Epigenetic Regulation in the Control of Flowering

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The timing of flowering has significant consequences for the reproductive success of plants. Plants need to gauge when both environmental and endogenous cues are optimal before undergoing the switch from vegetative to reproductive development. To achieve this, a complex regulatory network has evolved consisting of multiple pathways that quantitatively regulate a set of genes—the floral pathway integrators (Simpson and Dean 2002). The activity of these genes causes the transition of the shoot apical meristem to reproductive development and the production of flowers rather than leaves. The major environmental cues that regulate flowering are day length (photoperiod), light quality, and temperature.

Temperature plays different roles in the flowering process. First, in all plants, ambient temperature during the growing season affects growth rate and time to flower (Blázquez et al. 2003). Second, many plant species, particularly those growing in high latitudes, require a prolonged period (weeks to several months) of cold temperature (2–10ºC) before they will flower. This process, called vernalization, is an adaptation to ensure that plants overwinter before flowering, thus aligning flowering and seed production with the favorable environmental conditions of spring. A requirement for vernalization has also been extensively selected for in many crop plants, including wheat, *Brassica* species (Fig. 1A), barley (Fig. 1B), and sugar beet (Fig. 1C). Indeed, breeders have been so successful at introducing a strong vernalization requirement into crops that many accessions of wheat and oilseed rape will not flower without vernalization and so, if sown in spring, would stay vegetative through the whole summer, only flowering the following year after winter. The small garden weed *Arabidopsis thaliana* is an ideal model to study the molecular basis of this process as many *Arabidopsis thaliana* ecotypes also have a vernalization requirement and these have a very different growth form without vernalization to the classic rapid-cycling ecotypes used in the lab (Fig. 2C). The physiological properties of vernalization in *Arabidopsis* match those of all other plants: It is a quantitative response, with increasing weeks of cold progressively accelerating flowering (Figs. 1E, 2A); it is reversible if vernalized seeds are subsequently subjected to a brief period of heat stress; and it is not graft-transmissible (unlike the photoperiodic flowering signal). Importantly, the vernalization response is mitotically stable; the prolonged cold stimulus happens at one stage of development with flowering often occurring many months later. Also, cuttings from vernalized plants "remember" that they have experienced winter and flower at the appropriately early time. However, the process is reset at some point during meiosis or seed development so that seedlings need to be vernalized each generation to align flowering with spring.

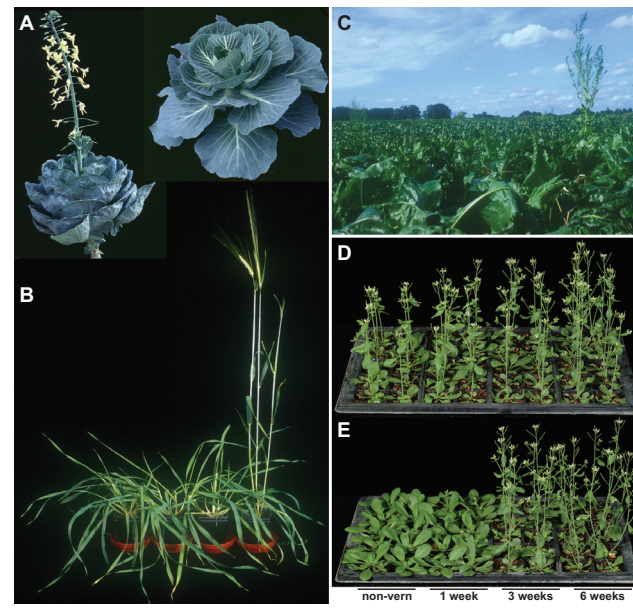

Figure 1. Vernalization requirement and response in crops and *Arabidopsis thaliana*. (*A*) Vernalization requirement is a trait bred in *Brassicas*. Pictured are cabbage (*Brassica oleracea ssp. capitata*) plants that were either vernalized as young plants (*left*) or not (*right*). (*B*) Barley varieties with a vernalization requirement (winter varieties, *left*) and a spring variety that has no vernalization requirement (*right*), all grown without vernalization. (*C*) Sugar beet plants require vernalization to flower, so are sown in late spring in order that they remain vegetative. An increasing problem in sugar beet fields is weed beet (the flowering plant in the picture), plants that have broken the vernalization requirement and flowered. (*D*) Both of the *Arabidopsis thaliana* laboratory strains Columbia or Landsberg *erecta* (pictured) do not have a strong vernalization requirement. Nonvernalized Landsberg *erecta* (*left*, nonvern) flower early with a similar number of leaves (vegetative phase) as vernalized L*er* (*right*, 6 weeks vernalization). (*E*) An active copy of the gene *FRIGIDA* (*FRI*) confers a strong vernalization requirement. *Arabidopsis thaliana* laboratory strains Columbia or Landsberg *erecta* (L*er*) have mutations in their *FRIGIDA* genes. Nonvernalized L*er* plants containing active *FRIGIDA* (*left*) are late flowering but this late flowering may be suppressed if vernalized (*right*). (*D*,*E:* Reprinted, with permission, from Henderson et al. 2003 [© Annual Reviews: www.annualreviews.org].)

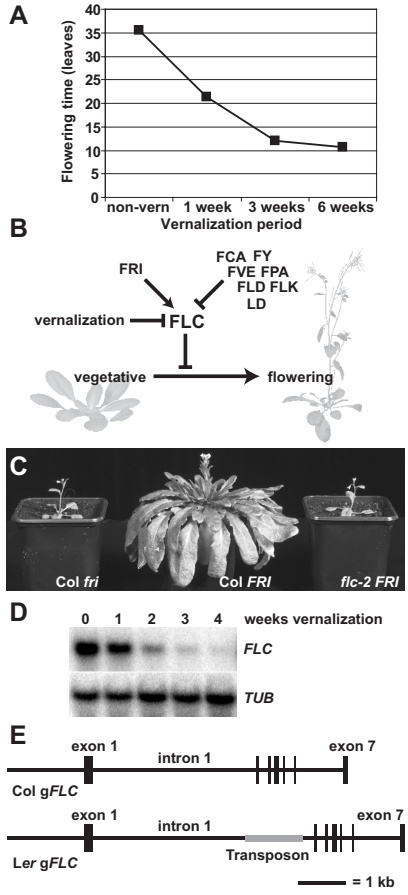

Figure 2. The role of FLC in vernalization requirement and response. (*A*) A response curve for *FRI*-containing Landsberg *erecta* (L*er*) showing the effect of increasing vernalization on flowering time. In all cases, SE was less than one leaf. (*B*) The pathways controlling expression of the MADS-box floral repressor FLC. The vernalization requirement conferred by mutations in *FCA*, *FY*, *FVE*, *FPA*, *FLC*, *LD*, *FLK*, or active *FRI* genes is by elevation of *FLC* levels that are suppressed by vernalization. (*C*) Wild-type *Arabidopsis thaliana* ecotype Columbia with inactive *fri* (*left*), Columbia containing an active copy of *FRI* (*middle*), Columbia containing active *FRIGIDA* but with a fast-neutron deletion of *FLC* (*flc-2* mutation) (*right*). (*D*) Northern blot showing *FLC* levels in *fca-1* declines with increasing vernalization periods. *fca-1* seeds were nonvernalized or vernalized for 1–4 weeks and then harvested after 14 days growth. The blot was reprobed with β-TUBULIN as a loading control (TUB). (*E*) The structure of *FLC* differs in Columbia and L*er*; a transposon in intron 1 of L*er* FLC reduces the steady-state *FLC* RNA levels in L*er* vs. Col backgrounds but does not affect the response to vernalization.

FLC IS A CENTRAL PLAYER IN THE VERNALIZATION PROCESS

Analysis of the genetic basis of vernalization requirement in natural accessions revealed that *FLC*, which encodes a MADS box transcriptional regulator, plays a central role in vernalization (Michaels and Amasino 1999; Sheldon et al. 1999). Increasing levels of FLC progressively delay flowering in a "rheostat"-like mechanism. FLC is thought to antagonize the activation of the floral pathway integrator genes (Simpson and Dean 2002; Boss et al. 2004), and evidence that this occurs directly has been found for SOC1/AGL20 (Hepworth et al. 2002). Pathways that confer a vernalization requirement increase levels of *FLC* expression (Fig. 2B). This is the case for *FRIGIDA* (*FRI*), a major determinant of flowering-time variation and vernalization requirement in natural *Arabidopsis thaliana* ecotypes (Fig. 2C) (Johanson et al. 2000) and for a series of mutations whose late-flowering phenotype can be corrected by vernalization (e.g., *fca*, *fy*) (Koornneef et al. 1991). Vernalization antagonizes the activities of these pathways by decreasing levels of *FLC* expression (Fig. 2D). An in vivo analysis of various *FLC* transgenes carrying deletions has identified *cis*-elements in the *FLC* promoter and first intron required to mediate regulation by these different pathways (Sheldon et al. 2002). Different *Arabidopsis* ecotypes used in the molecular analysis show molecular variation at or near these *cis*-elements that influences *FLC* regulation. For example, compared to the Columbia allele (the accession for which the entire genomic sequence is available), C24 (used in the analyses by Sheldon et al. 2002) is missing one-half of a 30-bp tandem repeat in intron 1 (Gazzani et al. 2003) and Landsberg *erecta* (the background in which most of the original late-flowering mutants were identified) has a Mutator-like transposable element at the 3′ end of intron 1 (Fig. 2E) (Gazzani et al. 2003; Michaels et al. 2003). The presence of the Mutator-element restrains the upregulation of *FLC* levels in response to FRIGIDA or mutations such as *fca*, *fy* but does not affect the decrease in flowering time in response to vernalization.

vrn MUTANTS IDENTIFY GENES REQUIRED TO MAINTAIN *FLC* REPRESSION

In order to define the molecular events occurring during the downregulation of *FLC* levels during the cold and the subsequent maintenance of *FLC* repression, a series of mutants were defined that have a reduced response to vernalization based on a late flowering after vernalization phenotype (*vrn1 to vrn7* for reduced *ver*nalization) (Chandler et al. 1996). Another group isolated the *vin3* mutant (*v*ernalization *in*sensitive) (Sung and Amasino 2004), which is allelic to *vrn7*. Phenotypically *vrn* mutants look quite normal apart from delayed flowering (Fig. 3B), and they are all reduced in response to vernalization (Fig. 3C). Analysis of *FLC* levels in *vrn* mutants showed that the decrease in *FLC* after prolonged cold is almost as in wild type. However, unlike wild type, *FLC* levels increase again during subsequent growth in all the *vrn* mutants (Fig. 3D). This result demonstrates that the major function of the *VRN* genes is in the maintenance of the repressed *FLC* state.

VRN GENES IMPLICATE CHROMATIN REMODELING IN *FLC* REGULATION

The first *VRN* gene to be cloned, *VRN2*, encodes a protein with homology to Su(z)12, a zinc finger protein that is a component of the E(z)/ESC Polycomb complex in *Drosophila* and humans (Fig. 4A) (Gendall et al.

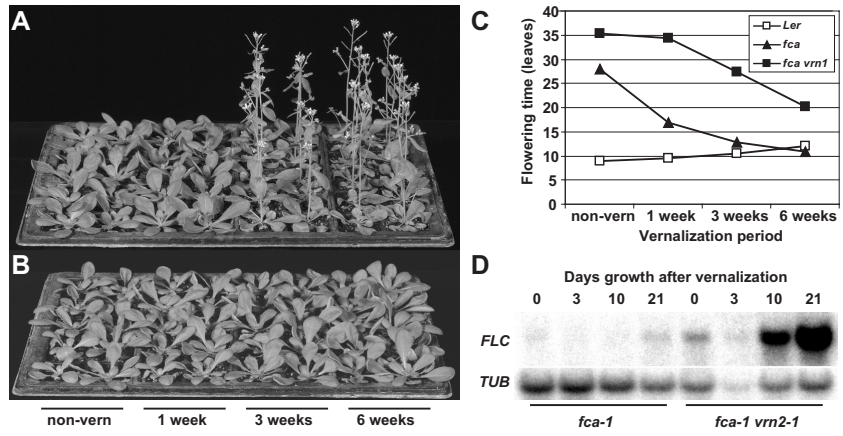

Figure 3. *VRN* genes are required to maintain suppression of *FLC* after vernalization. (*A*) Mutations of the *FCA* gene in L*er* (*fca-1* pictured) cause a strong vernalization requirement. Nonvernalized *fca-1* seeds (*left*, non-vern) produce late-flowering plants with a long vegetative phase. However as *fca-1* seeds are vernalized for progressively longer times (to *right*) their vegetative phase becomes much shorter and if vernalized long enough they produce similar numbers of leaves to wild-type L*er* (*right*, 6 weeks vernalization, see L*er* in Fig. 1*D*). (*B*) Vernalization response of *vrn1 fca-1* double mutants nonvernalized or vernalized as seeds for 1, 3, or 6 weeks. *vrn* mutants typically have a reduced response to vernalization and no pleiotropic phenotypes. (*C*) Flowering time is easily assayed as leaf number. L*er*, *fca-1*, and *vrn1 fca-1* mutants were nonvernalized or vernalized for 1, 3, or 6 weeks and their flowering time measured by counting the total number of leaves. In all cases, SE was less than two leaves. (*D*) The reduced vernalization response of *vrn* mutants is due to a failure to maintain suppression of *FLC* after vernalization during subsequent growth in long days. Seeds from *fca-1* and *vrn2 fca-1* were vernalized for 8 weeks and harvested immediately after the cold (0 days) and at different stages of subsequent growth. *FLC* levels in *fca-1* remain stably repressed after vernalization, but in *vrn2 fca-1 FLC* expression is initially repressed after vernalization but returns during subsequent growth. (*A*,*B*: Reprinted, with permission, from Henderson et al. 2003 [©Annual Reviews: www.annualreviews.org].)

2001; Czermin et al. 2002; Müller et al. 2002). The Polycomb complex has been shown in *Drosophila* to repress gene expression, including the HOX genes, by maintaining silent chromatin states. This appears to be analogous to the role VRN2 has in the stable repression of *FLC*.

VRN1 encodes a protein with two plant-specific DNA-binding domains that bind a range of DNA sequences in vitro (Levy et al. 2002). *VIN3* encodes a protein with a PHD (plant homeodomain) and a fibronectin III domain (Sung and Amasino 2004). PHD proteins have been found in chromatin complexes associated with histone deacety-

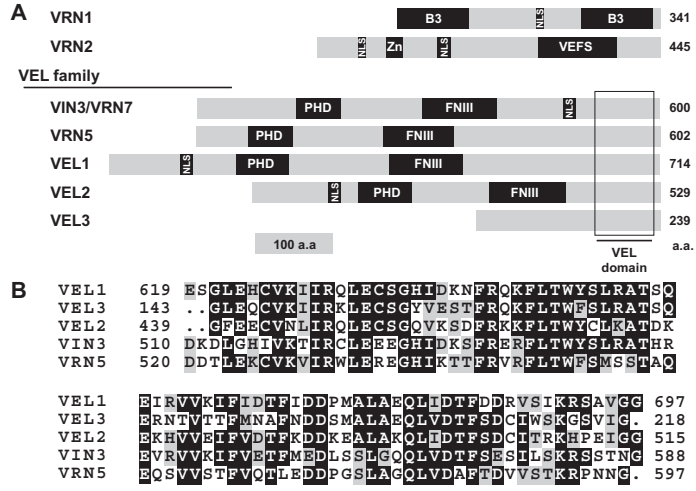

Figure 4. VRN proteins and the VEL (*VERNALIZATION5/VIN3*-Like) family. (*A*) Overview of genes involved in vernalization. Mutants in *VRN1*, *VRN2*, *VRN5*, and *VIN3/VRN7* were isolated in screens for mutants with reduced vernalization responses. VRN1 protein has strong in vitro DNA-binding properties and contains a putative nuclear localization sequence (NLS) and two plant-specific DNA-binding B3 domains. VRN2 shares domains with Su(z)12 and other PcG transcriptional regulators including a zinc finger (Zn), two NLSs, and a region highly conserved in VRN2, EMF2, FIS2, and SU(Z)12 (VEFS). *VRN5* and *VIN3/VRN7* are members of the *VEL* (*VERNALIZATION5/VIN3-Like*) gene family consisting of five members in *Arabidopsis*, which also includes *VEL1*, *VEL2*, and *VEL3*. With the exception of *VEL3*, all *VEL* genes carry a PHD (plant homeodomain) and a fibronectin III domain (FNIII). *VEL3* is much shorter than the other members but like all VEL proteins carries the highly conserved carboxyl terminus unique to the VEL family. (*B*) Pileup of the VEL domain unique to members of the VEL family. The gene numbers are At4G30200 for *VEL1*, At2G18870 for *VEL3*, At2G18880 for *VEL2*, At5g57380 for *VIN3*, and At3g24440 for *VRN5*.

lation and recently some PHD fingers have been found to function as nuclear phosphoinositide receptors, opening up the possibility that phosphoinositide signaling may play a role during the prolonged cold phase of vernalization (Gozani et al. 2003). During our screens for mutants with reduced vernalization responses we isolated a mutant *vrn5*, which has lesions in a *VIN3* homolog (T. Greb et al., unpubl.). *VRN5* and *VIN3* define a small gene family consisting of five members in *Arabidopsis* that we have called the *VEL* (VE*RNALIZATION5/VIN3*-L*ike*) gene family (Fig. 4A). This opens up the question whether other *VEL* family members are involved in vernalization. With the exception of *VEL3* all members carry a PHD finger as mentioned above and a fibronectin III domain that has been characterized mainly in animals as a protein–protein interaction domain (Potts and Campbell 1996). At their carboxyl terminus all *VEL* genes carry a highly conserved domain that shows no similarities to any described protein motif from plants or animals (Fig. 4B). Therefore we term the *VEL*-specific carboxy-terminal domain the "VEL domain." According to the available annotation, *VEL3* is much smaller and only contains the VEL domain. Furthermore, no EST or cDNA clone is reported for *VEL3*. It is yet to be determined whether *VEL3* is a functional gene. Lesions in *VRN5* and *VIN3* have shown that at least two members of the *VEL* family are involved in the repression of *FLC*. Further experiments will clarify the role of other *VEL* genes in the vernalization response. A functional molecular analysis of the newly defined *VEL* domain will be especially interesting.

USE OF AN *FLC:luciferase* TRANSLATIONAL FUSION TO SELECT SPECIFICALLY FOR MUTANTS IN *FLC* REGULATION

In order to saturate for mutations disrupting the maintenance of *FLC* repression and to target genes required early in the cold-induced repression, we have established a mutagenesis strategy based on *FLC* expression rather than flowering time. The firefly luciferase coding sequence was cloned into exon 6 of an *FLC* clone from Columbia DNA resulting in a translational fusion (Fig. 5A). The *FLC:luciferase* fusion was transformed into *Arabidopsis thaliana* Landsberg *erecta*, which had previously been transformed with a functional *FRIGIDA* allele. Transformants were screened by genetic segregation and Southern blots for lines containing one simple T-DNA insert (to avoid complications induced by silencing phenomena associated with complex T-DNA integration). Of those, a line expressing a high level of *FLC:luciferase* in the presence of *FRI* was selected and the repression of *FLC:luciferase* expression after vernalization was confirmed by luciferase assays (Fig. 6C–E) and northern analysis (Fig. 6F).

In order to determine the functionality of the *FLC:luciferase* translational fusion it was also transformed into *flc-3 FRI*, a Columbia null *FLC* mutant with active *FRIGIDA* introgressed from a late-flowering *Arabidopsis* ecotype (Michaels and Amasino 1999). The flowering time of these transformants was compared to *flc-3 FRI* and wild-type Columbia containing active *FRI* (Fig.

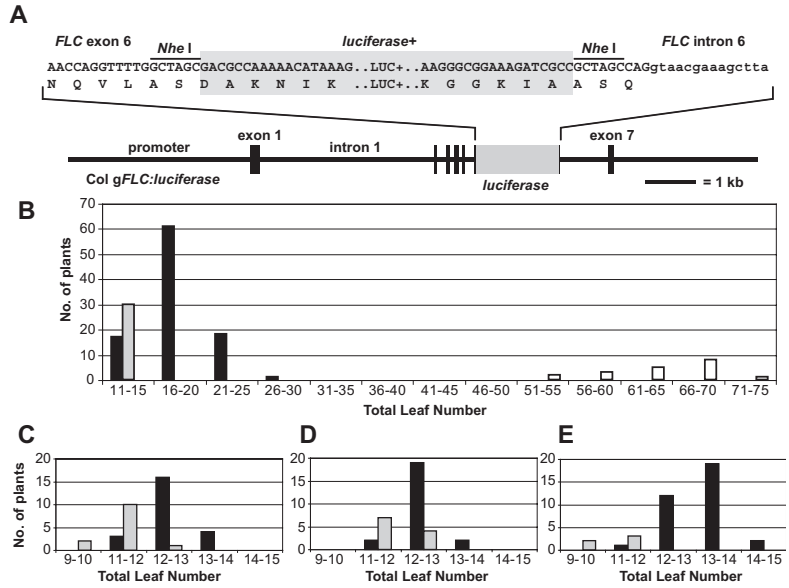

Figure 5. Structure of the genomic *FLC:luciferase* protein fusion construct and its functionality in planta. (*A*) The firefly luciferase (codon modified Luc+, Promega) coding region was inserted in-frame at the *Nhe*I site at the end of Columbia *FLC* exon 6 of a 12.1-kb *Sac*I *FLC* clone from Columbia DNA and cloned into an *Agrobacterium* binary vector pSLJ755I6 (Jones et al. 1992) and transformed into *Arabidopsis thaliana* Landsberg *erecta,* which had previously been transformed with a functional *FRIGIDA* allele. (*B*) The FLC:luciferase fusion has very little effect on flowering time. The flowering time of 100 transgenic *flc-3 FRI* lines containing the FLC:luciferase fusion (*black bars*) were slightly later than *flc-3 FRI* (*gray bars*), but far from the flowering time of *FLC FRI* (*open bars*). (*C–E*) Subsequent analysis of the next generation of three lines that flowered with 18, 23, and 25 leaves in the T1 (*C–E*, respectively) showed that *flc-3 FRI* containing the *FLC:luciferase* transgene (*black bars*) flowered at the same time or, at most, four leaves later than nontransgenic *flc-3 FRI* segregants (*gray bars*), indicating the FLC:luciferase fusion has very weak or negligible effect on flowering time.

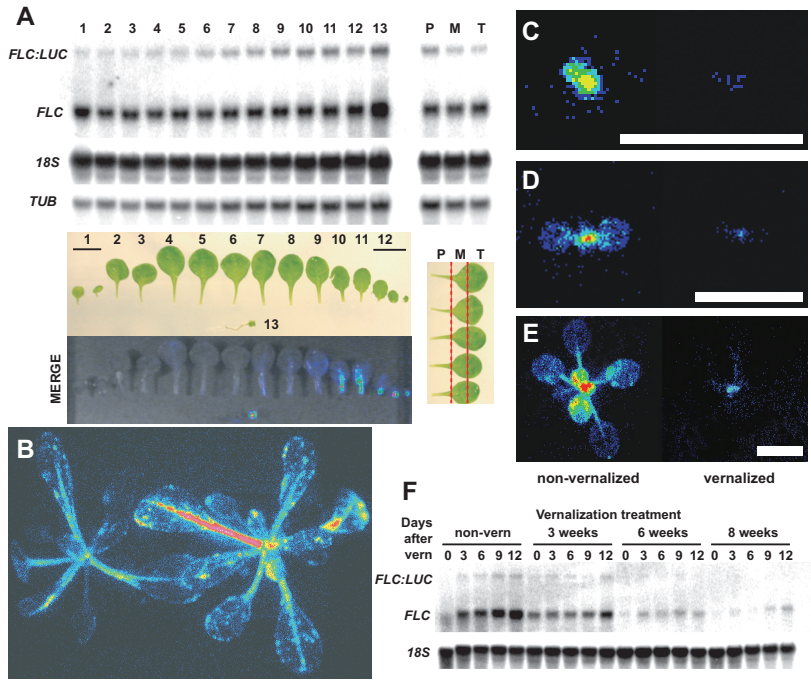

Figure 6. Analysis of *FLC:luciferase* mRNA and FLC:luciferase expression pattern in the L*er FRIGIDA FLC:luciferase* line. (*A*) Northern blot analysis of *FLC* and *FLC:luciferase* mRNA in leaves of different ages. Leaves from ten nonvernalized plants were grouped based on age (*1*, cotyledons; *2–12*, oldest to youngest leaves; and *13*, remaining plant center, root, and apical meristem) as well as into petioles, middle, and tips (P, M, T) and the RNA from these was probed with *FLC,* before stripping and reprobing with β-*TUBULIN* (*TUB*) and then *18S rDNA* probes. (*B*) L*er FRIGIDA FLC:luciferase* (*left*) and the same line containing a *vrn2* mutation (*right*) after a nonsaturating 4-week vernalization and 4 weeks of growth. (*C*) During vernalization, seeds germinate so the L*er FRIGIDA FLC:luciferase* seedling on the *right* was vernalized for 6 weeks and luciferase levels were imaged simultaneously with a nonvernalized seedling at a similar stage (3 days growth). Scale bar (for *C–E*), 1 cm. (*D*) Nonvernalized seedling (10 days, *left*) compared to the same line 7 days after vernalization (*right*). (*E*) Nonvernalized seedling (19 days, *left*) compared to the same line 16 days after vernalization (*right*). (*F*) Northern analysis to test the response of *FLC* and *FLC:luciferase* in the screen conditions. Seedlings were grown for 6 days before different vernalization treatments (non-vern, 3, 6, 8 weeks) and then harvested at different days of subsequent growth in long days.

5B–E). The flowering time of 100 different T1 transgenics suggested that the *FLC:luciferase* fusion is only weakly functional in delaying flowering time (Fig. 5B). Reexamination of the transgenic and nontransgenic segregants in the T2 generation of three individuals (Fig. 5C–E) revealed that, at most, the *FLC:luciferase* fusion may delay flowering by one or two leaves—thus the *FLC:luciferase* fusion mimics the endogenous *FLC* expression without significantly delaying flowering.

The FLC:luciferase signal was strongest in the apical meristem, stronger in younger leaves than older leaves, and stronger in petioles (leaf stems) than leaf blades (Fig. 6A,B,E). To determine if this was an artifact of the reporter or a consequence of differences in spatiotemporal expression of *FLC*, leaves of ten nonvernalized *FLC:luciferase FRI* plants were separated into age and tissue classes and RNA was isolated. Northern blots showed that both endogenous *FLC* and *FLC:luciferase* mRNA expression do not change dramatically with age or across the leaf blade (Fig. 6A). The most likely explanation for the difference in mRNA levels and strength of luciferase reporter signal is cell-size variation in the different tissues. For instance, in older leaves cells are larger and so if the amount of protein per cell is the same in young and old leaves, the reporter signal will be weaker in old leaves because the FLC:luciferase protein is spread over a wider area.

To obtain mutants defective in *FLC* suppression, L*er FLC:luciferase FRI* seeds were mutagenized with 0.3% ethyl methyl sulfonate (EMS) for 9 hours and M2 seeds were harvested from 25–35 M1 plants and pooled. M2 seeds were sterilized and sown on media lacking glucose. Plates were chilled for 2 days at 4ºC to synchronize germination and then grown for 6 days at 20ºC (16-hr photoperiod). After 6 days of growth, the seeds had germinated, the cotyledons had fully expanded, and the first true leaf pair was forming. The plates were transferred to a cabinet for 6 weeks of vernalization (4ºC, 8-hr photoperiod) and were imaged for luciferase activity immediately after the cold to isolate mutants defective in the downregulation of FLC:luciferase expression during vernalization. The remaining plants were grown for a further 2 weeks at 20ºC (16-hr photoperiod) to isolate mutants defective in the maintenance of low FLC:luciferase levels after vernalization.

The first mutants that were investigated were those which, in addition to their inability to suppress FLC:luciferase subsequent to vernalization, were late flowering, indicating that the suppression of endogenous *FLC* was also defective. Using this screen we isolated three new

noncomplementing mutations we named *vrn7*. Subsequent mapping of this gene reduced it to a 420-kb region containing 56 genes, including the recently published *VIN3* gene (Sung and Amasino 2004). Sequencing confirmed that *VRN7* was *VIN3* (J. Mylne and C. Dean, unpubl.). In addition to the *vrn7/vin3* alleles, we also isolated six alleles of *vrn5*.

HISTONE MODIFICATIONS ASSOCIATED WITH EPIGENETIC SILENCING OF *FLC*

The maintenance of *FLC* repression following vernalization suggested that this gene may be epigenetically silenced. Epigenetic silencing of genes is mediated by numerous covalent modifications of both DNA and histones (Bird 2002; Fischle et al. 2003). Previous work on the epigenetic regulation of vernalization focused on the role of DNA cytosine methylation in control of vernalization (Sheldon et al. 1999), but to date there is no evidence of a direct link between DNA methylation and vernalization. However, recent data have demonstrated a more important role for histone modifications at the *FLC* locus during vernalization (Bastow et al. 2004; Sung and Amasino 2004). Specific residues of histone H3 tails can be modified by acetylation and methylation and changes in these modifications serve as part of a "histone code" specifying active or repressed gene activity states (Fischle et al. 2003). Vernalization was found to increase histone H3 deacetylation in the 5′-region of *FLC*, a modification typically associated with gene repression (Fischle et al. 2003; Sung and Amasino 2004). Vernalization also induced increases in methylation of histone H3 lysine residues 9 and 27, modifications associated with repressed gene states (Bastow et al. 2004; Sung and Amasino 2004). These marks are bound by further mediators of gene silencing, which include Heterochromatin protein 1 in animals (Orlando 2003); however, what these components are in plants is as yet unclear. Interestingly, the histone modifications that are observed at *FLC* are localized to specific regions of the gene (Bastow et al. 2004), colocalizing with sequences shown to be involved in the regulation of *FLC* by vernalization (Sheldon et al. 2002).

CHANGES IN THE HISTONE MODIFICATION IN *vrn/vin* MUTANTS SUGGEST A SEQUENCE OF EVENTS

The vernalization-mediated repression of *FLC* and coincident decrease in histone acetylation and increase in H3 K9 and K27 methylation observed in wild-type *Arabidopsis* are not found in *vin3* mutants (Sung and Amasino 2004). Similarly, in *vrn2* mutants increases in methylation of histone H3 at lysine residues 27 and 9 do not occur at the *FLC* locus during vernalization (Fig. 7) (Bastow et al. 2004; Sung and Amasino 2004). Unlike

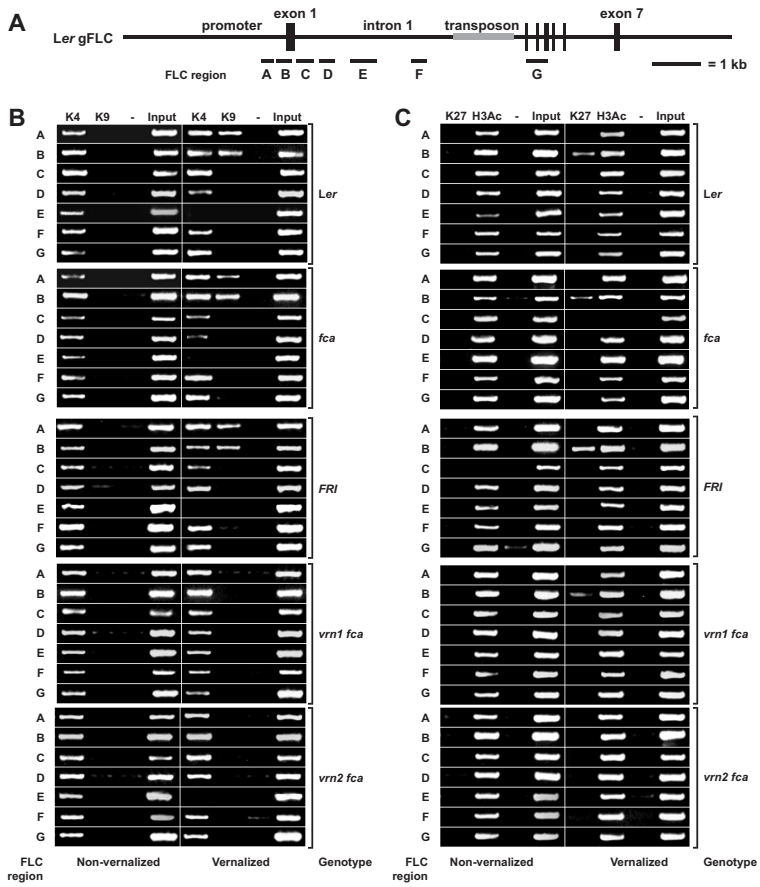

Figure 7. Histone modifications at *FLC* chromatin: PCR analysis of chromatin immunoprecipitates obtained with antibodies specific for H3 dimethyl K4, H3 dimethyl K9, H3 dimethyl K27, and acetylated H3 (Upstate). (*A*) Genomic structure of L*er FLC* and the regions tested (*A–G*) in ChIP assays. (*B*) H3 dimethyl K4 and H3 dimethyl K9 histone modifications at *FLC* are associated with vernalization and are dependent on the *VRN* genes. In ChIP analyses of *FLC* in L*er*, *fca-1,* L*er FRI*, *vrn1 fca-1*, and *vrn2 fca-1,* the vernalization-induced changes in H3 dimethyl K9 and H3 dimethyl K4, were lost in *vrn1 fca-1;* regions A and B did not show increased H3 dimethyl K9; and region E was precipitated by anti-H3 dimethyl K4. In vernalized *vrn2 fca-1,* no increase of H3 dimethyl K9 in regions A and B was found; however, the vernalization-induced loss of H3 dimethyl K4 from region E was maintained or restored only weakly. (*C*) The H3 dimethyl K27 histone modification at *FLC* is associated with vernalization and is dependent on VRN2, but not VRN1. The vernalization-induced H3 dimethyl K27 in region B was retained in *vrn1 fca-1*. In vernalized *vrn2 fca-1,* no increase of H3 dimethyl K27 in region B was found. Precipitation using the antibody to acetylated H3 is also shown. The reason for the low representation of region C only in nonvernalized L*er FRI* is not known. (*B*,*C*: Adapted from supplementary Figures 1 and 2 in Bastow et al. 2004.)

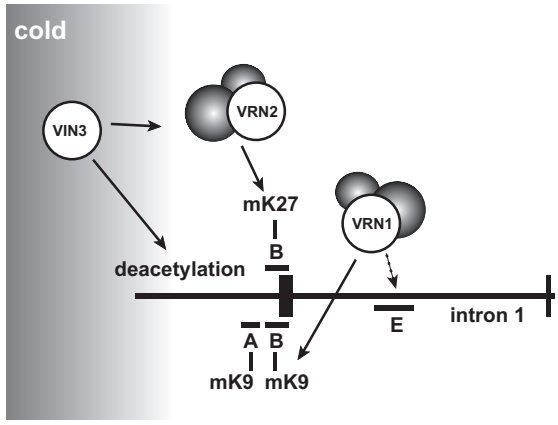

Figure 8. Potential sequence of events at the *FLC* locus during vernalization. Prolonged cold induces a series of events including *VIN3* expression and these result in *FLC* histone deacetylation. This is followed by VRN2 activity causing H3 dimethylation of lysine 27 (and possibly lysine 9) near the 5′ end of *FLC*, followed by, or in parallel with, VRN1 activity causing H3 dimethylation of lysine 9.

VRN2, VRN1 is required only for increases in histone H3 lysine 9 methylation in response to vernalization and not methylation of lysine 27 (Fig. 7) (Bastow et al. 2004; Sung and Amasino 2004). This suggests that it may function downstream or independently of VRN2 during *FLC* repression. Intriguingly, *VIN3* expression increases with cold and only significantly accumulates after a period of cold effective for vernalization (Sung and Amasino 2004). Therefore the sequence of events may entail cold activation of VIN3/VRN7 function resulting in histone deacetylation at *FLC*, followed by VRN2 activity causing H3 dimethylation of lysine 27 (and possibly lysine 9) in specific domains, followed by, or in parallel with, VRN1 activity causing H3 dimethylation of lysine 9 (Fig. 8). The double mutant phenotype of *vrn1* and *vrn2* is much stronger than either single mutant phenotype supporting parallel activities or multiple targets (T. Gendall and C. Dean, unpubl.).

Understanding how prolonged cold induces expression of *VIN3/VRN7* is a key question. The presence of the PHD finger, shown recently to act as a nuclear phosphoinositide receptor, also suggests a role for cold-induced signaling in the induction of VIN3/VRN7 activity. This is consistent with overexpression of *VIN3* being able to rescue the *vin3* mutation but not being able to lead to vernalization-independent early flowering (Sung and Amasino 2004). Constitutive expression of *VRN2* did not lead to pleiotropy or vernalization-independent flowering (T. Gendall and C. Dean, unpubl.); however, overexpression of VRN1 protein revealed a vernalization-independent function for VRN1 (Levy et al. 2002). When constitutively expressed, VRN1 caused *FLC*-independent early flowering and pleiotropic phenotypes, predominantly through a dramatic elevation of levels of the floral pathway integrator *FT*, and demonstrated that VRN1 requires vernalization-specific factors to target *FLC* (Levy et al. 2002).

INTEGRATION OF DIFFERENT FLOWERING PATHWAYS

The vernalization pathway is only one of many that regulate *FLC* levels. In turn, *FLC* is only one of many regulators of the floral pathway integrators, key regulators in the decision to flower. Therefore, an important aspect for future research will be establishing how the predominance of these different pathways is established and what changes these interactions during development or on the sensing of changed environmental cues. We are investigating this by analyzing how FCA/FY and FRIGIDA interact with the vernalization pathway (Fig. 2B). FCA and FY are two interacting proteins that promote flowering and encode an RNA-binding protein and a polyadenylation factor, respectively (Macknight et al. 1997; Simpson et al. 2003). The role of these posttranscriptional regulators in *FLC* expression raises some interesting questions. They may function to repress *FLC* directly or indirectly. However, they may also be components of a posttranscriptional mechanism that feeds back to regulate *FLC* transcriptionally, perhaps via chromatin regulation. There are now many examples where noncoding RNAs act to direct chromatin modifications (Verdel et al. 2004). Whatever their activities, both FCA/FY and vernalization function antagonistically with FRIGIDA, a novel protein that strongly upregulates *FLC* RNA levels (Johanson et al. 2000). Experiments are under way to address whether FRIGIDA also regulates *FLC* chromatin structure. The ability to screen for mutants in order to identify novel genes involved in flowering, together with the large resource of natural variants of *Arabidopsis thaliana* collected from a wide range of environments, will enable a detailed dissection of the molecular interactions of all these pathways.

ACKNOWLEDGMENTS

The authors would like to thank Tony Gendall for Northern blots images used in Figures 2D and 3D. Thanks to Judith Irwin for the image in Figure 1A, David Laurie for the image in Figure 1B, and Andrew Davis for the image in Figure 1C. Thanks also to Catherine Baxter for helpful comments on the manuscript. C.D. and C.L. are funded by a Biotechnology and Biological Sciences Research Council (BBSRC) core strategic grant to the John Innes Centre; J.M. is funded by European Commission grant QLK5-CT-2001-01412 and T.G. is funded by BBSRC grant 208/G15996.

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