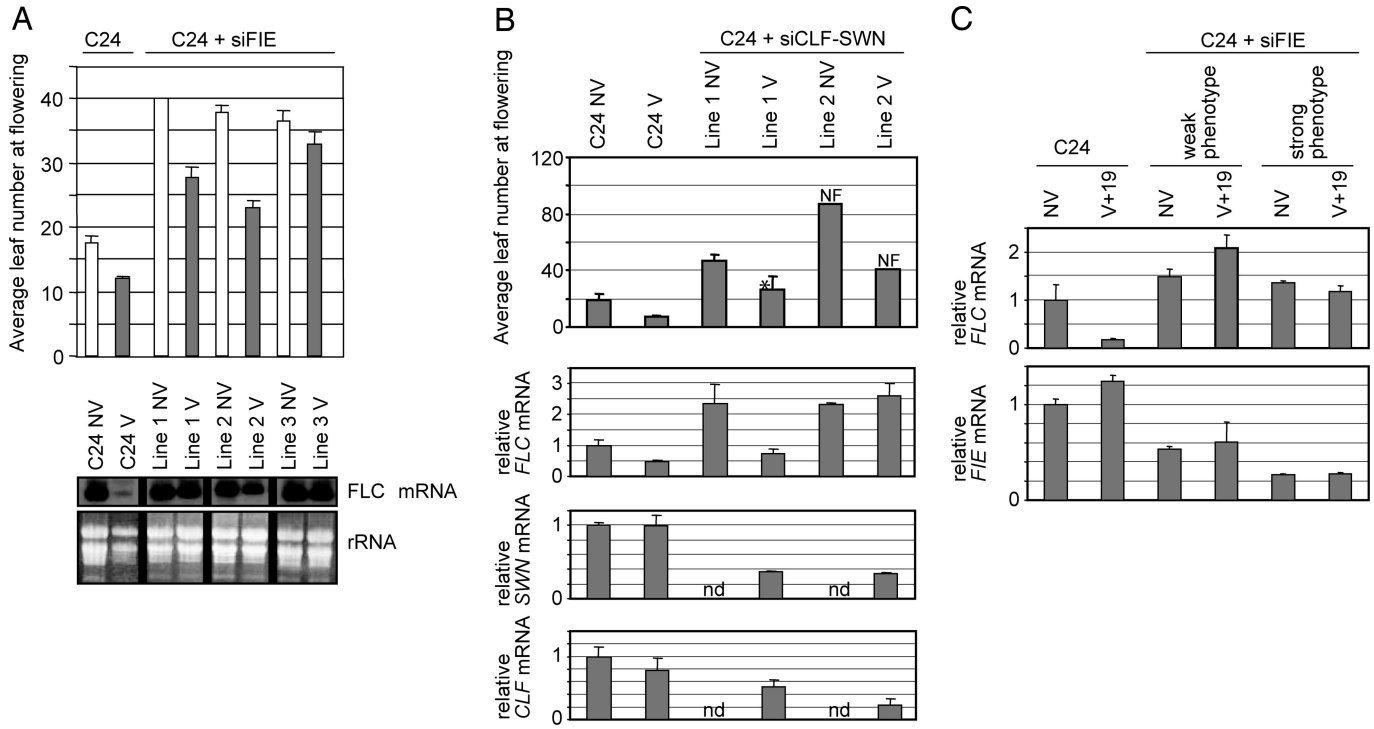

Fig. 5. PcG proteins are required for vernalization-mediated repression of *FLC*. (*A*) Average leaf number at flowering of three T2 siFIE lines grown in LD conditions (NV) or 12 LD followed by 4 weeks of vernalization and returned to LD conditions (V) and FLC mRNA extracted after 12 LD (NV) or 12 LD plus 4 weeks vernalization (V) for the same lines. (*B*) Average leaf number at flowering and FLC, SWN, and CLF mRNA content of C24 wild-type and T2 plants carrying the siCLF-SWN transgene. Plants were either grown in LD conditions (NV) or vernalized for 4 weeks as a seed before growth under LD conditions (V). mRNAs were quantified by quantitative RT-PCR normalized to FDH. *, 3 of 19 line 1 V plants not flowered, 7 of 7 line 2 NV plants not flowered, and 10 of 12 line 2 V plants not flowered (NF) at the termination of the experiment; nd, not determined. (*C*) FLC and FIE mRNAs (measured by quantitative RT-PCR, normalized to FDH) in C24 wild-type, weak, and strong T1 siFIE plants grown for 19 LD (NV) or vernalized 4 weeks as a seed and grown for 19 LD (V + 19). Plants were killed for RNA isolation, and flowering time was not determined.

tates from the double-transformed plants. The highest amount of HA-tagged protein was coimmunoprecipitated in plants sampled at the end of the vernalization treatment (Fig. 4*A*). The coimmunoprecipitation of the HA-tagged proteins was not sensitive to micrococcal nuclease (Fig. 4*B*), showing that the protein interactions probably are not DNA-dependent. In reciprocal experiments, VRN2-FLAG was detected in HA immunoprecipitates from VRN2-FLAG + SWN-HA, VRN2-FLAG + FIE-HA, and VRN2-FLAG + CLF-HA plants (Fig. 4*C*). A small amount of FIE-HA protein was reproducibly FLAG immunoprecipitated from a control line containing FIE-HA only (Fig. 4*A*). To confirm the VRN2 and FIE interaction, an affinity purification was carried out on a complemented *vrn2-1 fca-1* + VRN2-TAP line (Fig. 1). Endogenous FIE protein was copurified with VRN2-TAP in vernalized plants but not in nonvernalized plants or a nontransformed control (Fig. 4*D*). These data demonstrate that VRN2 forms one or more complexes involving FIE, CLF, and SWN, which increase in abundance with vernalization.

VRN2 and VIN3 Can Be Part of the Same Protein Complex. Because VIN3 expression and the size of protein complexes containing VIN3 mirrored the expression pattern and size of complexes containing PRC2-like components, we carried out immunoprecipitation from a *vrn2-1 fca-1* + VRN2-FLAG + VIN3-HA line to test whether VRN2 and VIN3 are in the same complex. The VRN2-FLAG and VIN3-HA proteins coimmunoprecipitate by using either FLAG or HA immunoprecipitation (Fig. 4 *A–C*); therefore, we conclude they are part of the same complex.

FIE, CLF, and SWN Are Required for *FLC* Repression by Vernalization. Because VRN2 is required for the vernalization response and is associated with other PRC2-like proteins in a high molecular mass complex, we tested whether function of a PRC2-like complex is required for the vernalization response by removing PRC2 components by RNAi. T1 plants generated with a *FIE* RNAi construct (siFIE) had a range of phenotypes from morphologically normal but late flowering to small, infertile plants with severe morphological defects, similar to those reported for FIE cosuppression (31) and *clf swn* double mutants (29). RNAi constructs targeting *CLF* and *SWN* individually did not markedly alter the vernalization response of the moderately late flowering ecotype C24 (data not shown), consistent with the functional redundancy of these genes (29). Plants transformed with an RNAi construct targeting both *CLF* and *SWN* (siCLF-SWN) were morphologically normal but late-flowering. The late-flowering phenotypes of T2 siFIE (morphologically normal phenotype) and siCLF-SWN plants were only weakly responsive to vernalization (Fig. 5 *A* and *B*). FLC mRNA expression measured by quantitative RT-PCR (Fig. 5 *B* and *C*) was slightly increased in both siFIE and siCLF-SWN plants grown without vernalization, suggesting a role in maintaining the correct level of *FLC* expression in nonvernalized conditions. *FLC* mRNA expression was not repressed to the same extent as WT C24 in vernalized plants (Fig. 5 *A–C*). The residual vernalization response in the siFIE and siCLF-SWN plants is likely due to the target genes being only partly silenced. Because homologues of both ESC and E(Z) are required for the repression of *FLC* by vernalization, we conclude that a PRC2-like complex is required for the response.

Discussion

We have shown that VRN2 is part of a PRC2-like complex in plants and that this complex acts to regulate *FLC* expression in both nonvernalized and vernalized conditions. Moreover, we

show that the PHD protein VIN3 can be in the same complex as VRN2. The complex and the component proteins accumulate during cold treatment and return to prevernalized levels when plants are returned to the normal temperatures. The increased abundance of this complex in the cold is consistent with both VRN2 and VIN3 having a role in the initiation of FLC repression by vernalization.

During vernalization, expression of *VIN3* is induced and VIN3 protein accumulates. Vernalized plants also contain increased amounts of the VRN2, FIE, CLF, and SWN proteins with no change in the cognate mRNAs, consistent with increased translation of the mRNAs or stability of the proteins in the low-temperature conditions. The VRN2, FIE, CLF, SWN, and VIN3 proteins are all part of high molecular mass protein complexes *in planta*. The abundance of the PRC2-like complexes involving VRN2, FIE, CLF, SWN, and VIN3 all increase during vernalization and return to the prevernalization levels once plants are returned to the normal temperatures. One explanation for our observations is that VIN3, expressed in vernalized plants, associates with VRN2 in a PRC2-like complex, reducing the rate at which the component proteins are turned over. Because PRC2 protein homologues other than VRN2 (FIE, CLF, and SWN) also are required for vernalization-mediated repression of *FLC*, we conclude that VRN2 acts as part of a PRC2-like complex.

Our analysis of the molecular masses of complexes show that the PRC2 core protein homologues, VRN2, FIE, CLF, and SWN, are distributed across a range of ≈600 kDa to 1,000 kDa in vernalized plants, with only FIE present as a probable monomeric protein. This size distribution is likely to reflect a population of PRC2-like complexes with different subunit composition. This size distribution differs from the FIS complex of 650 kDa that contains MEA, FIS2, FIE, and MSI1 observed in developing siliques (19). The size distribution of PRC2-like complexes we observed may be analogous to 600 and 1,000 kDa PRC2 complexes in *Drosophila*, where the larger complex contains the additional proteins PCL and the histone deacetylase RPD3. Interaction data suggests that PCL, a PHD domain protein, recruits RPD3 to the 1,000-kDa complex (16). VIN3 may be the equivalent of PCL in the VRN2 complex because it also contains a PHD domain, and evidence suggests it recruits a histone deacetylase (7). The VIN3 protein appears to be more abundant at the 1,000 kDa size relative to the size distribution of VRN2, FIE, CLF, and SWN (Fig. 3). Therefore, the 1,000-kDa but not the 600-kDa complexes may include VIN3. The size difference between the 600- and 1,000-kDa complexes is sufficient to include a histone deacetylase and VIN3.

The sizes of complexes containing VRN2 did not appear to alter reproducibly between nonvernalized and vernalized plants (Fig. 3 *A* and *B*) as might be expected if VIN3 and associated proteins were added to 600-kDa PRC2-like core complexes to form 1,000-kDa complexes in vernalized plants. The VIN3 expressed at a low level in nonvernalized plants is in complexes of 1,000 kDa (Fig. 3) and coimmunoprecipitates with VRN2 (Fig. 4*A*). Therefore, VIN3 can be part of 1,000-kDa PRC2-like complexes in nonvernalized plants and vernalized plants. It is possible that the increase in abundance of complexes containing VIN3 in vernalized plants is sufficient for *FLC* repression. However overexpression of VIN3 does not substitute for the vernalization response (7). Assuming that the overexpressed VIN3 assembles into a complex, the failure of overexpressed VIN3 to mimic the vernalization response implies that some other cold-regulated factor, such as protein modification or signaling molecule, is required to make the complex active during vernalization.

In *vrn2* mutant plants that have not been vernalized, *FLC* expression is higher and flowering time is later than in the corresponding wild type (5); this is also the case when FIE or CLF and SWN functions are reduced. This result suggests that even in nonvernalized plants, a PRC2-like complex containing VRN2, FIE, and CLF or SWN is acting to control *FLC* expression.

Deacetylation of H3 at *FLC* during vernalization requires VIN3 but not VRN2 (7). In *vrn2* mutants, it is possible that VIN3 can mediate histone deacetylation without being in a PRC2-like complex. Alternatively, VIN3 still may act in a PRC2-like complex that includes a mutant VRN2 protein or a Su(Z)12 homologue (such as EMF2) substituting for VRN2 activity. Methylation of H3K27 at *FLC* after vernalization requires both VIN3 and VRN2 (7, 21). Deacetylation of H3 by VIN3 may be required before a PRC-like complex containing VRN2 can methylate H3K27. Alternatively, if VIN3 accumulation is needed for the VRN2 complex to accumulate in *vin3* mutants, a lack of VRN2 complex may preclude H3K27 methylation. VIN3 (7) and reportedly VRN2 (21) are associated with FLC chromatin. VIN3 binds to a region of *FLC* intron 1 that overlaps with a "vernalization response element" that is required for maintenance of *FLC* repression by LIKE HETEROCHROMATIN PROTEIN 1 after vernalization (32). This region of the *FLC* gene may be the equivalent of the *Drosophila* polycomb response element. The VRN2–VIN3 complex may bring these histone deacetylase and histone methyltransferase activities together at the *FLC* locus, providing a coordinated mechanism for the epigenetic modifications associated with the vernalization-mediated repression of the *FLC* gene.

Materials and Methods

Plasmid Constructs. pHA-GW was made by insertion of a 6xHA encoding fragment (Fig. 1*B*), generated from oligonucleotides, into pWBVec2a (33), followed by insertion of the Gateway rfC cassette (Invitrogen, Carlsbad, CA). pTAP-GW was made by PCR amplifying the XhoI cassette used in pHellsgate8 (34) with the primers NcoRI and NcoR2 (5′-GGGCCATGGACAAGTTTGTACAAAAAAGCTG and 5′-GGGCCATGGAGACCACTTTGTACAAG) and cloning the product into the NcoI site of the pGREEN1479 plasmid containing the TAP coding sequence from pBS1479 (35). pHA-GW and pTAP-GW were sequenced across the junction of the Gateway cassette and the epitope tag to confirm that an ORF would be maintained.

Genomic fragments consisting of 1 kb of promoter and the entire protein coding region, including introns, to the nucleotide preceding the stop codon were amplified from C24 genomic DNA (primer sequences in Table 1, which is published as supporting information on the PNAS web site) and used to generate Gateway entry clones either by addition of Gateway attB1 and attB2 sites to the primers (VRN2) or by using the pENTR/D-TOPO vector (Invitrogen). The resulting genomic clones were sequence-verified and used to generate epitope-tag constructs in the pFLAG-GW, pHA-GW, and pTAP-GW vectors (Fig. 1 *A* and *B*).

The siFIE construct was generated by using a 314-bp fragment from the first exon of the FIE gene (primers in Table 1) with pHellsgate8 (34).

Plant Methods. *Arabidopsis* seeds were stratified and grown on MS + 3% sucrose agar plates. Long day (LD) conditions were a 16-h day at 21°C under fluorescent lights. Vernalization was at 4°C under low light conditions (2). Plant material sampled from vernalized treatments was harvested at 4°C. Transformation was by the floral dip method (36). Plants transformed with the constructs based on pFLAG-GW, pHA-GW, or pTAP-GW destination vector backbones were selected on MS agar plates with 50 mg/liter kanamycin, 15 mg/liter hygromycin, or 10 mg/liter glufosinate, respectively.

Protein Methods. Proteins were separated on 8 or 10% SDS/PAGE gels. Proteins were transferred to Immobilon P mem-

brane (Millipore, Bedford, MA) and detected by chemiluminescence. Detection of FLAG-tagged proteins was by 1:20,000 FLAG M2 antibody (Sigma, St Louis, MO) followed by 1:16,000 anti-mouse Ig HRP conjugate (Chemicon, Melbourne, Australia) or 1:5,000 FLAG M2-HRP conjugate (Sigma). HA-tagged proteins were detected by 1:40,000 HA-7 antibody (Sigma) followed by 1:16,000 anti-mouse Ig-HRP or 1:8,000 HA-7-HRP conjugate (Sigma). α-Tubulin was detected by 1:10,000 tubulin antibody B 5-1-2 (Sigma) followed by 1:16,000 anti-mouse Ig-HRP.

To raise antibodies against FIE, an internally deleted *FIE* cDNA clone was amplified (primer sequences in Table 1), and the region encoding amino acids 1–63 and 273–369 was expressed in BL21-RIL cells by using pDEST-ProEx and the recombinant protein purified as described in ref. 37. The recombinant protein was used to raise polyclonal antibodies in rabbits. In Western blots with plant material, the anti-FIE serum was used at a 1:3,000 dilution.

Estimates of the size of protein complexes containing the epitope-tagged proteins by size exclusion chromatography and immunoprecipitation were conducted essentially as described (ref. 24; *Supporting Text*, which is published as supporting information on the PNAS web site).

VRN2-TAP affinity purification was carried out by extracting total protein from 14-day-old *vrn2-1 fca-1* +VRN2-TAP or *vrn2-1 fca-1* plants (1 g) by using calmodulin (CaM)-binding buffer (35). VRN2-TAP and any associated proteins were isolated on CaM-agarose beads (Stratagene, La Jolla, CA), eluted with SDS/PAGE buffer, and the proteins from 2 mg of total protein were used for Western blot analysis.

mRNA Expression Analysis. RNA was extracted from plant tissue by using TRIzol (Invitrogen). Gel blots were carried out with 10 μg of total RNA separated on 1.2% formaldehyde-agarose gels and transferred to Hybond N membrane (GE Biosciences). The blot was probed with an antisense RNA probe generated from FLC cDNA (2) and visualized by a phosphorimager. cDNA was synthesized from 3 μg of total RNA by using SuperScript III reverse transcriptase (Invitrogen) and an oligo dT primer according to the manufacturer's instructions. Quantitative PCR was carried out as described in *Supporting Text* by using primers detailed in Table 1.

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