

The *VERNALIZATION 2* Gene Mediates the Epigenetic Regulation of Vernalization in *Arabidopsis*

Anthony R. Gendall, Yaron Y. Levy,² Allison Wilson, and Caroline Dean¹

Department of Cell and Developmental Biology
John Innes Centre
Colney Lane
Norwich NR4 7UH
United Kingdom

Summary

The acceleration of flowering by a long period of low temperature, vernalization, is an adaptation that ensures plants overwinter before flowering. Vernalization induces a developmental state that is mitotically stable, suggesting that it may have an epigenetic basis. The *VERNALIZATION2* (*VRN2*) gene mediates vernalization and encodes a nuclear-localized zinc finger protein with similarity to Polycomb group (PcG) proteins of plants and animals. In wild-type *Arabidopsis*, vernalization results in the stable reduction of the levels of the floral repressor *FLC*. In *vrn2* mutants, *FLC* expression is downregulated normally in response to vernalization, but instead of remaining low, *FLC* mRNA levels increase when plants are returned to normal temperatures. *VRN2* function therefore stably maintains *FLC* repression after a cold treatment, serving as a mechanism for the cellular memory of vernalization.

Introduction

Plants regulate developmental transitions in response to a variety of both external stimuli and endogenous internal cues. Genetic studies of the transition to flowering in the model plant *Arabidopsis* have revealed four major floral promotion pathways (Reeves and Coupland, 2000; Simpson et al., 1999). The photoperiod and vernalization pathways integrate environmental signals into the flowering decision, while the autonomous and gibberellin (GA) pathways appear to act largely independently of external cues. These pathways form a quantitative network that regulates the timing of the transition from a vegetative to reproductive phase of development (Koornneef et al., 1998; Simpson et al., 1999). The vernalization pathway promotes flowering in response to a long period of cold temperature, usually experienced as plants overwinter. Despite having a low-temperature stimulus, vernalization is distinct from the other cold responses of acclimation and freezing tolerance (Thomashow, 1999).

Vernalization has long been recognized as a key process in the floral transition (reviewed in Chouard, 1960). Physiological studies have revealed a number of properties of vernalization common to many plant species (for reviews see Lang, 1952; Michaels and Amasino, 2000;

Napp-Zinn, 1987; Sheldon et al., 2000a). Vernalization is a quantitative response with increasing periods of low temperature causing progressively earlier flowering until a saturation point is reached. Although all dividing cells are thought to undergo vernalization, only the shoot apical meristem needs to be vernalized to accelerate flowering (Metzger, 1988; Schwabe, 1954). Vernalization is nongraft transmissible and mitotically stable (Schwabe, 1954), with its effects persisting through mitosis but, importantly, not through meiosis. Vernalization does not directly induce flowering, as plants that are vernalized as seedlings do not flower immediately upon the return to normal temperatures, but often weeks later. Thus, there is a clear separation in time between the perception of the cold temperature and the response—the conversion from a vegetative to reproductive meristem. An element of cellular memory is clearly involved in vernalization, with the shoot meristem “remembering” through multiple cell divisions that vernalization has occurred. This has led to the proposition that vernalization has an epigenetic basis.

In naturally occurring *Arabidopsis* accessions, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) determine the requirement for vernalization (Koornneef et al., 1994; Lee et al., 1994). *FRI* encodes a novel protein that increases the mRNA level of the MADS-domain gene *FLC* (Johanson et al., 2000; Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* acts as a strong floral repressor by negatively regulating the expression of genes that promote the floral transition including *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1/AGAMOUS-LIKE 20* (*SOC1/AGL20*) and *FT* (Lee et al., 2000; Samach et al., 2000). Vernalization promotes flowering by reducing *FLC* mRNA levels, thereby antagonizing *FRI* function (Michaels and Amasino, 1999; Sheldon et al., 1999). The extent of this reduction is proportional to the duration of vernalization and is closely correlated with flowering time. *FLC* expression is also downregulated by the action of genes of the autonomous floral promotion pathway (*FCA*, *LUMINIDEPENDENS* (*LD*), *FVE*, and *FPA*). Mutations in these genes cause increased *FLC* levels and a late-flowering phenotype that can be reversed by vernalization. Thus, *FLC* is a convergence point of multiple floral pathways.

We have previously identified several *VERNALIZATION* (*VRN*) genes, which mediate the vernalization response in *Arabidopsis* (Chandler et al., 1996). *vrn* mutants appear to be unable to reduce *FLC* mRNA in response to low temperature, suggesting that they encode regulators of *FLC* expression (Sheldon et al., 2000b). Here, we show that one of these genes, *VRN2*, encodes a nuclear-localized zinc finger protein similar to the *Drosophila* Polycomb group (PcG) protein Suppressor of zeste 12 (SU[Z]12), and to two developmental repressors in *Arabidopsis*, FERTILIZATION-INDEPENDENT SEED 2 (FIS2) and EMBRYONIC FLOWER 2 (EMF2). *VRN2* does not appear to be required for the vernalization-induced decrease of *FLC* mRNA, but is essential for the stable repression of *FLC* later in development. This suggests that *VRN2* has a PcG-like func-

¹ Correspondence: caroline.dean@bbsrc.ac.uk

² Present Address: Department of Plant Research, Risø National Laboratory, Frederiksborgvej 339, DK-4000 Roskilde, Denmark.

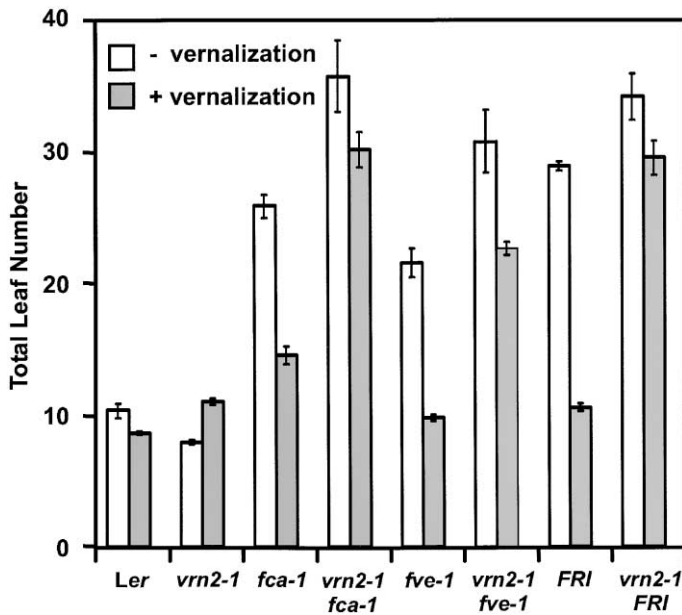


Figure 1. *vrn2* Reduces the Vernalization Response of Late Flowering Genotypes

Plants were grown under vernalization conditions for 4 weeks or stratified for 2 days (4°C, short days) before transferring to normal growth conditions (20°C, long days). Flowering time was estimated by counting the total leaf number, comprising rosette leaves and cauline leaves on the main inflorescence. Data shown are mean \pm SE, of 15–20 plants per treatment.

tion that maintains the repressed state of a key developmental regulator.

Results

vrn2 Mutations Affect the Vernalization Response of Late Flowering Mutants

The *vrn2-1* mutant was originally isolated in the late flowering, vernalization-responsive *fca-1* background following a screen for mutants that exhibited a reduced vernalization response (Chandler et al., 1996). The *vrn2-1 fca-1* double mutant exhibits a clear reduction in its vernalization response, which correlates with increased levels of *FLC* mRNA (relative to *fca-1*) after vernalization (Sheldon et al., 2000b). To determine if the *vrn2-1* mutation was indeed a true regulator of vernalization response and not a specific modifier of *fca-1*, we generated additional double mutants of *vrn2-1* and the late flowering, vernalization-responsive genotypes *fve-1* and *FRI* and measured their vernalization response (Figure 1). When combined with dominant *FRI* or recessive *fve* alleles, *vrn2-1* mutants showed a significant reduction in the vernalization response (Figure 1). This effect was most pronounced in the *vrn2-1 FRI* double mutant, which showed only a 13% decrease in leaf number after a vernalization treatment, compared to the 63% reduction observed in the *FRI* line. Thus, *VRN2* is clearly involved in mediating the activity of the vernalization pathway and is not solely an enhancer of *fca-1*.

In the absence of vernalization, *vrn2-1 fca-1* double mutants flowered later than *fca-1*, indicating *VRN2* has floral-promotive functions in an *fca-1* background (Chandler et al., 1996). To analyze this further, the flowering time of *vrn2-1* in a wild-type background was analyzed and found to be similar to the wild-type Landsberg *erecta* (*Ler*) control (Figure 1). This was also true for a second allele of *vrn2*, in the form of the *rpp5-2* mutant that carries a large deletion that includes *VRN2* (A.R.G. and J. Parker, data not shown). Thus, the additional

functions of *VRN2* are only revealed in the presence of mutations in *FCA* or *FVE* or dominant alleles of *FRI*, all of which lead to increased levels of *FLC* mRNA.

Positional Cloning of *VRN2*

A mapping population was generated by crossing *vrn2-1 fca-1* (in the *Ler* background) to *fca-10* (Wassileskija background) and *VRN2* mapped to the long arm of chromosome IV. Three recombinants in a population of 499 plants positioned *VRN2* between the marker CC36F6 and g4539, an interval of 245 kb. Two markers, TGCAPS1 and TGCAPS2 were approximately 220 kb apart and cosegregated with *VRN2* (Figure 2A). This low frequency of recombination is roughly centered on the *RPP5* locus that regulates resistance to the fungus *Peronospora parasitica* (Parker et al., 1998). We chose to undertake a large-scale cosmid complementation experiment. A contig was assembled by end sequencing cosmids from a *Ler* genomic library and subclones of the Columbia yeast artificial chromosome (YAC) clone EW16B10 that spans this region (Figure 2B) (Bancroft et al., 1997). These cosmids were introduced into *vrn2-1 fca-1* plants via *Agrobacterium*-mediated transformation, and four cosmids that complemented the *vrn2-1* mutant phenotype were identified (Figure 2B). Two complementing cosmids and two overlapping but noncomplementing cosmids defined a region of approximately 14 kb that encompasses *VRN2* (Figure 2C).

A detailed analysis and reannotation of this region (accession numbers AF180942 and AL161545) (Mayer et al., 1999; Noël et al., 1999) revealed that it contained only two complete putative open reading frames (ORFs) (Figure 2C). Using primers based on the sequence of an annotated cDNA clone in this region (5KMY) (Bevan et al., 1998) and RT-PCR, the expression of one of these genes was confirmed. Sequencing of products from three independent RT-PCR reactions amplifying this 1718 bp cDNA revealed a point mutation, a G to A change at position 1195, only in cDNA derived from

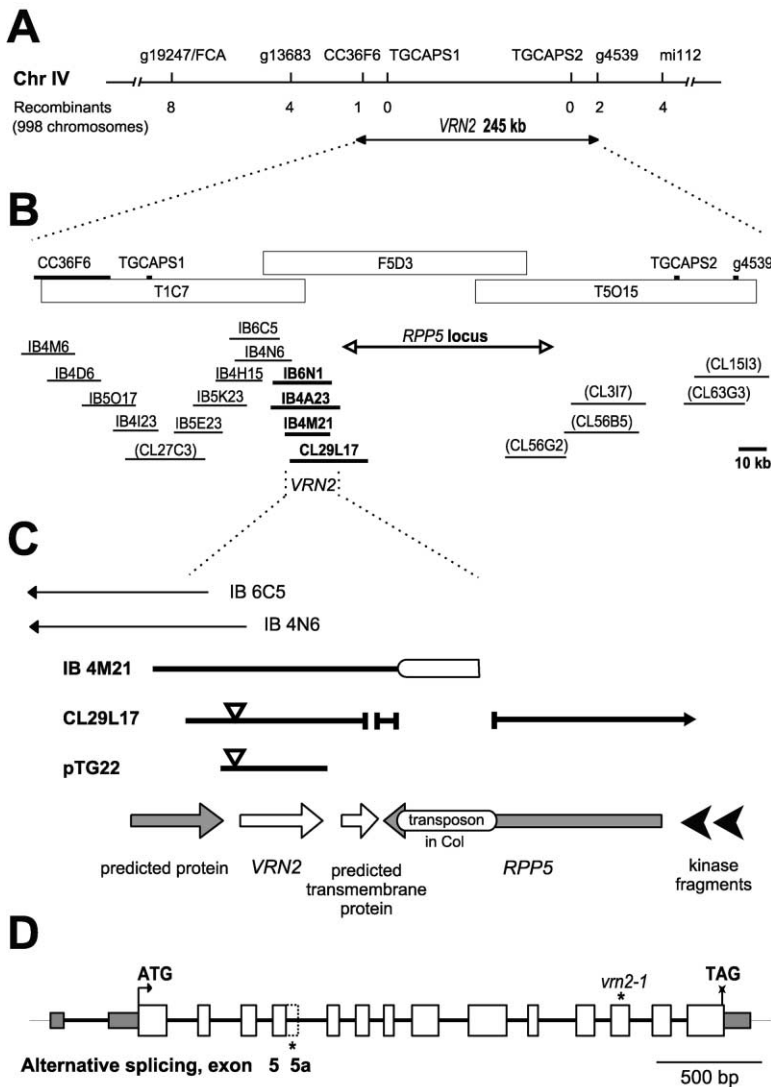


Figure 2. Cloning of *VRN2*

(A) Genetic mapping of *VRN2*. *VRN2* was positioned between the RFLP marker CC36F6 and CAPS marker g4539 in a segregating F2 population derived from the cross *vrn2-1 fca-1* (*Ler*) × *fca-10* (WS).

(B) Physical map near *VRN2*. Bacterial artificial chromosome (BAC) clones are shown as open rectangles, with markers indicated above. Cosmids that complemented the reduced vernalization response of *vrn2-1 fca-1* mutants are shown as thick lines. Cosmids derived from the Columbia YAC EW16B10 and *Ler* genomic DNA are prefixed IB and CL, respectively. A complementing subclone of CL29L17, pTG22, is also shown. Cosmids in brackets were positioned but not tested for complementation.

(C) Identification of *VRN2*. Predicted genes completely encompassed by the complementing cosmids IB4M21 and CL29L17 are shown as open arrows. Filled arrows indicate partial or interrupted genes. A retrotransposon insertion in Columbia is shown as an open oval. The genomic sequence of *Ler* relative to Columbia is indicated, with an insertion represented by an open triangle, and deletions by gaps.

(D) Structure of the *VRN2* gene. Coding regions are shown as open boxes, untranslated regions as filled boxes, and introns as thick lines. The predicted start and stop codons are indicated by ATG and TAG, respectively. The alternative exon of *VRN2'* is indicated by 5a, and the position of stop codons introduced by the *vrn2-1* mutation and the alternate exon are shown by asterisks. The introns of *VRN2* range from 81 to 234 nucleotides in length with an average of 137 nucleotides, all with canonical splice-site junctions. The 5' untranslated region comprises the first 230 nucleotides.

vrn2-1 fca-1 mutant plants. As this mutation introduces a premature stop codon in the predicted ORF, it appeared likely that this gene was *VRN2*. Sequencing of the other predicted gene, a putative transmembrane protein, revealed no mutations in *vrn2-1 fca-1* plants. A PCR-based assay specifically detected the G to A change only in *vrn2-1 fca-1* mutant plants, confirming the presence of this mutation in the genome (data not shown). A subcloned 6 kb fragment of the complementing CL29L17 cosmid (pTG22) corresponding to the genomic region of the putative *VRN2* gene was able to rescue the reduced vernalization response phenotype of *vrn2-1 fca-1* mutants. As this genomic fragment contains no other predicted ORFs, it confirms this gene is *VRN2* (Figure 2C).

The structure of the *VRN2* gene was determined by comparing the sequence of the *VRN2* cDNA (accession number AF284500) to the genomic sequence (Figure 2D). This analysis revealed that the 1718 nucleotide *VRN2* cDNA is encoded by 15 exons, the first of which is noncoding. The *vrn2-1* G to A transition results in the conversion of a TGG tryptophan codon to a TGA stop codon, truncating the predicted protein after amino

acid 322 (Figure 2D). Examination of the *VRN2* cDNA sequence derived from cloned RT-PCR products revealed the presence of an alternatively spliced transcript (*VRN2'*) of 1740 nucleotides (accession number AF284501) (Figure 2D). The additional 22 nucleotides of *VRN2'* (which include an in-frame stop codon) arise from the use of an alternate 5' splice donor site at the end of the fifth exon, producing a novel exon 5a (Figure 2D). This alternate transcript is predicted to encode a nonfunctional truncated form of the *VRN2* protein of only 107 amino acids that lacks almost all the predicted functional domains. The *VRN2'* transcript represents a small proportion of the total *VRN2* mRNA, as it was undetectable by semiquantitative RT-PCR and was only observed after sequencing cloned RT-PCR products (data not shown).

VRN2 Is Similar to Putative Transcriptional Regulators

The *VRN2* open reading frame is predicted to encode a 445 amino acid protein, of approximately 51 kDa, with an in-frame stop codon present nine nucleotides up-

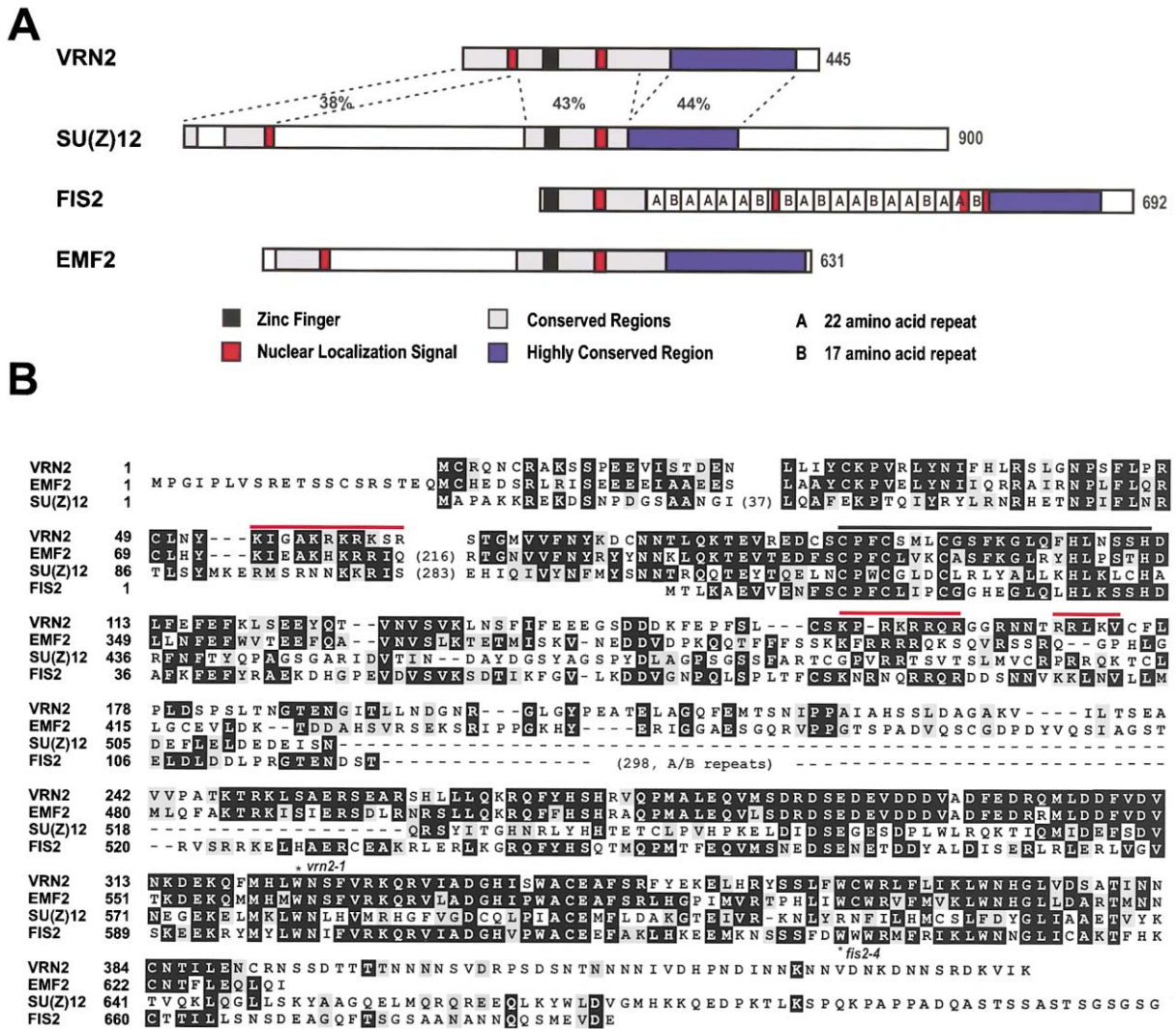


Figure 3. VRN2 Resembles PcG Transcriptional Regulators

(A) VRN2 shares a number of motifs and domains with FIS2, EMF2, and SU(Z)12. A schematic alignment of VRN2 (accession no. AF284500), FIS2 (accession no. AF096096), SU(Z)12 (accession no. AF149047), and EMF2 (GenBank accession number AB023044) is shown with regions showing similarity shaded in gray. Percentages refer to amino acid similarity between VRN2 and SU(Z)12. The conserved N-terminal region is indicated in green, the zinc finger motif is indicated in black, and positions of putative NLSs/clusters of basic residues indicated in red. The position of the highly conserved acidic region and the LWN repeats is shown in blue. The 22 amino acid A and 17 amino acid B repeats of FIS2 are indicated. Regions with no significant similarity are unshaded.

(B) Sequence alignment VRN2, EMF2, FIS2, and SU(Z)12. An alignment of the four proteins is shown, with numbers referring to amino acid number. Identical residues are shown as white type on a black background, while similar residues are shaded with gray. Dashes indicate gaps introduced to optimize the alignment, with regions excluded for clarity indicated in brackets. The putative NLSs of VRN2 are indicated by red overlining, and the zinc finger motif is shown by a black overline. The position of the *vrn2-1* mutation (a stop codon at amino acid 323) and the *fis2-4* mutation (a stop codon at position 637) are indicated.

stream of the predicted initiating methionine codon. The VRN2 protein shows significant homology to the *Arabidopsis* proteins FERTILIZATION-INDEPENDENT SEED 2 (FIS2) (Luo et al., 1999) and EMBRYONIC FLOWER2 (EMF2) (Yoshida et al., 2001), and the *Drosophila* PcG protein Suppressor of zeste 12 (SU[Z]12) (Figure 3A) (Birve et al., 2001). *fis2* mutants display gametophytic maternal-effect lethality and endosperm development in the absence of fertilization, suggesting that one function of FIS2 is to repress endosperm development before fertilization (Luo et al., 1999). EMF2 also encodes

a putative floral repressor, as *emf2* mutants exhibit a very early flowering phenotype, producing abnormal flowers almost immediately after germination (Yang et al., 1995). *emf2* mutants are also deficient in the maintenance of a normal inflorescence, with terminal flowers produced in place of the normal indeterminate structure (Chen et al., 1997; Yang et al., 1995). Mutations in SU(Z)12 cause embryonic lethality, and embryos rescued from homozygous mutant germ line clones show severe homeotic transformations. *su(z)12* mutations enhance the phenotype of mutations in Polycomb-group

genes including *Polycomb* (*PC*), *Polycomb-like* (*PCL*), and *Sex comb on midleg* (*SCM*), and in some cases act as dominant suppressors of variegation (Birve et al., 2001).

Overall, the structures of VRN2, FIS2, EMF2, and SU(Z)12 are similar with respect to the order of regions that show similarity, but not to their spacing (Figure 3A). VRN2, EMF2, and SU(Z)12 share an N-terminal-conserved region of approximately 60 amino acids, which includes a putative nuclear localization signal (NLS). In SU(Z)12 there are 37 additional amino acids toward the start of this region. In EMF2 and SU(Z)12 this N-terminal domain is followed by a weakly conserved region (14% identity and 23% similarity over 281 amino acids) that is not present in VRN2 or FIS2 (Figure 3A and 3B). The second recognizable motif present in VRN2, at amino acids 90–111, is a C2H2-type zinc finger that is also found in FIS2, EMF2, and SU(Z)12 (Figure 3B). The carboxy-terminal half of VRN2 is comprised of a region conserved in all four proteins, from amino acid 246 to 390 of VRN2. The first half of this region is characterized by a large proportion of the acidic amino acids aspartate and glutamate (Figure 3B). The second half of this region in VRN2 includes two repeats of the sequence leucine-tryptophan-asparagine (LWN) but otherwise bears no obvious functional motifs. Two pieces of evidence suggest that these motifs may be functionally important. First, the *vrn2-1* mutation results in a stop codon in the first of these LWN motifs. Second, the *fis2-4* mutation introduces a stop codon prior to the second of these motifs, truncating the protein after amino acid 636 (Luo et al., 1999) (Figure 3B).

In summary, the similarity between VRN2 and SU(Z)12 suggests that VRN2 may mediate the response to vernalization in a manner similar to the action of PcG proteins by contributing to the maintenance of transcriptional repression, perhaps through an effect on chromatin structure.

VRN2 Expression Is Not Altered by Vernalization

Given that VRN2 has a major effect on flowering time after a long period of low temperature, it was possible that VRN2 activity might itself be regulated by vernalization. We therefore examined the expression of VRN2 by Northern blot analysis. VRN2 mRNA could not be detected using total RNA isolated from vernalized or nonvernalized plants, or from plants of a wide range of developmental stages or a variety of tissues (data not shown). However, VRN2 mRNA was detectable in polyA⁺-enriched RNA fractions (Figure 4A), suggesting either that VRN2 mRNA is a low abundance transcript or is expressed in a small subset of cells. The observed size of the transcript was ~1.7 kb, in close agreement with the size predicted from the VRN2 cDNA. The VRN2 transcript was detectable in *vrn2-1* mutants, both in *Ler* and *fca-1* backgrounds at approximately wild-type levels. It was completely absent in plants carrying the *rpp5-2* mutation, which results in the complete deletion of VRN2 (A.R.G. and J. Parker, unpublished results) (Figure 4A).

The level of VRN2 transcript was not altered in *fca-1* plants after a 3 week vernalization treatment, and as such did not parallel the previously observed changes

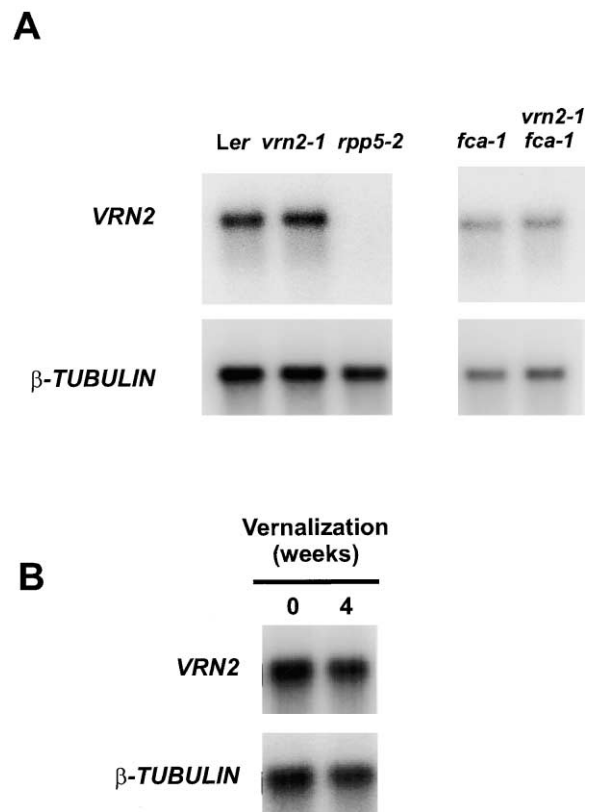


Figure 4. VRN2 Expression

(A) VRN2 mRNA expression in *vrn2* mutants. Northern blot analysis was performed using approximately 2 μg of polyA⁺ RNA isolated from nonvernalized 14-day-old plants of the indicated genotypes and probed with a VRN2-specific probe. The blot was then stripped and reprobed with a β-TUBULIN probe as a loading control.

(B) Effect of vernalization on VRN2 Expression. Northern blot analysis was performed using approximately 2 μg of polyA⁺ RNA isolated from 14-day-old *fca-1* seedlings that were previously vernalized for 4 weeks or untreated. Blots were probed as in (A).

in *FLC* mRNA (Figure 4B) (Michaels and Amasino, 1999; Sheldon et al., 1999). These data indicate that VRN2 is expressed in the absence of vernalization and suggest that if VRN2 activity is regulated by vernalization, it must occur posttranscriptionally.

VRN2 Is Nuclear Localized

The subcellular localization of VRN2 was determined using a chimeric green fluorescent protein (GFP)-VRN2 fusion protein and a transient transfection assay into onion epidermal cells (Varagona et al., 1992). The GFP control transfection produced cells that exhibited uniformly distributed fluorescence (Figure 5A). In contrast, cells transfected with a GFP:VRN2-expressing plasmid exhibited only nuclear fluorescence (Figures 5C and 5D). This fluorescence was not uniformly distributed throughout the nucleus, but appeared to be concentrated in discrete regions (Figure 5E). We also observed similar patterns of localization when VRN2 was fused to the N terminus of GFP or β-glucuronidase (GUS) (data not shown). These results strongly suggest that VRN2 is nuclear localized, implying that the putative NLSs may indeed be func-

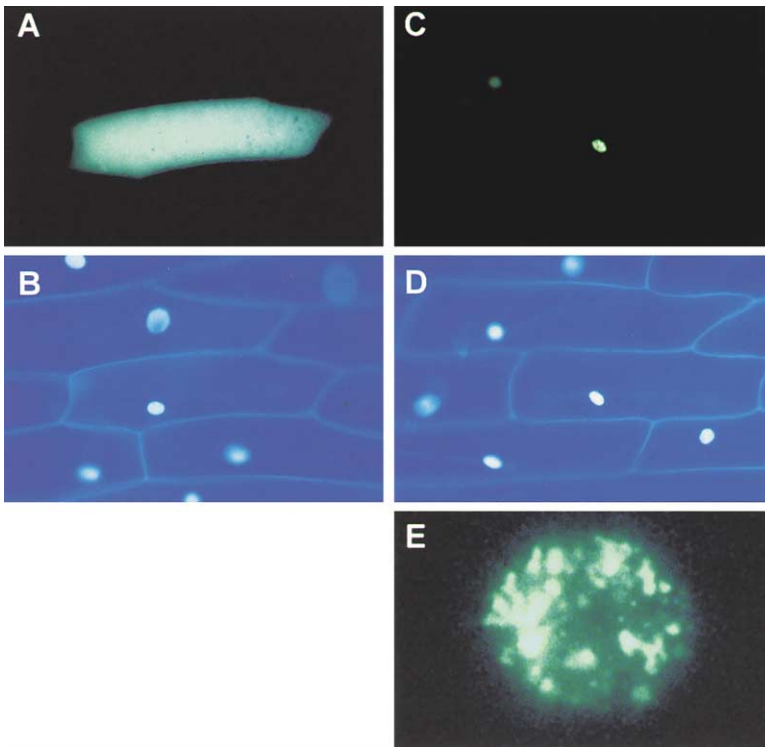


Figure 5. GFP:VRN2 Fusions are Nuclear Localized

(A), (C), and (E) GFP fluorescence in GFP-(A) or GFP:VRN2-expressing (C) and (E) onion epidermal cells. (E) shows discrete nuclear speckles in a cell transfected with a GFP:VRN2 expressing plasmid.

(B) and (D) DAPI (4', 6-diamidino-2-phenylindole) staining of the cells shown in (A) and (C). GFP or GFP:VRN2 fusions were placed under the control of the CaMV 35S promoter. Onion epidermal peels were bombarded with DNA-coated gold particles and GFP expression visualized 12 hr later.

tional. This result is also consistent with the hypothesis that VRN2 may directly affect transcription.

VRN2 Function Is Required for the Stable Repression of *FLC*

In animals, PcG proteins are expressed throughout development but act only in a subset of cells to stably repress the transcription of particular genes that were initially repressed by other factors (for reviews see Brock and van Lohuizen, 2001; Pirotta, 1998). PcG proteins are not required for the establishment of an expression pattern, but are required for the maintenance of this pattern throughout subsequent development and over many cell divisions. If VRN2 functions as a PcG-like protein in *Arabidopsis*, then it should behave in a similar manner. Previous studies indicated that *vrn2-1 fca-1* plants have elevated *FLC* mRNA levels after vernalization, indicating that *FLC* regulation is perturbed in these mutants, and implying that VRN2 is required for the downregulation of *FLC* expression (Sheldon et al., 2000b). However, the discovery that VRN2 encodes a PcG-like protein suggests that VRN2 is more likely to function in the maintenance of *FLC* expression. We therefore investigated in detail the regulation of *FLC* expression in *vrn2-1* mutants.

Unlike previous studies (Sheldon et al., 2000b), we examined *FLC* mRNA expression in *vrn2-1 fca-1* mutants in detail throughout development and after differing periods of vernalization. By harvesting plants at different stages of development after the transfer from vernalization conditions to normal growth conditions, it became apparent that the profile of *FLC* mRNA levels in *vrn2-1 fca-1* mutants is more complex than was ap-

parent by examining a single time point (Figure 6A). In *fca-1* mutants, *FLC* mRNA decreases following a vernalization treatment, and this reduction is observed as soon as plants are harvested after the shift to normal growth conditions (Figure 6A; 3 and 6 weeks vernalization, day 0). This is consistent with previous observations in an *FRI* background that demonstrated *FLC* mRNA levels decline during the low-temperature treatment (Sheldon et al., 2000b). Furthermore, this low level of *FLC* expression is maintained throughout subsequent development in *fca-1* mutants (Figure 6A; 3 and 6 weeks vernalization, days 0–20). In contrast, *vrn2-1 fca-1* mutants show a different profile of *FLC* mRNA levels (Figure 6A). Similar to *fca-1* plants, *vrn2-1 fca-1* mutants exhibit reduced *FLC* mRNA levels immediately after vernalization (Figure 6A; 3 and 6 weeks vernalization, day 0), but this low level of *FLC* mRNA is not maintained throughout development (days 0–20). Elevated levels of *FLC* mRNA are detectable in *vrn2-1 fca-1* mutants 10 days after removal from vernalization, and by 20 days of growth at normal temperature, the level of *FLC* had further increased. The regulation of *FLC* mRNA levels was qualitatively similar for plants that had received an extended vernalization treatment of 12 weeks, with an increase in *FLC* mRNA detectable at 10 days after the transfer from vernalization (Figure 6B). These data indicate that, contrary to a previous suggestion (Sheldon et al., 2000b), VRN2 is not required for the downregulation of *FLC* mRNA induced by vernalization, but is essential for the stable repression of *FLC* mRNA levels. The *vrn2-1* mutation therefore uncovers a novel, complex aspect of *FLC* regulation.

VRN2 Affects the DNase I Accessibility of *FLC*

The homology and functional similarity of VRN2 to PcG proteins suggests that it might act to alter the chromatin

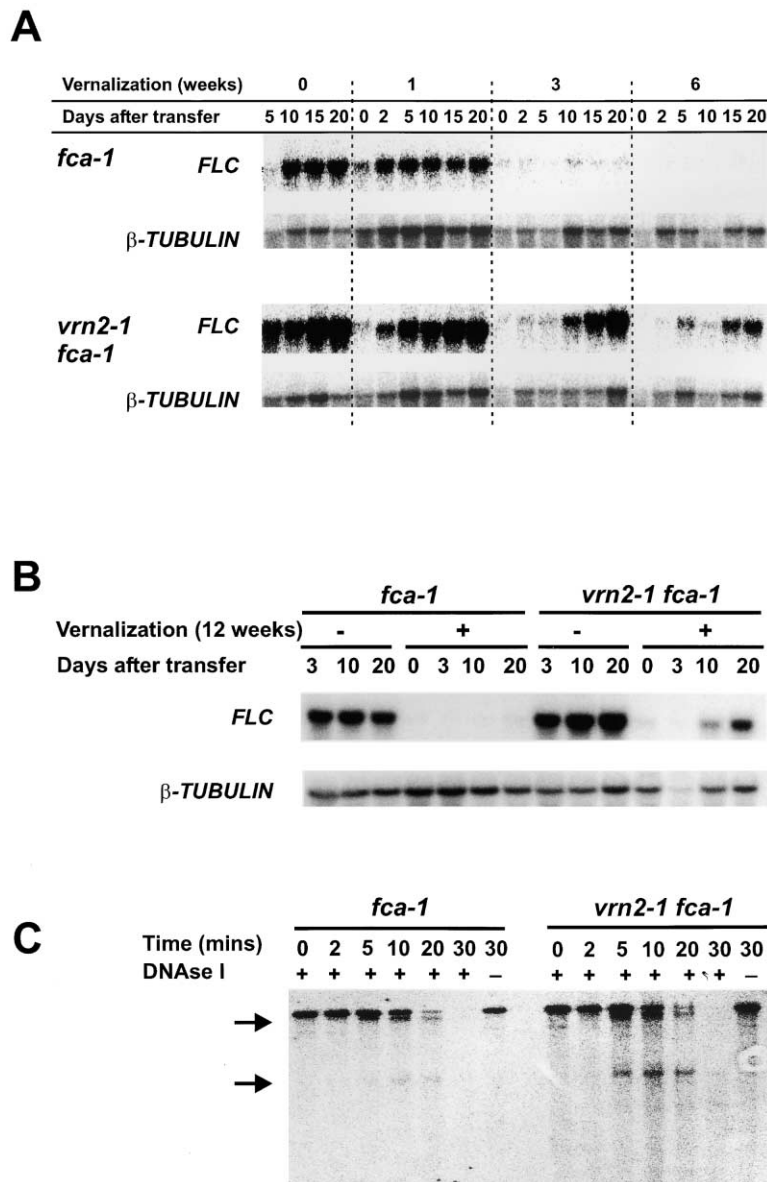


Figure 6. VRN2 Mediates Stable FLC Repression and DNase I Sensitivity Following Vernalization

(A) Seeds were grown on agar plates under vernalization conditions for the indicated periods before being transferred to normal growth conditions. Plants were harvested at the indicated times after the transfer, with day 0 being immediately after the shift to normal growth temperature. Total RNA was isolated and probed with an *FLC*-specific probe. After exposure, the blot was stripped and re-hybridized to a β -TUBULIN probe.

(B) As above, but seeds were grown under vernalization conditions for 12 weeks (+) or stratified for 2 days (-) and then transferred to normal growth conditions.

(C) Nuclei isolated from vernalized plants were treated with DNase I. Following extraction the DNA was digested to completion with NcoI and blotted. The blot was probed with a 370 bp fragment of *FLC* intron 1 adjacent to exon 1. The lower arrow points to a 3.3 kb fragment indicating a major hypersensitive site within the first intron of *FLC*. The ratio of this fragment to the full-length *FLC* NcoI fragment at the 20 min time point is 2-fold higher in *vrn2-1 fca-1* relative to *fca-1*. The ratio of the 3.3 kb fragment to a *FRIGIDA* fragment (that should not show a differential sensitivity to DNaseI in *vrn2*) progressively increased in *vrn2-1 fca-1* relative to *fca-1* over the first 20 min of the timecourse (data not shown). The upper arrow corresponds to a DNaseI hypersensitive site distal to the 3' end of the coding region of *FLC*.

organization of *FLC*. We tested this by examining the DNase I sensitivity of *FLC* in *fca-1* and *vrn2-1 fca-1* seedlings that had been subjected to 4 weeks vernalization (Figure 6C). *FLC* DNase I sensitivity was enhanced in plants carrying the *vrn2-1* mutation suggesting that VRN2 mediates changes in the chromatin organization of *FLC* following vernalization. A major hypersensitive site mapped within the first intron of the *FLC* gene. This is consistent with the requirement for sequences in addition to the promoter and 3' region of *FLC* in marker gene fusions to obtain expression that mimics the normal pattern of *FLC* RNA regulation (C. Lister and C.D., unpublished data).

Discussion

Our data indicate that a gene required for a normal vernalization response in *Arabidopsis*, *VRN2*, exhibits sequence homology to a PcG gene of *Drosophila*. Fur-

thermore, *VRN2* is not required for the regulation of *FLC* expression that occurs during a cold treatment period, but is essential for maintaining its correct repression (Figure 6), indicating that VRN2 behaves in a functionally similar way to PcG proteins. The implication of these data is that the stable VRN2-dependent repression of *FLC* contributes to the epigenetic basis of vernalization.

A characteristic feature of vernalization is the clear temporal separation between the low-temperature stimulus and the transition to flowering—in some species this “memory” of vernalization can be maintained for up to 300 days (Lang, 1965). This has been interpreted to indicate that vernalization induces a developmental state that is clonally inherited by the descendants of cells that were actively dividing at low temperatures (Wellensiek, 1964), with the induced state eventually favoring the transition to flowering. The maintenance of the vernalized state through multiple cell divisions is reminiscent of epigenetic phenomena (Riggs et al., 1996;

Wolffe and Matzke, 1999). The best-characterized system of epigenetic gene regulation is the control of homeotic gene expression by PcG proteins during *Drosophila* development (Brock and van Lohuizen, 2001; Müller and Leutz, 2001). In this case, after the initial expression pattern of a homeotic gene is established by the segmentation genes, including the gap genes, it is maintained in a repressed state throughout subsequent development by PcG proteins. PcG proteins are expressed throughout development and act in large protein complexes to silence gene expression, in a mechanism that probably involves chromatin remodelling (Brock and van Lohuizen, 2001; Müller and Leutz, 2001).

The discovery that *VRN2* is a member of a small gene family suggests that other *VRN2*-like proteins may also act to maintain stable patterns of gene repression. The genetic screens that identified *FIS2* also identified *MEDEA* (*MEA*) and *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), which encode proteins similar to the *Drosophila* PcG proteins Enhancer of zeste (*E[Z]*) and extra sex combs (*ESC*), respectively (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999; Ohad et al., 1999). *MEA* and *FIE* directly interact in vitro, although a direct interaction between *FIS2* and *MEA* or *FIE* could not be detected (Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000). It has been proposed that *FIS2* regulates gene repression by indirectly interacting with the *MEA/FIE* protein complex, perhaps recruiting the *MEA/FIE* complex to target genes (Luo et al., 2000; Yadegari et al., 2000). *VRN2* might be a component of, or be required for, the assembly or maintenance of a large protein complex, similar to one of the two distinct PcG complexes in *Drosophila* (Ng et al., 2000; Shao et al., 1999; Tie et al., 2001). *EMF2* also affects gene expression in a manner consistent with a PcG-like function, as *emf2* mutants exhibit ectopic expression of the floral homeotic genes *APETALA1* and *AGAMOUS* (Chen et al., 1997).

It has been suggested that the promotive effects of vernalization are mediated by a reduction in DNA methylation of as yet unidentified floral regulators (Burn et al., 1993). However, extensive hypomethylation of the *Arabidopsis* genome in the C24 accession, induced by an antisense DNA methyltransferase (α MET), does not fully substitute for a vernalization treatment (Finnegan et al., 1998; Sheldon et al., 1999). In the Columbia accession an α MET transgene delays flowering (Ronemus et al., 1996). Plants with reduced histone deacetylation also show flowering time changes (Tian and Chen, 2001). These global genome changes cause a range of phenotypes in addition to altering flowering time, so it is not clear if they are acting upon genes normally involved in flowering time control. Whether methylation or deacetylation changes play a role in chromatin-based regulation of *FLC* remains to be established.

The observation that *VRN2* encodes a PcG-like gene raises several new issues for the understanding of the mechanism of vernalization. What causes the initial reduction in *FLC* expression during a cold treatment is unknown. The explanation for the quantitative nature of vernalization has also been elusive. All cells in the shoot meristem may respond equally to vernalization in a quantitative manner, or perceive and respond to vernalization differently because of intrinsic differences. This

may be due to being in different phases of the cell cycle or different positions within the meristem. The observations that *VRN2* is expressed at the same level independently of a vernalization treatment (Figure 4B), and *VRN2* has floral promotive functions in the absence of vernalization (Figure 1), might indicate that a *VRN2*-containing complex is constitutively present and has some role in the absence of vernalization but is further activated or recruited only following a long period of growth at low temperatures. Alternatively, it may be recruited or formed when plants return to normal temperatures. However, *VRN2* is not essential for the repression of *FLC* during a vernalization treatment, nor for the stable repression of *FLC* early in development (Figure 6). A detailed analysis of the requirement for *VRN2* at different stages of vernalization, using a system of inducible *VRN2* activity, may clarify the situation. It is also important to determine if *VRN2* regulates the expression of genes other than *FLC*, and if it does this as a component of a larger protein complex or as an isolated DNA binding factor. The isolation of a number of distinct *vrn* mutants reveals that the response to vernalization is mediated by several genes (Chandler et al., 1996). Characterization of *VRN1* reveals that at least one of these genes functionally overlaps with *VRN2* (Y.Y.L., A.R.G., and C.D., unpublished data). It will be essential to determine if this similarity is the result of a direct interaction between *VRN* proteins.

Experimental Procedures

Plant Growth Conditions, Vernalization Treatments, and Flowering Time Analyses

The *Ler*, *fca-1*, and *fve-1* lines were all originally obtained from M. Koornneef (Wageningen University, The Netherlands). *rpp5-2* was a gift from J. Parker (Sainsbury Laboratory, Norwich, UK), while the *fca-10* allele was a gift from R. Amasino (University of Wisconsin). The *vrn2-1 fca-1* double mutant has been described and was used at the 4th backcross (Chandler et al., 1996). The *FRI* line used was a *Ler* line containing the JU223 cosmid (Johanson et al., 2000). Vernalization treatments were conducted by sowing seeds on moist soil and growth at 4°C under short day (SD) conditions (8 hr light: 16 hr dark) in white light (approximately 10 μ mol photons $m^{-2} s^{-1}$). Nonvernalized control seeds were stratified to break dormancy for 2 days under vernalization conditions. For flowering time measurements, plants were transferred into individual wells of sectioned trays and grown in controlled environment rooms at 20°C under long day (LD) conditions (16 hr light: 8 hr dark, with fluorescent lights at approximately 100 μ mol photons $m^{-2} s^{-1}$). Flowering time was measured by recording the number of rosette leaves and added to the number of cauline leaves on the main stem.

Genetic and Physical Mapping

The *VRN2* gene was mapped using late flowering, vernalization-nonresponsive F2 plants from a cross between *vrn2-1 fca-1* (*Ler* background) to *fca-10* (Wassilewskija background), similar to that used to map *VRN1* (Chandler et al., 1996). Genomic DNA was prepared from 499 F2 plants that were late after vernalization. Recombinants near *VRN2* were initially selected with markers g19247 and g4539. Fine mapping was performed with markers TGCAPS1, TGCAPS2, (for details of new markers see TAIR database <http://www.Arabidopsis.org>), and RFLP markers CC36F6 (Bancroft et al., 1997), g13683, and mi122 (Schmidt et al., 1996).

Cosmid Complementation

A binary cosmid contig was assembled by end sequencing Columbia cosmids, derived from YAC EW16B10, in p04541 (Bancroft et al., 1997) and aligning them to the genomic sequence. Additional *Ler* cosmids in the p04541 vector were identified by hybridization

to the inserts of BACs T5015 and T1C7, or VRN2-specific probes. Cosmids were introduced into *Agrobacterium* strain C58C1 pGV2260 by triparental mating, and *vrn2-1 fca-1* plants transformed by floral dip (Clough and Bent, 1998). Primary T1 transformants were selected on GM media without sucrose (Valvekens et al., 1988) supplemented with 50 µg/ml kanamycin. Cosmids were scored as complementing if T2 segregating populations segregated 3:1 early:late flowering plants following a 4 week vernalization treatment. This complementation was confirmed in the next generation with homozygous T3 plants. pTG22 is a 5.95 kb EcoRI-SpeI fragment of CL29L17 in the SLJ75515 vector (<http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/jonathan-jones/plasmid-list/plasmid.htm>). pTG22 transformants were selected on soil after spraying with BASTA herbicide.

Gene Prediction and Identification

Genes were predicted with Genscan and Net PlantGene2 (Hebsgaard et al., 1998). Protein sequences were analyzed with PROSITE (Bairoch et al., 1997), PSORT (Nakai and Kanehisa, 1992), and ψ-BLAST searches (Altschul et al., 1997). Initial protein alignments were generated using the Clustal program with MegAlign (Lasergene) and adjusted manually. Genomic DNA or first strand cDNA was amplified with the ExpandHiFi PCR kit (Roche), and gel-purified products were directly sequenced with BIGDYE (Amersham) using an ABI377. Sequences were compiled using the SeqMan program within the DNASTar package (Lasergene). PCR products from three independent reactions were sequenced, and all contained the same nucleotide substitution in *vrn2-1 fca-1* mutants. The presence of this mutation was also confirmed with a dCAPS marker (details available upon request).

RNA Analysis

RNA was fractionated on 1.2% agarose formaldehyde-containing gels and transferred to nylon membranes. The FLC probe was a 403 bp PCR-amplified cDNA fragment lacking MADS-domain sequences (corresponding to nucleotides 298–700 of the FLC cDNA) (Sheldon et al., 1999). The VRN2 probe was a 585 bp HindIII fragment of the VRN2 cDNA comprising nucleotides 2–586. Loading was normalized by stripping in boiling 0.5% SDS and rehybridization with a β-TUBULIN coding-region probe (Snustad et al., 1992). Blots were exposed to PhosphorImager screens (Molecular Dynamics)

DNase I Sensitivity Assays

DNase I sensitivity assays were performed essentially as described in van Blokland et al. (1997). Plants were vernalized for 4 weeks, and grown in LDs for 20 days prior to harvesting. The FLC probe used corresponds to nucleotides 295–665 of the FLC gene (accession number AF116528). The FRIGIDA probe used was a 1.8 kb EcoRI fragment of the cDNA (Johanson et al., 2000). Bands were quantified using ImageQuant.

Transient Subcellular Localization

VRN2 fusions to GFP were made using the pAVA120 vector (von Arnim et al., 1998). VRN2 coding regions were amplified by PCR and subcloned as a C-terminal fusion to GFP using restriction sites introduced on the PCR primers, and placed under the control of the 35S promoter, and all constructs sequenced. Transient transfections of onion epidermal cells were performed as described (Vargona et al., 1992), except 10 µg of DNA was used to coat 2 mg of 1.6 µm gold particles. Twelve hours after bombardment, nuclei were counter-stained for 10 min with 10 mg/ml DAPI. Epidermal peels were mounted on slides in 50% glycerol and visualized using a Nikon E-800 fluorescence microscope.

Acknowledgments

We thank R. Schmidt and P. Piffanelli for the sequence of the 5K cDNA, I. Bancroft for YAC-subclone cosmids, and L. Nöel, J. Parker, and J. Jones for *rpp5-2* seed and *Ler* sequences prior to publication. We are grateful to N. Yoshida, A. Birve, Å. Rasmuson-Lestander, and J. Müller for sharing data prior to publication, and to G. Coupland, R. Macknight, and R. Sablowski for comments on the manuscript. We would like to acknowledge M. Smith for assistance in the green-

house, and K. Torney and G. Simpson for help with Figure 6. Thanks also to members of the Dean lab, particularly G. Simpson, for discussions and comments on the manuscript. This work was supported by BBSRC grant 208/MOL4649 to C.D. and a BBSRC core strategic grant to the JIC.

Received March 19, 2001; revised September 24, 2001.

References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Bairoch, A., Bucher, P., and Hofmann, K. (1997). The PROSITE database, its status in 1997. *Nucleic Acids Res.* 25, 217–221.
- Bancroft, I., Love, K., Bent, E., Sherson, S., Lister, C., Cobbett, C., Goodman, H.M., and Dean, C. (1997). A strategy involving the use of high redundancy YAC subclone libraries facilitates the contiguous representation in cosmid and BAC clones of 1.7 Mb of the genome of the plant *Arabidopsis thaliana*. *Weeds World* 4, 1–9, <http://nasc.nott.ac.uk:8300/Vol8304i/bancroft.html>.
- Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., et al. (1998). Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* 391, 485–488.
- Birve, A., Sengupta, A.K., Beuchle, D., Larsson, J., Kennison, J., Rasmuson-Lestander, Å., and Müller, J. (2001). *Su(z)12*, a novel *Drosophila* Polycomb group gene that is conserved in vertebrates and plants. *Development* 128, 3371–3379.
- Brock, H.W., and van Lohuizen, M. (2001). The Polycomb group—no longer an exclusive club? *Curr. Op. Genes Dev.* 11, 175–181.
- Burn, J.E., Bagnall, D.J., Metzger, J.D., Dennis, E.S., and Peacock, W.J. (1993). DNA methylation, vernalization, and the initiation of flowering. *Proc. Natl. Acad. Sci. USA* 90, 287–291.
- Chandler, J., Wilson, A., and Dean, C. (1996). *Arabidopsis* mutants showing an altered response to vernalization. *Plant J.* 10, 637–644.
- Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 94, 4223–4228.
- Chen, L., Cheng, J.-C., Castle, L., and Sung, Z.R. (1997). *EMF* genes regulate *Arabidopsis* inflorescence development. *Plant Cell* 9, 2011–2024.
- Chouard, P. (1960). Vernalization and its relations to dormancy. *Annu. Rev. Plant Physiol.* 11, 191–237.
- Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743.
- Finnegan, E.J., Genger, R.K., Kovac, K., Peacock, W.J., and Dennis, E.S. (1998). DNA methylation and the promotion of flowering by vernalization. *Proc. Natl. Acad. Sci. USA* 95, 5824–5829.
- Grossniklaus, U., Vielle-Calzada, J.-P., Hoepfner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by *MEDEA*, a *Polycomb* group gene in *Arabidopsis*. *Science* 280, 446–450.
- Hebsgaard, S.M., Korning, P.G., Tolstrup, N., Engelbrecht, J., Rouzé, P., and Brunak, S. (1998). Splice site prediction in *Arabidopsis thaliana* pre-mRNA by combining local and global sequence information. *Nucleic Acids Res.* 24, 3439–3452.
- Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* 290, 344–347.
- Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinnery, J., Wells, D., Katz, A., Margossian, L., Harada, J.J., Goldberg, R.B., et al. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 96, 4186–4191.
- Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M., and Soppe, W.

- (1998). Genetic control of flowering time in *Arabidopsis*. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 345–370.
- Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W., and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg *erecta* wild-type. *Plant J.* **6**, 911–919.
- Lang, A. (1952). Physiology of Flowering. *Annu. Rev. Plant Physiol.* **3**, 265–306.
- Lang, A. (1965). Physiology of flower initiation. In *Encyclopedia of Plant Physiology*, W. Ruhland, ed. (Berlin: Springer Verlag), pp. 1380–1536.
- Lee, H., Suh, S.-S., Park, E., Cho, E., Ahn, J.H., Kim, S.-G., Lee, J.S., Kwon, Y.M., and Lee, I. (2000). The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes Dev.* **14**, 2366–2376.
- Lee, I., Michaels, S.D., Masshardt, A.S., and Amasino, R.M. (1994). The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDENS* is suppressed in the Landsberg *erecta* strain of *Arabidopsis*. *Plant J.* **6**, 903–909.
- Luo, M., Bilodeau, P., Dennis, E., Peacock, W.J., and Chaudhury, A. (2000). Expression and parent-of-origin effects for *FIS2*, *MEA*, and *FIE* in the endosperm and embryo of developing *Arabidopsis* seeds. *Proc. Natl. Acad. Sci. USA* **97**, 10637–10642.
- Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J., and Chaudhury, A.M. (1999). Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **96**, 296–301.
- Mayer, K., Schüller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Düsterhöft, A., Stiekma, W., Entian, K.-D., Terryn, N., et al. (1999). Sequence analysis of chromosome 4 of the plant *Arabidopsis thaliana*. *Nature* **402**, 769–777.
- Metzger, J.D. (1988). Localization of the site of perception of thermo-inductive temperatures in *Thlaspi arvense* L. *Plant Physiol.* **88**, 424–428.
- Michaels, S.D., and Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949–956.
- Michaels, S.D., and Amasino, R.M. (2000). Memories of winter: vernalization and the competence to flower. *Plant Cell Environ.* **23**, 1145–1153.
- Müller, C., and Leutz, A. (2001). Chromatin remodelling in development and disease. *Curr. Op. Genes Dev.* **11**, 167–174.
- Nakai, K., and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14**, 897–911.
- Napp-Zinn, K. (1987). Vernalization—environmental and genetic regulation. In *Manipulation of Flowering*, J.G. Atherton, ed. (London: Butterworths), pp. 123–132.
- Ng, J., Hart, C.M., Morgan, K., and Simon, J.A. (2000). A *Drosophila* ESC-E(Z) protein complex is distinct from other polycomb group complexes and contains covalently modified ESC. *Mol. Cell Biol.* **20**, 3069–3078.
- Nöel, L., Moores, T.L., van der Biezen, E.A., Parniske, M., Daniels, M.J., Parker, J.E., and Jones, J.D.G. (1999). Pronounced intraspecific haplotype divergence at the *RPP5* complex disease resistance locus of *Arabidopsis*. *Plant Cell* **11**, 2099–2111.
- Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J., Goldberg, R.B., and Fischer, R.L. (1999). Mutations in *FIE*, a WD polycomb group gene, allow endosperm development without fertilization. *Plant Cell* **11**, 407–415.
- Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schmidt, R., Van der Biezen, E.A., Moores, T., Dean, C., Daniels, M.J., and Jones, J.D.G. (1998). The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the Toll and Interleukin-1 receptors with *N* and *L6*. *Plant Cell* **9**, 1–17.
- Pirotta, V. (1998). Polycomb the genome: PcG, trxG, and chromatin silencing. *Cell* **93**, 333–336.
- Reeves, P.H., and Coupland, G. (2000). Response of plant development to environment: control of flowering by daylength and temperature. *Curr. Op. Plant Biol.* **3**, 37–42.
- Riggs, A., Martienssen, R., and Russo, V. (1996). Introduction. In *Epigenetic Mechanisms of Gene Regulation*, A. Riggs, R. Martienssen, and V. Russo, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 1–4.
- Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis*. *Science* **273**, 654–657.
- Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. *Science* **288**, 1613–1616.
- Schmidt, R., West, J., Cnops, G., Love, K., Balestrazzi, A., and Dean, C. (1996). Detailed description of four YAC contigs representing 17Mb of chromosome 4 of *Arabidopsis thaliana* ecotype Columbia. *Plant J.* **9**, 755–765.
- Schwabe, W.W. (1954). Factors controlling flowering in the Chrysanthemum IV. The site of vernalization and translocation of the stimulus. *J. Exp. Bot.* **5**, 389–400.
- Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.-T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a polycomb complex. *Cell* **98**, 37–46.
- Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11**, 445–458.
- Sheldon, C.C., Finnegan, E.J., Rouse, D.T., Tadege, M., Bagnall, D.J., Helliwell, C.A., Peacock, W.J., and Dennis, E.S. (2000a). The control of flowering by vernalization. *Curr. Op. Plant Biol.* **3**, 418–422.
- Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (2000b). The molecular basis of vernalization: the central role of *FLOWERING LOCUS C (FLC)*. *Proc. Natl. Acad. Sci. USA* **97**, 3753–3758.
- Simpson, G.G., Gendall, A.R., and Dean, C. (1999). When to switch to flowering. *Annu. Rev. Cell Dev. Biol.* **99**, 519–550.
- Snustad, D.P., Haas, N.A., Kopczak, S.D., and Silflow, C.D. (1992). The small genome of *Arabidopsis* contains at least nine expressed β -tubulin genes. *Plant Cell* **4**, 549–556.
- Spillane, C., MacDougall, C., Stock, C., Kohler, C., Vielle-Calzada, J., Nunes, S., Grossniklaus, U., and Goodrich, J. (2000). Interaction of the *Arabidopsis* Polycomb group proteins FIE and MEA mediates their common phenotypes. *Curr. Biol.* **10**, 1535–1538.
- Thomashow, M.F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 571–599.
- Tian, L., and Chen, Z. (2001). Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. *Proc. Natl. Acad. Sci. USA* **98**, 200–205.
- Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E., and Harte, P. (2001). The *Drosophila* Polycomb Group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. *Development* **128**, 275–286.
- Valvekens, D., Van Montagu, M., and Van Lisjebettens, M. (1988). *Agrobacterium tumefaciens*—mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* **85**, 5536–5540.
- van Blokland, R., ten Lohuis, M., and Meyer, P. (1997). Condensation of chromatin in transcriptional regions of an inactivated plant transgene: evidence for an active role of transcription in gene silencing. *Mol. Gen. Genet.* **257**, 1–13.
- Varagona, M.J., Schmidt, R.J., and Raikhel, N.V. (1992). Nuclear localization signals required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell* **4**, 1213–1227.
- von Arnim, A.G., Deng, X.-W., and Stacey, M.G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene* **221**, 35–43.
- Wellensiek, S.J. (1964). Dividing cells as the prerequisite for vernalization. *Plant Physiol.* **39**, 832–835.
- Wolffe, A., and Matzke, M. (1999). Epigenetics: regulation through repression. *Science* **286**, 481–486.

Yadegari, R., Kinoshita, T., Lotan, O., Cohen, G., Katz, A., Choi, Y., Katz, A., Nakashima, K., Harada, J.J., Goldberg, R.B., et al. (2000). Mutations in the *FIE* and *MEA* genes that encode interacting Polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. *Plant Cell* 12, 2367–2381.

Yang, C.-H., Chen, L.-J., and Sung, Z.R. (1995). Genetic regulation of shoot development in *Arabidopsis*: role of the *EMF* genes. *Dev. Biol.* 169, 421–435.

Yoshida, N. Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R. and Takahashi, S. (2001). *EMBRYONIC FLOWER2*, a novel Polycomb group protein homolog, mediates shoot development and flowering in *Arabidopsis*. *Plant Cell*, in press.

Accession Numbers

The following accession numbers have been deposited in GenBank: *VRN2*, AF284500; and *VRN2'*, AF284501.