

The PHD Finger Protein VRN5 Functions in the Epigenetic Silencing of *Arabidopsis FLC*

Thomas Greb,^{1,2,5} Joshua S. Mylne,^{1,3,5}
Pedro Crevillen,¹ Nuno Geraldo,¹ Hailong An,¹
Anthony R. Gendall,^{1,4} and Caroline Dean^{1,*}

¹Department of Cell and Developmental Biology
John Innes Centre
Norwich, NR4 7UH
United Kingdom

Summary

Vernalization, the acceleration of flowering by the prolonged cold of winter, ensures that plants flower in favorable spring conditions. During vernalization in *Arabidopsis*, cold temperatures repress *FLOWERING LOCUS C (FLC)* expression [1, 2] in a mechanism involving *VERNALIZATION INSENSITIVE 3 (VIN3)* [3], and this repression is epigenetically maintained by a Polycomb-like chromatin regulation involving *VERNALIZATION 2 (VRN2)*, a Su(z)12 homolog, *VERNALIZATION 1 (VRN1)*, and *LIKE-HETEROCHROMATIN PROTEIN 1* [4–8]. In order to further elaborate how cold repression triggers epigenetic silencing, we have targeted mutations that result in *FLC* misexpression both at the end of the prolonged cold and after subsequent development. This identified *VERNALIZATION 5 (VRN5)*, a PHD finger protein and homolog of *VIN3*. Our results suggest that during the prolonged cold, *VRN5* and *VIN3* form a heterodimer necessary for establishing the vernalization-induced chromatin modifications, histone deacetylation, and H3 lysine 27 trimethylation required for the epigenetic silencing of *FLC*. Double mutant and *FLC* misexpression analyses reveal additional *VRN5* functions, both *FLC*-dependent and -independent, and indicate a spatial complexity to *FLC* epigenetic silencing with *VRN5* acting as a common component in multiple pathways.

Results and Discussion

To isolate mutants affected in the cold-induced repression of *FLC*, we mutagenized an *Arabidopsis* line carrying *FRIGIDA* and an *FLC-LUCIFERASE (FLC-LUC)* translational fusion [9] with EMS. The translational fusion mirrors endogenous *FLC* expression in all currently analyzed aspects, namely response to vernalization, up-regulation by *FRIGIDA*, and *fca* mutations. M2 seedlings that showed high *FLC* expression were identified in populations of seedlings either just exposed to 6 weeks of

cold temperatures or exposed to the cold and then grown for a subsequent 2 weeks at 20°C. Analysis of those mutants showing higher *FLC-LUC* activity than the wild-type at both stages identified two new *vin3* alleles. The *vin3-6* mutation causes a change in the splicing site at the beginning of intron 3 (G1244A), and the *vin3-7* mutation introduces a premature stop codon in the second exon (G716A, numbers counted from start codon). In addition, six alleles of a complementation group (*vrn5-2* to *vrn5-7*) allelic to a γ -ray-induced mutant isolated from a flowering time screen in an *fca-1* background (*vrn5-1*) were identified. All *vrn5* mutants showed a clear reduction in their vernalization response based on flowering time assays and high *FLC* expression after vernalization (Figures 1A and 1B). Interestingly, *vrn5-6* confers a relatively weak phenotype relative to the other alleles despite the fact it carries an early stop mutation. All the alleles also showed subtle additional phenotypes, which did not require a functional *FLC*, including mild leaf curling, an increase in mean petal number, early flowering in short-day conditions, and distorted siliques showing greater indeterminacy (see Figure S1 and Table S1 in the Supplemental Data available with this article online). These data suggest *VRN5* has targets in addition to *FLC*.

VRN5 was identified by map-based cloning, complementation, and analysis of the lesions in eight alleles including the Columbia T-DNA insertion allele (SALK_136506) *vrn5-8*. *VRN5* (At3g24440) encodes a protein of 602 amino acids [9] carrying a fibronectin III protein interaction domain, a PHD finger and is a homolog of *VIN3* [3]. Independent *vrn5* mutations disrupt the coding sequence by prevention of splicing (*vrn5-1* Δ 430–446, *vrn5-3* and *vrn5-5* AG>AA at end of intron 1) or generation of stop codons (*vrn5-2* and *vrn5-4* Q139*, *vrn5-6* W47*, *vrn5-7* W144*). Two other *Arabidopsis* genes, named *VEL1* (At4g30200) and *VEL2* (At2g18880), and a partial gene fragment *VEL3* (At2g18870) are homologous to *VRN5* and *VIN3*, and together they have been grouped into the *VEL (VERNALIZATION5/VIN3-like)* gene family [9]. The four intact genes share a conserved C terminus that shows no homology to a characterized protein domain or homology to any other protein in the database. Because none of the available T-DNA insertion mutants for *VEL1* or *VEL2* led to loss of function, we generated hairpin constructs targeting specifically either *VEL1* or *VEL2* (*VEL1*: bp 139–443, *VEL2*: bp 47–359). *VEL1* RNA levels were reduced by up to 85% (data not shown), but flowering time after vernalization was not delayed in 72 transformants. Together with the absence of *vel1* mutations from the *FLC-LUC* screen, this suggests that *VEL1* may not play a role in vernalization. Reduction of *VEL2* expression could not be detected after analysis of 68 transformants so additional experiments will be required for investigating the role of *VEL2* in vernalization. PHD fingers are found in many chromatin-associated proteins, often in more than one copy, and have recently been shown to bind

*Correspondence: caroline.dean@bbsrc.ac.uk

²Present address: Gregor-Mendel-Institute, Dr. Bohr-Gasse 3, 1030 Vienna, Austria.

³Present address: Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia.

⁴Present address: Department of Botany, La Trobe University, Bundoora, Victoria 3083, Australia.

⁵These authors contributed equally to this work.

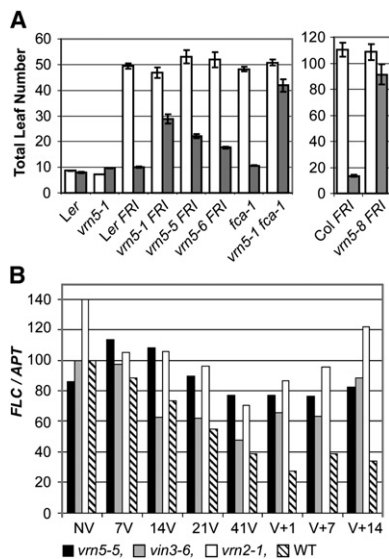


Figure 1. The Role of VRN5 in FLC and Flowering Time Regulation
(A) Flowering time measured as total leaf number. Plants grown in extended short days as described [24]. White bars represent nonvernalized plants, and gray bars represent plants that were vernalized 6 weeks. Error bars represent SE of 20 plants. At least two independent experiments gave the same results.
(B) FLC mRNA levels assayed with northern blots, relative to ADENOSINE PHOSPHORIBOSYL TRANSFERASE (APT -At1g27450 used interchangeably as a reference transcript in northern analysis with β -TUBULIN). *FRI vrn5-5* (black bars), *FRI vrn3-6* (gray bars), *FRI vrn2-1* (white bars), and *FRI* (dashed bars) plants were grown on GM plates for 7 days nonvernalized (NV) and then were either vernalized for 7, 14, 21, or 41 days in the cold then grown for 1, 7, or 14 days at 20°C. Experiments were repeated at least twice, and results from a typical experiment are shown.

methyl-lysine residues with high specificity for H3K4me3 [10, 11]. However, a conserved hydrophobic region required for forming the pocket necessary for the trimethyl group is not conserved in the VRN5 or VIN3 PHD fingers. PHD fingers have also been shown to bind phosphoinositides [12], suggesting a mechanism for how environmental signals might induce chromatin changes.

VIN3 mRNA levels increase during the prolonged cold [3]; therefore, we analyzed the expression profile of VRN5. Unlike VIN3, VRN5 is expressed at a similar level before, during, and after a prolonged cold treatment (Figure 2A). VEL1 expression also is relatively unchanged, whereas VEL2 RNA appears to increase with the prolonged cold. RT-PCR on independent RNA samples showed that VEL2 RNA is detectable in nonvernalized tissue and relative to β -TUBULIN increases progressively during the cold (Figure S2A). These expression profiles, together with those of VRN1 and VRN2, were not significantly changed in *vrn1*, *vrn2*, *vrn5*, and *vin3* mutant backgrounds (example in Figure S2B). Thus, there is no indication of cross-regulation of the VIN/VRN genes. The expression of VRN5 was confirmed by analysis of a complementing genomic VRN5-GUS fusion, which showed that VRN5 accumulates in shoot and root apices and generally in the leaf (Figure 2B and Figure S2C). VIN3 is likewise expressed in shoot and root tips [3] so the expression domains

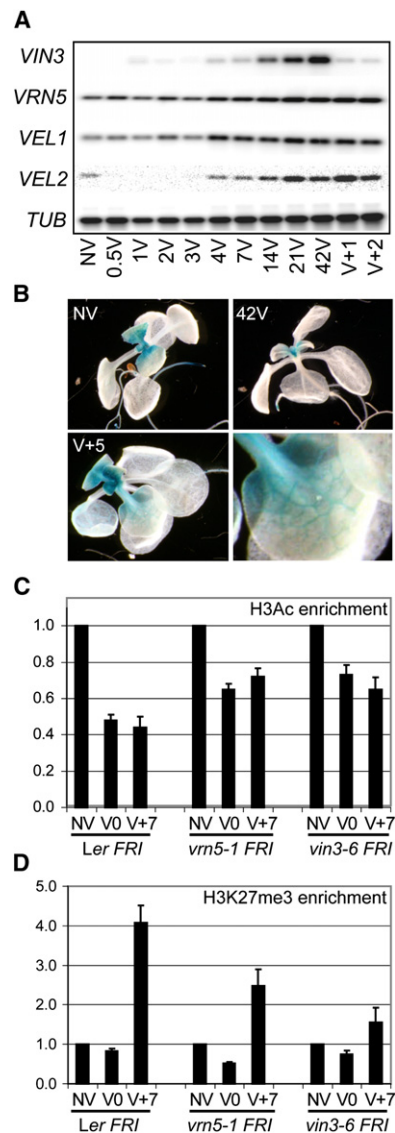


Figure 2. Expression of VRN5 and Its Role in FLC Chromatin Regulation

(A) Semiquantitative RT-PCR analysis with VIN3, VRN5, VEL1, and VEL2 expression at different stages of vernalization. *FRI* plants were grown on GM plates for 7 days nonvernalized (NV), then were either transferred to a cold environment for 0.5, 1, 2, 3, 4, 7, 14, 21, or 42 days or given 42 days in the cold then grown for 1 or 2 days at 20°C. PCRs were 20 cycles and were followed by Southern analysis with gene-specific probes.

(B) Analysis of VRN5 expression by a complementing VRN5-GUS translational fusion after NV, 42 days in a cold environment (42V) or 42 days in the cold including 5 days at 20°C (V+5). The picture bottom right is a magnification of the V+5 picture for illustrating VRN5 expression in the leaf vasculature.

(C) Chromatin immunoprecipitation analyzing H3 acetylation (lysine 9 and 14) in the promoter region A of FLC [8]. ACTIN amplification was used for normalizing DNA concentrations between the samples.

(D) Analysis of histone H3 lysine 27 trimethylation (H3K27me3) in the same FLC promoter region. Ta3 amplification was used for normalizing DNA concentrations. The diagrams in (C) and (D) summarize data from at least two independent experiments for each analysis and three PCR assays experiment. Values indicate ratio FLC/ACTIN or Ta3 amplification normalized to nonvernalized samples. NV, nonvernalized; V0, vernalized for 6 weeks; V+7, vernalized for 6 weeks and grown for 7 days further at 20°C.

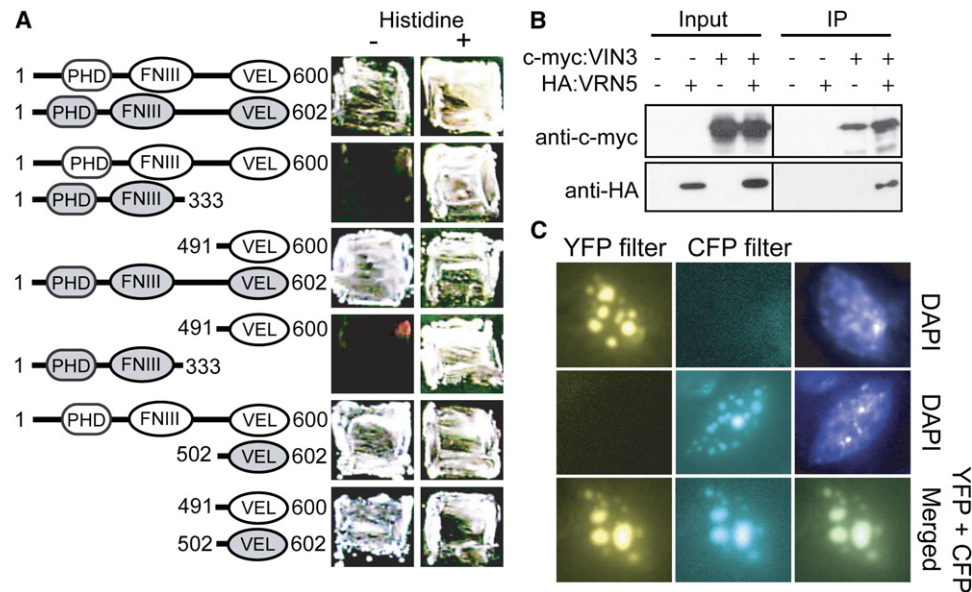


Figure 3. Interactions between VRN5 and VIN3 Proteins

(A) VIN3 (white) and VRN5 (gray) protein interaction in yeast mediated by the conserved C terminus named the VEL domain. Interaction was tested by growing of transformed strains on selective medium with 0.5 mM 3-AT and without histidine. None of the proteins were able to self-activate (data not shown). Numbers indicate start and end point of the protein sequence in different experiments.

(B) Investigation of the interaction between VRN5 and VIN3 in transient assays in *Nicotiana benthamiana*. Protein extracts were precipitated with anti-c-myc Sepharose, and after washing, the eluate was analyzed in western blots with anti-c-myc and anti-HA, respectively.

(C) Transient overexpression of VRN5-YFP (upper row and lower row) and VIN3-CFP (middle row and lower row). Constructs were introduced into *Arabidopsis* leaf cells by particle bombardment.

of both genes overlap, but only during long periods of the cold.

Yeast-two-hybrid experiments revealed that VEL proteins have a widespread ability to form homodimers and heterodimers in yeast (Figure 3A and Figure S3). For example, VIN3 but not VRN5 is able to form homodimers, but VIN3/VRN5 effectively heterodimerize. By using deletion constructs, we found the C-terminal domain conserved in VEL family members [9] to mediate the protein-protein interaction (Figure 3A). None of the VEL proteins interacted with VRN1 or VRN2, and VRN1 and VRN2 did not interact with each other (data not shown). To test the in planta physical interaction of VRN5 and VIN3, we transiently expressed epitope-tagged versions of both proteins in tobacco leaves [13]. The VRN5 protein was precipitated with an antibody raised against the c-myc-epitope fused to VIN3 (Figure 3B), showing that VIN3 and VRN5 proteins interact in plant cells. This was supported by analysis of their subcellular localization after transient expression in *Arabidopsis* leaves. VRN5-YFP and VIN3-CFP fusion proteins localized to speckles within the nucleus (Figure 3C). The patterns overlapped completely when both proteins were expressed in the same cell, suggesting subnuclear colocalization of the two proteins (Figure 3C). However, it is possible that the localization in speckles may be due to overexpression of the two proteins. A *pVRN5:VRN5-YFP* fusion, able to complement the *vrn5-1* mutation, also localized to the nucleus (Figures S2C and S4B), but no obvious subnuclear localization was detected. The analysis of the physical interaction of endogenous VRN5/VIN3 proteins during vernalization was hampered by their very low expression.

Because the overlapping expression patterns and physical interaction of VIN3 and VRN5 suggested that they function together in vivo to regulate *FLC*, the histone-modification profiles of *vin3* and *vrn5* mutants were analyzed by chromatin immunoprecipitation. Both mutants showed a similar reduction in vernalization-induced histone H3 deacetylation (Figure 2C and Figure S5), supporting their functioning together in vivo. They were also impaired in the establishment of histone methylation, namely histone H3 lysine 27 trimethylation (H3K27me3, Figure 2D and Figure S5), which is characteristic of Polycomb-repressed genes [14] and which shows maximal accumulation after the plants are returned to ambient temperatures [3].

The interaction and histone-modification data suggest a model for vernalization where VIN3, induced at the shoot apex by the prolonged cold, heterodimerizes with VRN5, and the formation of a dimer carrying two PHD fingers triggers a series of epigenetic modifications at *FLC*. The *Drosophila* Polycomb-like protein PCL contains two PHD fingers, and both are required for recruiting the histone deacetylase RPD3 to Polycomb targets [15]. Requirement of two different PHD fingers could explain the requirement for a VRN5-VIN3 heterodimer. The recent demonstration of colocalization of VIN3, VRN2, and CLF/SWN-E(z) homologs in a large molecular mass (~1000 kDa) protein complex [16] suggests that VRN5/VIN3 interact with a VRN2-PRC2-like complex for initiation of the sequence of events leading to epigenetic silencing. Such a model would predict that *vin3* and *vrn5* would not be additive when combined and each would be epistatic to the other *vrn* mutations. We have tested this model by analyzing VIN/VRN gene

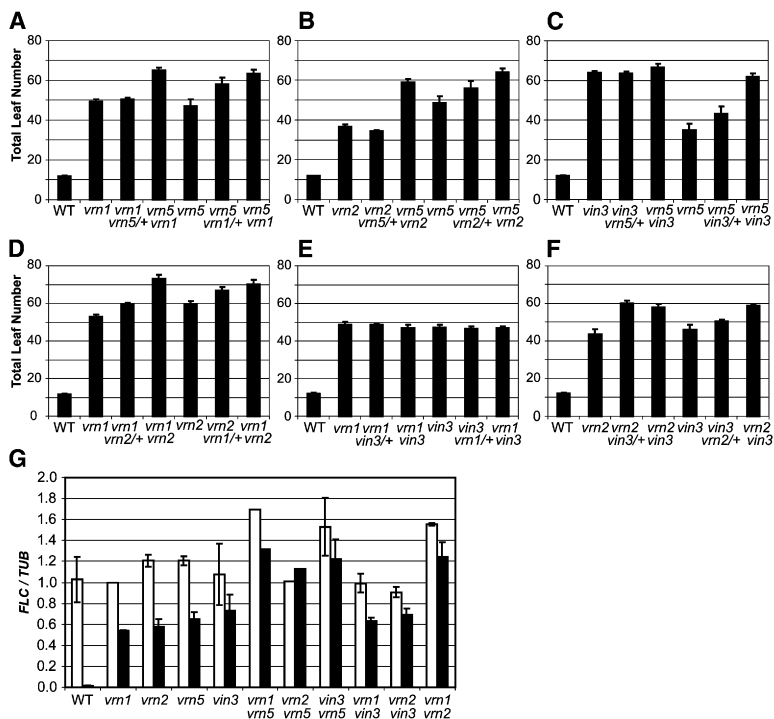


Figure 4. Flowering Time and *FLC* Transcript Levels in Double Mutants

(A–F) Flowering time, measured as total leaf number of the wild-type and plants homozygous for single and double mutants or heterozygous for one mutation. (A) shows *vrn1*, *vrn5* combinations, (B) shows *vrn2*, *vrn5* combinations, (C) shows *vin3*, *vrn5* combinations, (D) shows *vrn1*, *vrn2* combinations, (E) shows *vrn1*, *vin3* combinations, and (F) shows *vrn2*, *vin3* combinations. All the plants are also homozygous for an active *FRI* allele. Plants grown in extended short days in conditions described in [24]. Error bars represent SE of 12 plants on average with five plants being the lowest number. At least two independent experiments gave the same results. (G) *FLC* transcript levels analyzed from northern-blot experiments (relative to β -*TUBULIN*) in nonvernalized (white) and vernalized (black) plants. Values shown are expressed relative to *vrn1* data, and those with error bars are mean \pm SE values from three experiments. The alleles used were *vrn1*-2, *vrn2*-1, *vrn5*-5, and *vin3*-6.

interactions. Double mutants were generated with strong loss-of-function alleles, and flowering time and *FLC* levels were analyzed. In contrast to the prediction, *vin3 vrn5* double mutants did contain higher *FLC* levels after vernalization than either parent (Figure 4G). However, *vin3 vrn5* did not flower later than either parent (Figure 4C) so misexpression of other targets of *VRN5* may be contributing to *vin3 vrn5* flowering time. The higher *FLC* expression in the double mutant suggests that in addition to functioning as a heterodimer, one or both proteins have other functions in *FLC* regulation. In combination with any of the other *vrn/vin* mutants, *vrn5* further delayed flowering (Figures 4A and 4B) and increased *FLC* expression (Figure 4G). In contrast, the *vin3vrn1* double mutant neither flowered later (Figure 4E) nor had higher *FLC* expression (Figure 4G) compared to the single *vin3* and *vrn1* mutants. Overall, the simplest interpretation of the double-mutant analysis is that *VIN3* and *VRN1* function in the same genetic pathway and *VRN5* and *VRN2* function in additional pathways. Analysis of the homozygous and heterozygous combinations showed that plants lacking *VRN5* are more sensitive to gene dosage of *VRN1*, *VRN2*, and *VIN3* (Figures 4A–4C).

Analysis of *FLC-LUC* misexpression after vernalization in the mutant backgrounds revealed an additional complexity to *FLC* silencing. In *vrn5*, *FLC* expression remained relatively unchanged compared to the nonvernalized pattern and was high in the apex, petioles, and leaves. In *vin3*, however, *FLC* expression was predominantly in the apex and petioles, and the leaf expression was not as high as in *vrn5* (Figure 5 and Figures S6A and S6B). In *vrn1*, *FLC* misexpression resembled that of *vin3* and was mainly apical, whereas in *vrn2*, *FLC* expression was significantly reduced in the apex and leaves and was strongest in the petioles and vasculature (Figure 5

and Figures S6A and S6B). Taken together, the double-mutant and misexpression analyses suggest that different *VIN/VRN* proteins quantitatively differ in their spatial contribution to *FLC* silencing. *VIN3* and *VRN1* appear to have a prominent role in the shoot apical region with *VRN2* loss having the greatest effect on *FLC* expression in petioles and vasculature. *EMBRYONIC FLOWER 2*, another *Su(z)12* homolog, has been shown to function semiredundantly with *VRN2* [17] so it may partially cover for loss of *VRN2* function in the apical region. *VRN5* is required in the apex, petioles, and vasculature, and leaves so it appears to be a common component of the vernalization machinery in different parts of the plant. One prediction from this work is that *VEL2*, whose expression is upregulated by the prolonged cold, may heterodimerize with *VRN5* and mediate the vernalization-induced silencing of *FLC* in the leaves.

Our analysis has identified a requirement for the PHD finger protein *VRN5* in the vernalization-mediated epigenetic silencing of *FLC* in many parts of the plant. *VRN5* is

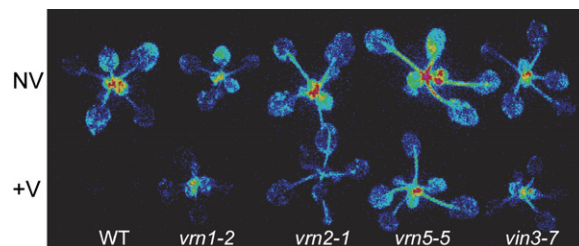


Figure 5. *FLC-LUC* Expression Pattern in *FRI* and *FRI vrn* Plants with and without Vernalization
NV stands for nonvernalized plants that are 23 days old; +V plants were vernalized for 9 weeks and then grown at 20°C for 18 additional days.

a member of a gene family in *Arabidopsis* that is well conserved, and clear homologs can be identified in monocot genomes (J. Cockram, D. Laurie, and C.D., unpublished data). Although VRN5 appears to be a common component, our data suggest that other VIN/VRN proteins quantitatively differ in their spatial contribution to *FLC* epigenetic silencing. This spatial complexity in subunit composition of Polycomb complexes is not unusual because multiple Polycomb complexes with differing composition and targets have been described in *Drosophila* and humans [15, 18, 19]. Further analysis of the *Arabidopsis* complexes may provide insight into their evolution. The four homologs of the VEL family plus three *Arabidopsis* E(z) and Su(z)12 isoforms give the potential for enormous complexity in the *Arabidopsis* Polycomb machinery, and future biochemical analyses will have to be adapted so that the spatial contribution of these different pathways can be dissected. The recent discovery that *FLC* functions independently in both the apex and vasculature and represses different targets, *FLOWERING D (FD)* and *SUPPRESSOR OF CONSTANS 1 (SOC1)* in the apex and *FLOWERING T (FT)* in the vasculature [20], may provide some rationale for the complexity. The evolution of vernalization pathways with a degree of spatial specificity may have provided effective silencing of a major floral repressor as well as maintained a degree of plasticity that provides fitness advantage in certain environments.

Experimental Procedures

Seeds and Growth Conditions

Unless otherwise specified, seeds were sown on soil and vernalized for six weeks at 4°C under short-day conditions (8 hr light, 16 hr darkness) before being transferred to long-day conditions (16 hr light at 20°C, 8 hr darkness at 16°C). For nonvernalized samples, seeds were sown on soil one week before the end of the cold treatment of vernalized plants, incubated at 4°C for two days, and then transferred to long-day conditions. Luciferase imaging was as described in [9].

The *flc-5* mutation was isolated in *Ler* background and is an AG-to-AA change at the splice recognition site in the second *Ler FLC* intron. The fact that *flc-5* behaves as a functional null allele is shown by the finding that *flc-5 FRI* plants grown in extended short days flower at the same time or earlier than wild-type *Ler (FLC fri)* (data not shown). The *flc-5* mutation was genotyped by PCR amplification with dCAPs primer JM40 (5'-AACCATAGTTCAGAGCTTTTGACTGAGATC-3') and JM41 (5'-TTAAAGCCTTGTAATACAAACATT-3'). Subsequent digestion with BglII cuts the *flc-5* mutant product for yielding 193 + 27 bp, whereas the wild-type remains uncut at 220 bp. *FLC* mRNA expression in *flc-5 FRI* is greatly reduced and what little mRNA is present is misspliced (data not shown).

Protein-Interaction Analysis

Yeast two-hybrid analysis was undertaken as described in [9]. For *in planta* analysis, a *VRN5* gene was isolated from a *Ler* cosmid library [21] and a 5.5 kb Swal fragment from 1681 bp upstream of the start codon to 1809 bp downstream of the stop codon was cloned into pGreenII0229. NcoI and XmaI sites were generated at the C terminus of the *VRN5* open reading frame by site-directed mutagenesis and were used for introducing the PCR-amplified open reading frames of the *EYFP* gene (amplified from *pEYFP*, Clontech, *pVRN5:VRN5-EYFP*) or the *GUS* gene (*pVRN5:VRN5-GUS*). For transient overexpression of HA-VRN5 and *c-myc*-VIN3 (derived from PCR cloning the cDNA from Landsberg *erecta*) in *N. benthamiana* leaves, HA-tag and 6× *c-myc*-tag were fused as N-terminal fusions to *VIN3* or *VRN5* open reading frames and fragments were cloned into pBIN61 [13] (*p35S:HA-VRN5*, *p35S:6xc-myc-VIN3*). For transient overexpression of VRN5-EYFP and VIN3-ECFP in *Arabidopsis* leaf

cells, fragments with translational fusions were cloned similarly into pBIN61 (*p35S:VRN5-EYFP*, *p35S:VIN3-ECFP*).

Semiquantitative RT-PCR Experiments

Total RNA was extracted with TRIzol Reagent (Invitrogen Life Technologies). First-strand cDNA was synthesized with 2.0 μg of total RNA with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instruction. Primers used were as follows:

VIN3 F: 5'-ACTTGCTCGGATGCTGGAGAA-3'; R: 5'-GTAGAAG AAGACGGCTCCTCGG-3'
VRN5 F: 5'-GCCAATGCAGGTAGAGATTTAGTTC-3'; R: 5'-GCTGCTTTTCCAACATCCAAGA-3'
VEL1 F: 5'-CCAAGATGCAGCAAGATACAGCA-3'; R: 5'-CAGAAG AAGGATTAGAAGACAATGTGC-3'
VEL2 F: 5'-CACTGCCTTCTACTATCCAAGGATCAA-3'; R: 5'-CC CAAACTGCAGCTTCTGA-3'
TUBULIN F: 5'-CTCAAGAGTTCTCAGCAGTA-3'; R: 5'-TCA CCTTCTTCATCCGAGTT-3'

Chromatin Immunoprecipitation

Chromatin-immunoprecipitation assays (ChIP) were performed as previously described [22]. Chromatin preparations from *Arabidopsis Ler FRI*, *vrn5-1 FRI*, and *vin3-6 FRI* seedlings were immunoprecipitated with either anti-acetyl-histone H3 (lysine 9 and 14) (Upstate Biotechnology catalog no. 06-599) or anti-trimethyl-histone H3 (lysine 27) (Thomas Jenuwein; rabbit 6523, 5 bleed) and magnetic Dynabeads Protein A (DynaL Biotech). ChIP DNA was analyzed by semiquantitative PCR and data (mean ± SE is shown for six assays). *FLC* promoter primers were *FLC-Af* (5'-AGCGAGTGGTTCTTTGTTT-3') and *FLC-Ar* (5'-CTTGTACTTTTGCAATGCC-3'). Actin and Ta3 primers were as described in [23].

Supplemental Data

Supplemental Data include six figures and can be found with this article online at <http://www.current-biology.com/cgi/content/full/17/1/73/DC1/>.

Acknowledgments

We thank members of the Dean laboratory for discussions, Vincent Dielen for help mapping *vrn5-1*, Clare Lister for help with luciferase quantification, Wladimir Tameling for donation of pBIN61 and other control plasmids, Thomas Jenuwein for antibodies to H3K27me3, Joe Ecker's laboratory for T-DNA insertion alleles, and Mervyn Smith for excellent care of *Arabidopsis* plants. This work was funded by Biotechnology and Biological Sciences Research Council Core grant to J.I.C., grant BB/C517633/1, Natural and Environmental Research Council grant NE/C507629/1, European Commission grant QLK5-CT-01412, and Marie Curie Category 30 fellowship to T.G., and a Gulbenkian Foundation studentship (to N.G.).

Received: October 18, 2006

Revised: November 13, 2006

Accepted: November 14, 2006

Published online: December 14, 2006

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