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THE NEED FOR WINTER IN THE SWITCH TO FLOWERING

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■ **Abstract** Vernalization is the process whereby the floral transition is promoted through exposure of plants to long periods of cold temperature or winter. A requirement for vernalization aligns flowering with the seasons to ensure that their reproductive phase occurs in favorable conditions. The mitotic stability of vernalization, suggestive of an epigenetic mechanism, has intrigued researchers for many years. Genetic analysis of the vernalization requirement in *Arabidopsis* has identified key floral repressor genes, *FRI* and *FLC*. The action of these floral repressors is antagonized by vernalization and the activity of a set of genes grouped into the autonomous floral pathway. Analysis of the vernalization pathway has defined a series of epigenetic regulators crucial for "cellular-memory" of the cold signal, whereas the autonomous pathway appears to function in part through posttranscriptional mechanisms. The mechanism of the vernalization requirement, which is now being explored in a range of plant species, should uncover the evolutionary origins of this key agronomic trait.

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INTRODUCTION

Seasonal Control of Flowering

Higher plant shoots develop through the postembryonic activity of stem cell populations, termed meristems (87). Shoot meristems function both in self-renewal and to generate lateral organs (87). After a certain period of vegetative development a switch to reproductive fate or flowering occurs, changing the nature of the meristem and the lateral organs it produces. Plant development is highly plastic in response to environmental cues and this characteristic is likely to reflect an adaptation to their sessile growth habit. Numerous external signals including photoperiod, light quality, and ambient temperature influence the timing of the switch to flowering (58, 84) (Figure 1). These cues vary geographically, within local microenvironments, and also seasonally. Temperate climates expose plants to pronounced seasonal change in their growing environment and adaptation of reproductive development to this annual cycle is evident in many species (3). In northern latitudes, long-day photoperiods and the long period of cold temperature experienced over winter are used as cues to predict the arrival of spring and summer conditions, favorable to seed set.

Considerable progress has been made on the molecular dissection of the floral pathways controlling photoperiodic control of the floral transition [for recent reviews, see (58, 84)]. Therefore, in this review we focus on the acceleration of flowering by a long period of cold, a process termed vernalization. We first review our understanding derived from physiological studies conducted in diverse angiosperm species, and then second, from the molecular genetic approaches taken in the model species *Arabidopsis thaliana*. Finally, we apply the genetic models derived for the vernalization requirement and response in *Arabidopsis* to crop species where the vernalization requirement is an important agronomic trait.

PHYSIOLOGY OF VERNALIZATION

The vernalization response has been documented in numerous flowering plant species and studied at the physiological level (55). Common to all vernalization responses is a means to perceive exposure to long periods of low temperature

and couple this to promotion of the floral transition. Comparative analysis of vernalization provides us with a number of general qualities, which may suggest the nature of the response's mechanism.

Cold Temperature Sensing

The thermosensory capacity of plants is demonstrated by several distinct responses: the acclimation to freezing temperatures, the effects of low temperature on bud dormancy and seed germination, and the effects of ambient temperature on flowering time (8, 93, 97). Relative to other temperature responses, vernalization displays unique characteristics. First, the response requires exposure to unusually long periods, usually weeks, of low temperature to be effective (35). Examples also exist of developmental competency to respond to vernalization; the ability to respond to cold temperature is only achieved around day 10 of growth in *Hyocyamus niger* (36).

Grafting experiments have played an important role in flowering time research and defined the transmissible "florigen" signal involved in photoperiodic induction (104). Grafting has played a similar role in revealing the site of cold perception during vernalization as the shoot apex (34). Although these studies indicated exposure of the apex to cold was required for vernalization, studies using regenerated somatic tissue suggest that cells outside the meristem are also able to respond to the signal (98). Furthermore, it appeared that mitotic activity of somatic tissue was an important factor for this response to occur (99). The connection between cell division and vernalization remains unclear.

Vernalization as a Quantitative Response

A striking characteristic of the vernalization response is its pronounced quantitative nature. Increasing exposure to low temperature leads to progressively accelerated flowering time (35). The response is generally saturable, reaching a point at which further exposure to cold does not lead to additional acceleration of flowering (35). A requirement for exposure to long periods of cold may allow plants to distinguish between the passage of winter and exposure to an unusually cold night. The gradual nature of the vernalization response may reflect the progressive accumulation of a floral promoter or removal of a repressor or both. It is also possible to devernalize a number of species, that is, the promotive effect of a vernalization treatment can be reversed by subsequent exposure of the plant to high temperature (5). An interplay also exists between vernalization and photoperiodic requirement. In wheat, exposure of plants to short-day photoperiods during winter, especially in combination with partial vernalization, can accelerate flowering (33).

Epigenetic Memory in Vernalization

During vernalization there is clear temporal separation between exposure of plants to the cold and their response in flowering time. After plants return to elevated temperatures it may be many weeks before flowering occurs (5). Hence, perception

of low temperatures must be "remembered" by the apex through numerous mitotic divisions. Cold treatment is believed to induce a developmental state that is clonally inherited through mitosis; ultimately, this state promotes the transition to flowering. Although remarkably stable through vegetative development and vegetative propagation, the vernalized state is reset at meiosis (5). Within the context of the multiple cues a plant integrates during the decision to flower, the vernalized state serves to provide competency to flower (13). For example, in wheat and rapeseed, vernalization is often an obligate requirement but is insufficient to induce flowering alone. Exposure to a subsequent long-day photoperiod is required to induce the floral switch (5).

The physiological characteristics of vernalization suggest that this response involves a form of epigenetic, cellular memory that is stable through multiple cell divisions. This state is induced through a thermosensory pathway with a strong quantitative element. Our understanding of the nature of this response at the molecular level has awaited a genetic approach in the model species *Arabidopsis thaliana* (58, 84). Although the initial mechanism of cold sensing remains unknown, genetic analysis has recently begun to provide insight into the basis of the vernalization requirement, the quantitative nature of the vernalization response, and the epigenetic aspects of its mitotic stability.

ARABIDOPSIS VERNALIZATION REQUIREMENT AND RESPONSE

Flowering-time Genetics in *Arabidopsis*

Two complementary approaches have been important in the analysis of flowering in *Arabidopsis*: the isolation of mutants compromised in either promotion or repression of flowering (32, 103) and the analysis of natural variation in flowering time that exists within wild accessions (1). A combination of these methods has allowed the development of our current model for flowering control; it consists of multiple input pathways that integrate to quantitatively regulate several key genes that control the floral switch (see Figure 2) (58, 84). This model reflects the multifactorial models for flowering-time control derived from physiological studies (4).

FLORAL REPRESSORS AND VERNALIZATION REQUIREMENT

Vernalization Requirement and *FRIGIDA*

Natural accessions of *Arabidopsis* display great variation in both flowering time and their response to vernalization. The majority of natural populations are winter annual strains that flower late without a vernalization treatment (63). Early work on the genetic basis of differences between winter and rapid cycling strains was

pioneered by Klaus Napp-Zinn in the 1950s (60). Crosses between the rapid cycling Limburg-5 and the winter annual Stockholm background established that winter annualism could be mapped as a monogenic trait to the *FRIGIDA* (*FRI*) locus (60). The importance of *FRI* became apparent as additional winter annual accessions were analyzed and other loci conferring the winter annual habit (*FLA, FLORENS*) were found to be alleles of *FRI* (11, 14, 31, 43, 52, 75). *FRI* is a single-copy gene encoding a novel protein with two potential coiled-coil domains (26). The importance of these putative protein-protein interaction domains to FRI function remains to be determined. The cellular function of FRI is unclear, although it is known to have a nuclear localization and is strongly expressed in meristematic regions (K. Torney, C. Lister & C. Dean, unpublished observations).

The allelic variation at *FRI* is an important determinant of flowering time in wild *Arabidopsis* populations (11, 43). Many early flowering, non-vernalization responsive ecotypes, for example, the rapid-cycling accessions Columbia and Landsberg *erecta*, carry recessive, nonfunctional alleles of *FRIGIDA* (26). The availability of the *FRI* sequence made it possible to analyze the basis of this recessivity. The Col *FRI* allele was found to carry a 16-bp deletion within exon one, resulting in a premature stop-codon in exon two (26). The L*er FRI* allele had acquired an insertion-deletion event at the beginning of the *FRI* open reading frame, removing the putative translation start-codon (26). Hence, loss-of-function mutations in *FRI* and early flowering appear to have arisen at least twice independently. Indeed, further analysis of wild accessions has extended the known number of naturally occurring loss-of-function *FRI* mutations (19, 39).

The evolutionary forces that have led to the independent fixation of early flowering mutations in *FRI* remain to be determined. Potential advantages to loss of *FRI* activity could be imagined in climates permissive to multiple generations within one year or to evade stressful conditions such as drought or very severe winters. No clear environmental associations have yet been found in patterns of *FRI* variation and it is possible that complex local selective forces determine the distributions observed (26). *FRI* sequence variation within natural populations is being analyzed to determine the selective pressures acting at this locus. The patterns observed suggest that the variation is adaptive and has been maintained by positive selection (39).

The Floral Repressor *FLOWERING LOCUS C*

Crosses of active *FRI* alleles to the early L*er* accession showed that active *FRI* alleles require a second dominant gene, *FLOWERING LOCUS C (FLC)*, to delay flowering (11, 31, 44). A combination of *FRI* and *FLC* confers late flowering, which is antagonized by a vernalization treatment (11, 31, 44). The epistatic relationship between *FRI* and *FLC* has been demonstrated by the complete suppression of *FRI* by a null mutation at *flc* (56). Although *FLC* activity is strongly enhanced by the presence of *FRI*, it is not absolutely dependent upon *FRI*. This is demonstrated by the fact that an *flc* mutant in the Col background flowers earlier than the parent

under noninductive SD photoperiods (56). Analysis of the late-flowering *Sy-0* accession, which forms aerial rosettes on the infloresence, has identified a second gene, *AERIAL ROSETTE1* (*ART1*), that acts independently from *FRI* to upregulate *FLC* (66a). In this background *ART1* and *FLC* act synergistically to delay flowering. How *ART1* and *FRI* function are related will be interesting to determine.

Molecular cloning of *FLC* showed it to encode a MADS-box transcription factor (54, 79). The presence of *FRI* greatly upregulates *FLC* mRNA, and this accumulation is quantitatively antagonized by vernalization (54, 79, 81). The levels of *FLC* message and protein correlate closely with flowering time (73). The semidominant effects of *FLC* have led to the proposal that it functions as a repressive "rheostat" in flowering time control (55). Repression of *FLC* is mitotically stable after vernalization and reset following meiosis, paralleling the known physiology of the response (54, 79, 81).

The mechanism by which *FLC* inhibits the floral transition appears to be through repression of a set of genes termed floral pathway integrators (56). Floral pathway integrator genes are common targets of multiple flowering-time pathways (84). Through inhibition of integrators *FLC* is able to antagonize the other floral promotive pathways. The presence of active *FRI* and *FLC* genes leads to potent repression of flowering under otherwise inductive conditions, e.g., long days, potentially explaining why *FRI* and *FLC* play such a major role in the natural variation in *Arabidopsis* flowering time (84). This repression activity may provide a means to distinguish the seasonal context of inductive photoperiods with reference to the prior passage of winter.

Examples of floral integrator targets of *FLC* are *FT* and *AGL20* [also called *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO*)] (27, 28, 74). These targets are regulated both positively by the photoperiod pathway transcription factor *CO* (*CONSTANS*) and negatively by *FLC* (22). To analyze the molecular basis of this pathway integration, a minimal *AGL20* promoter element responsive to both *CO* and *FLC* has been defined (22). Antagonistic regulation by the CO and FLC transcription factors maps to distinct *cis*-elements within this promoter. Recent work has identified a further MADS-box gene *AGL24* (*AGAMOUS-LIKE24*) acting downstream of *AGL20* as a dosage-dependent promoter of flowering. Interestingly, *AGL24* appears to promote *AGL20* expression and also to be upregulated by vernalization through *FLC* independent mechanisms (57, 102).

MADS-box transcription factors exist as a large, multigene family in *Arabidopsis* (71). Several members of this group function in floral development, acting in multimeric transcription complexes with additional MADS proteins (23). The existence of a similar FLC complex is currently unknown. Within the MADS-box family *FLC* shares strong similarity with a small clade of genes (68). A member of this clade, *FLOWERING LOCUS M* (*FLM*)/*AGL27*, also functions as a dosage-sensitive floral repressor, although it does not appear to be involved in the vernalization requirement (78).

Although the majority of natural variation in the vernalization requirement exists at *FRI*, allelic variation at *FLC* has also been discovered. The *FLC* allele

in the L*er* background is weak relative to the Col allele although they encode identical proteins (11, 31, 44, 81). *FLC* accumulates to a lower level when an active copy of *FRI* is introduced into L*er*, so this allele is compromised in its ability to respond to upstream signals (54, 79, 81). Sequencing of the genomic *FLC* locus reveals a transposon insertion within intron one of L*er-FLC* (19). As *cis*-sequences important for *FLC* regulation have been mapped to this region, this insertion may underlie the weakness of the L*er* allele (80). Although the L*er FLC* allele is clearly weak, it is not a complete null, as introgression of an active *FRI* allele still leads to a delay in flowering (11, 31, 44). A group of accessions has been identified that carry functional *FRI* alleles, yet are early flowering (26). These backgrounds are candidates for carrying loss-of-function *FLC* alleles. Indeed, this appears to be the case for the Shakhdara accession (19). Although loss of *FRI* or *FLC* function leads to the same phenotypic consequence with respect to the vernalization requirement, variation at *FLC* appears rarer in nature. Hence, loss of *FLC* function may confer a selective disadvantage relative to *FRI*.

VERNALIZATION RESPONSE

Vernalization and DNA Methylation

Once a vernalized state has been established at the plant's apex, it is stable through multiple cell divisions. The establishment and maintenance of silenced chromatin states during epigenetic regulation has been associated with a myriad of modifications at both the DNA and histone levels (6, 88). These modifications appear to interact in a complex code controlling activity of gene expression.

Of these modifications, DNA cytosine methylation has been studied intensively in relation to the vernalization response (18). Demethylation induced by 5-azacytadine treatment was found to partially substitute for cold treatment in several vernalization-requiring species and backgrounds, including *Arabidopsis* (10). Vernalization treatment itself was also reported to induce a global reduction in DNA methylation levels (10, 18). To test this model genetically, antisense expression of the cytosine methyltransferase, *MET1*, allowed recovery of plants with globally reduced DNA methylation. Reduction of *MET1* expression altered flowering time, but has been reported to both delay and accelerate flowering in different backgrounds (10, 18, 72). Indeed, reduction of *MET1* expression and 5-Aza treatment both lead to pleiotropic effects on plant development (72). Although these data did not provide a clear definition of the importance of cytosine methylation in vernalization, the identity of *FLC* as its major target allowed a direct test of this hypothesis. Analysis of *FLC* mRNA levels in *MET1* antisense backgrounds revealed a reduction in its accumulation (81); however, this is an indirect effect of the developmental perturbations caused by global loss of methylation as bisulfite sequencing has revealed no change of *FLC* DNA methylation status through vernalization (J. Finnegan, personal communication).

VRN Genes and Epigenetic Regulation of *FLC*

To elucidate the mechanism underlying vernalization, genetic screens have been performed to isolate mutants defective in this response. The late-flowering *fca* mutant shows a pronounced response to vernalization treatment and has been used as a background in which to screen (12). Several recessive *vernalization* (*vrn*) mutants have been identified and divided into distinct complementation groups (*vrn1*, *vrn2*, *vrn4*, *vrn5*) (12) (A.R. Gendall, Y.Y. Levy & C. Dean, unpublished observations).

The *vrn2* mutant was isolated as showing a strong reduction in the vernalization response (12). This reduction correlated with increased levels of *FLC* mRNA after cold treatment (81). Detailed analysis of *FLC* expression dynamics was particularly revealing with respect to *VRN2* function. Normally, *FLC* expression is silenced by vernalization treatment and once the cold stimulus is removed, silencing is maintained throughout subsequent development, although the level is reset after meiosis (81). In *vrn2* a distinct pattern was evident; cold treatment led to normal repression of *FLC*, but on returning to elevated temperatures, silencing was not maintained and progressive increases in *FLC* mRNA were observed (20). Hence, *VRN2* is not required for the initial cold-induced repression of *FLC*, but for stable maintenance of repression once the stimulus is removed. This demonstrates that *FLC* regulation is biphasic, comprising genetically separable phases for establishment and maintenance; and that loss of *VRN2* only affects the second phase.

VRN2 encodes a protein carrying an N-terminal C2H2 zinc-finger, nuclear localization sequences, and a C-terminal region conserved with a number of other proteins (20). Within the *Arabidopsis* genome these are *FERTILISATION INDEPENDENT SEED2* (*FIS2*) and *EMBRYONIC FLOWER2* (*EMF2*), genes characterized as developmental regulators acting to repress endosperm and flower development, respectively (49, 101). The connection between the generic function of these genes in developmental repression and their molecular mode of action will be interesting to determine. In addition, *VRN2* displays homology to a further protein, *Suppressor of Zeste-12* (*Su(Z)12*), a gene characterized as a Polycomb-Group developmental regulator in *Drosophila* (7). The Polycomb-Group genes function to maintain transcriptional silencing of homeotic genes during and after *Drosophila* embryogenesis; thus the *VRN2* function in repression of *FLC* parallels their function (65).

The Su(Z)12 protein has recently been characterized as part of a histone methyltransferase complex directed against histone 3 lysine 27 and possibly also histone 3 lysine 9 (15, 59). Other members of this complex include the SET domain and WD-repeat proteins Enhancer of Zeste, Extra Sex Combs, and NURF-55 (15, 59). The homology of VRN2 to Su(Z)12 and the presence of E(Z) and ESC homologs within the *Arabidopsis* genome raise obvious questions as to whether VRN2 acts in a similar complex to mediate epigenetic silencing at *FLC* through vernalization. Consistent with this model are the observed changes in DNaseI accessibility within the first *FLC* intron observed in the *fca-1 vrn-2* mutant after vernalization (20).

TABLE 1 The major field crops with vernalization requirement

Species	Common name	Species	Common name
Allium cepa	Onion	*Linum usitatissimum*	Flax
Avena sativa	Oat	*Lolium perenne*	Ryegrass
Beta vulgaris	Beet	*Papaver somniferum*	Poppy
Brassica napus	Rape	*Pisum sativum*	Pea
Brassica oleracea	Cauliflower	*Raphanus sativus*	Radish
Cicer arietinum	Chickpea	*Secale cereale*	Rye
Dactylis glomerata	Cocksfoot	*Spinacia oleracea*	Spinach
Daucus carota	Carrot	*Trifolium repens*	White clover
Hordeum vulgare	Barley	*Triticum aestivum*	Wheat
Lactuca sativa	Lettuce	*Vicia taba*	Faba bean
Lens culinaris	Lentil		

Sequences within this intron have been reported to be of importance for epigenetic repression of *FLC* after cold treatment (80). Hence, on the basis of such sequence and functional homology a strong candidate for the epigenetic change mediating *FLC* silencing would be histone methylation.

The *vrn1* mutation was isolated in the same screen as *vrn2* and they have been found to share many characteristics (12). The pattern of *FLC* derepression, observed in *vrn2*, has also been shown in *vrn1*, suggesting that the genes act together to maintain *FLC* silencing (45). Cloning of *VRN1* showed it encoded a protein containing two B3 domains, plant-specific domains that have been demonstrated in one example to mediate specific DNA binding (45, 90). These domains and the nuclear localization of VRN1 are consistent with its function in transcriptional repression. The plant-specific identity of VRN1 and its function in epigenetic regulation of *FLC* suggests that the mechanism at work is not identical to that characterized for Polycomb repression in *Drosophila*. Indeed, other components of Polycomb repression such as the PRC1 complex are not encoded in the *Arabidopsis* genome (65). Analysis of VRN1 function and its association with VRN2 to maintain the silencing of *FLC* will be very revealing with respect to evolutionary parallels and differences in chromatin regulation.

VRN1 has additional functions during vernalization and in control of flowering (45). Evidence for this comes from the fact that the *vrn1* mutation alone is late in some growth conditions and this is not associated with increased *FLC* levels (45, 81). Second, overexpression of *VRN1* leads to early flowering, again not mediated through changes in *FLC* mRNA (45). In both cases, changes in *VRN1* activity results in changes in expression of downstream floral integrators such as *FT* and *AGL20* (45). The existence of multiple targets for *VRN1* in flowering time control supports its in vitro, nonspecific DNA binding activity (45). A key question is how the cold signal is able to target VRN1 to the *FLC* locus. The existence of multiple targets for *VRN* genes is consistent with genetic analysis of *FLC* in vernalization

requirement (56, 69). *FLC*-independent mechanisms for floral promotion by cold temperature have been suggested as the *flc* null mutant still displays a vernalization response under SD (56). Furthermore, the triple *co-2 fca-1 ga1-3* mutant fails to flower unless vernalized (69). This indicates that even in the absence of the photoperiodic, autonomous, and gibberellin-promotive pathways, vernalization can still act directly to accelerate flowering, suggesting both *FLC*-dependent and -independent functions.

Cold Temperature Sensing

VRN1 and VRN2 appear to mediate maintenance of *FLC* repression, but loss of their function does not affect the initial establishment of repression (20, 45). The genes required for this and the upstream cold-sensing pathway remain uncharacterized. How this pathway recruits or activates the VRN proteins will also be of interest, as their expression is unaffected by vernalization (20, 45).

Candidate genes involved in cold sensing are those characterized as functioning during cold stress. Cold induces expression of *COR* genes, mediated by a transcription factor cascade involving the CBF1 protein (93). A role for the *VRN* genes in this pathway has been discounted as *COR* transcripts still show pronounced upregulation by cold in the *vrn* mutants (12). Cold signaling associated with vernalization has also been shown not to involve CBF1 or abscisic acid (47). Evidence for some cross-talk between vernalization and acclimation comes from *HOS1* (40). *HOS1* appears to act as an upstream activator of both the *CBF1*, *COR*, and *FLC* genes. This gene encodes a RING-finger protein suggested to function as an E3-ubiquitin ligase (40). Intriguingly, the localization of a HOS1:GFP fusion protein changes from cytoplasmic to nuclear after cold treatment (40). Screens directed to isolate regulators of the primary phase of *FLC* downregulation may identify components of this thermosensory pathway.

AUTONOMOUS PROMOTION OF FLOWERING

Autonomous Repression of *FLC*

In addition to the *FRI* and *FLC* genes, a further class of loci has been identified with strong effects on the vernalization requirement in *Arabidopsis*. Screens for flowering time mutations identified a group of six recessive, late-flowering mutants with a common physiology (*fca, fy, fpa, fve, ld, fld*) (32, 42, 76). Their late flowering occurs under both long-day and short-day conditions, distinguishing these genes from those controlling the photoperiodic pathway (32). Furthermore, this lateness is suppressed by vernalization and growth in far-red enriched light, similar to *FRI*, *FLC* genotypes (Figure 3) (41). This pathway was termed the autonomous pathway because of its apparent photoperiod-independent promotion of flowering.

The recessive nature of autonomous mutants suggests that normally the pathway functions to promote flowering. The suppression of their late flowering by

vernalization reveals that, at least in the rapid-cycling backgrounds, the autonomous pathway is acting in parallel with the vernalization pathway to promote flowering. This relationship suggested a role for the autonomous genes in repression of *FLC* and expression analysis of *FLC* showed that autonomous pathway mutants have higher levels of *FLC* RNA (54, 79). Hence, the autonomous pathway functions to limit the accumulation of *FLC* mRNA. Indeed, introduction of an *flc* null mutation or an antisense *FLC* transgene into autonomous backgrounds completely suppresses their late-flowering phenotype (56).

Genetic analysis has suggested that the function of this pathway may be facilitative rather than directly promotive. The triple *co-2 fca-1 ga1-3* mutant fails to flower unless vernalized (69); however, the *co-2 ga1-3* double mutant is also extremely late flowering, indicating that the autonomous pathway alone has little promotive activity (69). Conversely, the *fca-1* and other autonomous mutations are very late flowering (32). By repressing *FLC*, the autonomous pathway exerts a promotive effect by repressing a repressor, and facilitating the activity of other directly promotive pathways such as the photoperiodic and gibberellin pathways (62, 69).

The loss of autonomous pathway activity has the same consequences on *FLC* expression and the vernalization requirement as dominant alleles of *FRI*. However, analysis of natural variation in the vernalization requirement has yet to reveal loss-of-function changes in autonomous genes. Indeed, recessive loci contributing to the vernalization requirement appear to be rare in nature. The only reports are from the St X Li-5 crosses, where the recessive alleles *kryophila* and *juvenalis* contributed to a vernalization requirement (61), and in the Kiruna-2 ecotype, where a recessive, monogenic trait leads to winter annualism (11). Whether these loci depend upon *FLC* has not been investigated, but such loci may represent natural variation in the autonomous pathway. Crosses of Kir-2 to the autonomous mutants did not reveal allelism (11). Autonomous mutations may confer a selective disadvantage in the wild not seen for *FRI*. More extensive sampling and analysis of winter annual accessions will be required to determine whether natural variation within the autonomous pathway exists.

Molecular Partnership of *FCA* and *FY*

Double mutant analysis within the autonomous pathway demonstrated epistatic grouping of the members. Two subgroups can be distinguished; *fca*, *fy* and *fpa*, *fve*, with combinations of mutants between these groups displaying additional lateness (30). Recent progress in analysis has demonstrated the epistasis between *FCA* and *FY* as reflecting interaction between their gene products (85). *FCA* encodes a novel, nuclear RNA binding protein with two N-terminal RRM (RNA recognition motif)-type RNA binding domains and a C-terminal WW protein interaction domain (50, 89). The *Arabidopsis* genome contains many RRM proteins novel to the plant kingdom; however, very few have had their function within RNA metabolism determined (48). A role for FCA in RNA 3′-end processing has been revealed through the identification of its partner protein FY (85).

The *FY* gene encodes a highly conserved protein consisting of N-terminal WD-repeats and a proline-rich C-terminal extension (85). FY homologs exist in all sequenced eukaryotic genomes and the yeast homolog Pfs2p has a well-characterized role in 3′-end processing of pre-mRNA (64). Pfs2p functions to maintain the integrity of a large complex involved in the cleavage and polyadenylation of the 3′-ends of mRNA (64). In contrast to the conserved homologs, FY has acquired a novel proline-rich C terminus with which FCA interacts (85). A functional role for FY and FCA in 3′-end processing was uncovered through analysis of *FCA* autoregulation (67, 85). The *FCA* pre-mRNA is processed to produce four distinct transcripts, with alternative 3′-end processing/polyadenylation occurring either at a promoter-proximal site within intron 3 or at a distal site within the 3′-UTR (50, 51). Processing within intron 3 generates a truncated transcript (beta), which is inactive in flowering-time control (50). Increasing the levels of FCA through overexpression of the cDNA led to negative feedback regulation via promotion of processing at the promoter-proximal site. This autoregulation was found to depend upon FY and an intact FCA-WW domain, providing evidence that the sequence homology of FY to poly(A) factors is meaningful with respect to its function (67, 85). This mechanism of *FCA* autoregulation appears to be of biological consequence as altering its occurrence changes the balance between pathways influencing the vernalization requirement (67). Normally, active *FRI* alleles are epistatic to the autonomous pathway, delaying flowering. However, bypassing *FCA* autoregulation accelerates flowering even in the presence of *FRI*. This reveals that regulation of *FLC* by antagonistic pathways is quantitatively balanced. *FCA* auto-processing also presents a possible mechanism for the pathway to be regulated. Indeed, processing of FCA intron 3 occurs in a precise spatial and temporal pattern through development (51, 67). The determinants of this pattern are unknown but may provide insight into the activity of the autonomous pathway.

Within the context of *FCA* autoregulation, FCA and FY appear to function in 3′-end processing (67, 85). However, alternative processing of the *FLC* transcript has not yet been detected, nor is it known whether *FLC* is a direct floral target of FCA and FY. Although FCA and FY appear to function together during floral promotion, analysis of an allelic series of *fy* mutations have suggested additional roles for this gene. A null *fy* mutation is lethal and detailed analysis has revealed the late-flowering, viable alleles as hypomorphic (I.R. Henderson & C. Dean, unpublished observations). This suggests that FY has retained the ancestral, essential RNA processing function of its homologs. The novel role of FY in plants in controlling the floral transition appears to derive from the acquisition of a new C terminus and interacting partner FCA.

FPA, *FVE*, and *LD*

Cloning of the *FPA* gene has revealed that this gene encodes a second protein carrying RNA binding domains within the autonomous pathway (77). Although both FCA and FPA carry RRM-type RNA binding domains, whether they perform

similar functions in RNA metabolism is not known. *FPA* functions like the other genes in the autonomous pathway to repress *FLC* function, yet it is distinguished by additional phenotypes under short-day (SD) conditions (77). Pleiotropic functions for *FPA* are also suggested by the lethal interaction between *fpa* and *fy* mutations (30).

Two further autonomous genes have been cloned; *LUMINIDEPENDENS* and *FVE*, which encode a nuclear, homeodomain and a WD-repeat protein, respectively (9, 42). The LD protein is predicted to carry two nuclear localization sequences and a glutamine rich C terminus, suggestive of a function as a transcription factor (2). However, LD is atypical in that only four of the seven invariant homeobox residues are conserved (2, 95). In this respect note that homeodomain proteins have also been characterized as acting posttranscriptionally (17). The *ld* mutation and *fld* (defining another gene of the autonomous pathway that is as yet uncloned) are genetically distinct from the other autonomous loci in their relationship with different *FLC* alleles. Both mutations are completely suppressed by the weak L*er* *FLC* allele, whereas the other four mutations are not (31, 44, 76).

Ambient Temperature and Flowering Control

Aside from the promotive effects of cold temperature during vernalization, ambient temperature also exhibits an effect on flowering time, with elevated temperatures leading to accelerated flowering (8). Recent genetic analysis of flowering behavior at 23°C and 16°C has implicated components of the autonomous pathway in a thermosensory flowering pathway (8). Mutations in *FCA* and *FVE* did not show differential flowering times at 16°C and 23°C, characteristic of wild-type. *FCA* and *FVE* were proposed to promote flowering in response to temperature acting to up-regulate *FT* expression in a pathway that functioned independently of *FLC* (8). However, the ambient temperature effects may be mediated entirely through a phytochrome-cryptochrome-dependent pathway that up-regulates *FT* (8, 21). High levels of *FLC* mRNA caused by *fca* and *fve* mutations (*fy* and *fpa* causing lower *FLC* accumulation) might suppress the effects of this pathway by efficiently repressing *FT* expression.

Activators of *FLC*

Early flowering mutations have defined a large class of repressors of the floral transition. Genetic analysis has placed several of these floral repressors close to regulation of *FLC* activity. The *esd4* mutation has pleiotropic consequences on shoot architecture and silique shape and leads to early flowering (70). When combined with autonomous mutations, *esd4* reduces the accumulation of *FLC* mRNA (70). However, *esd4* effects on *FLC* only partially explain its flowering time and developmental phenotypes. *ESD4* encodes a nuclear, SUMO-directed protease and in the *esd4* mutant there are lower levels of free SUMO (G. Coupland, personal communication). Currently, in vivo targets of the SUMO machinery in plants are unknown but appear likely to include a regulator of *FLC* expression. Similarly,

the *efs* mutant displays early flowering under short-day conditions and pleiotropic defects in plant development, including reduced plant size, fertility, and apical dominance (86). Although pleiotropic, the *efs* mutant shows complete epistasis with the autonomous mutations *fca* and *fve* (86). This would place *EFS* in a similar genetic position as *FLC*, as a floral-inhibitory function counteracted by the activity of the autonomous pathway.

A further epistasis group of early flowering mutants has recently been characterized as activators of *FLC*. The *vip* (*vernalization independence*) mutants comprise at least seven loci that share an early flowering phenotype and pleiotropic growth defects potentially relating to ectopic *AGAMOUS* expression (96). Double mutants within the *vip* class show no synergistic phenotypic enhancement consistent with the products acting either within a linear genetic pathway or together within a large complex (96). The VIP4 protein shows some similarity to the yeast transcriptional activator Leo1, which functions within the Paf1 complex, and other Paf subunits within the *Arabidopsis* genome map to *VIP* loci (96, 105). Hence, the *VIP* genes may comprise a complex involved in transcriptional regulation of *FLC* and other targets. How these groups of *FLC* activating genes interface with the other upstream pathways will be interesting to determine. The *vip4* mutation nearly completely suppresses the *ld* autonomous mutation, which may indicate that *VIP* function is downstream of *LD* or that it acts independently (96).

THE VERNALIZATION REQUIREMENT IN CROP PLANTS

Forward Genetic Analysis in Crops

The vernalization requirement is an important agronomic trait in diverse crop species (see Table 1) and this has significantly extended the geographical range of where they are grown. Quantitative trait loci (QTL) analysis of flowering time and vernalization requirement has been undertaken in a number of *Brassica* species. Teutonico & Osborn first reported that two QTLs, *VFR1* and *VFR2*, contributed most of the variation in flowering time in *B. rapa* (92). Alignment of the maps between the *Brassica* species suggested that they corresponded to the QTLs *VFN1* and *VFN2* in *B. napus* (29, 66). Comparative mapping between *Brassica* and *A. thaliana* indicated that the region containing *VFR1* and *VFN1* showed colinearity with several regions of the *Arabidopsis* genome, including the top of chromosome 4 where *FRI* maps (29, 66). The genomic region containing *VFR2* and *VFN2* is homologous to a region on chromosome 5 of *A. thaliana* where *CO*, *EMF1*, *FY*, and *FLC* map (29, 66). High-resolution mapping has shown that V*RF2* is homologous to *FLC*, and five *FLC* orthologues genes, *BnFLC1–5*, isolated from the winter cultivar of *B. napus*, delay flowering in a rapid-cycling *Arabidopsis* accession (29, 91). These data support the idea that within the Brassicaceae the "*FRI-FLC*" mechanism has been conserved and controls the vernalization requirement.

Forward genetic analysis in wheat and barley has identified the *Vrn1* loci as conferring the vernalization requirement (in contrast to *Arabidopsis VRN* genes

that mediate vernalization response). *Vrn1* occurs as three homeologs *Vrn-A1*, *Vrn-B1*, and *Vrn-D1* in hexaploid bread wheat (*Triticum aestivum*) that map to identical regions of the group 5 chromosomes (82). Orthologous genes have been mapped in diploid wheat (*Triticum monococcum*, *Vrn-A^m1*) and barley (*Hordeum vulgare*, *Sgh2*, *Vrn-H1*) (16, 37). Dominant alleles of the *Vrn1* gene confer a spring growth habit (16, 37, 82, 83). The map-based cloning of wheat *Vrn1* has recently been completed (100). The locus was found to be completely linked to two MADS-box genes encoding proteins highly homologous to *Arabidopsis* AP1 and AGL2. No recombination was found between the two genes (called *AP1* and *AGL-G1*), so their expression was examined to determine which gene was most likely to correspond to *Vrn1*. *AP1* RNA was undetectable in winter accessions of *T. monococcum* until after six weeks of vernalization, whereas it was expressed independently of vernalization in spring accessions (100). *AGLG1* expression was detected in apices only after the transition to flowering and was not affected by vernalization. This expression profile in addition to the role of *Arabidopsis AP1* in floral meristem identity (46) suggested that *AP1* is the most likely candidate for wheat *Vrn1*. The *AP1* gene was analyzed from winter and spring wheat accessions to look for functional polymorphism. Although amino acid sequences were identical, a 20-bp indel was found in the promoter of spring accessions (100).

Wheat *Vrn2* has an opposite effect to *Vrn1*, with dominant alleles conferring a winter growth habit (83, 94). The *Vrn2* gene has been mapped in barley (*Vrn-H2*, *Sgh1*) to the 4H chromosome, and in einkorn wheat (*Vrn-A^m2*) to the *5A^m* chromosome (16, 37, 82, 83). The orthologous *Vrn2* genes have yet to be identified in bread wheat, although analysis of aneuploid lines of hexaploid wheat have identified genes with similar functions to *Vrn2* on group 1 and 6 chromosomes (24, 38). Genetic analysis has shown an epistatic interaction between the spring habit conferred by the *vrn-A^m2* allele and *Vrn-A^m1* (16, 37, 83, 94). The identification of the 20-bp indel in the promoter of *Vrn1*, together with this epistatic relationship, led to the proposal that Vrn2 acts to repress expression of *Vrn1* by binding to the *Vrn1* promoter region incorporating the 20-bp indel (100). Vernalization would act to reduce levels of Vrn2, thus relieving repression of *Vrn1*. Spring growth habit would result through loss of the Vrn2 binding site and thus loss of *Vrn1* repression. The imminent cloning of *Vrn2* by the Dubcovsky group will enable this mechanism to be tested (16).

Comparative Analysis of Vernalization Genes

The identification of genes conferring the vernalization requirement in *Arabidopsis* and cereals enables a comparative analysis of vernalization. The identification of *AP1* as the most likely candidate for wheat *Vrn1* is intriguing as *AP1* has not so far been associated with the vernalization requirement in *Arabidopsis* or been found as a target of FLC. The proposed characteristics of wheat *Vrn2*—encoding a floral repressor whose expression/function to repress a floral pathway integrator is antagonized by vernalization—could be describing an *Arabidopsis FLC* function.

However, no clear homolog of *FLC*, *FRI*, or *Arabidopsis VRN* genes has been found in cereals (D. Laurie, personal communication). Identification may be difficult if gene divergence has occurred or the genes have been lost. As rice does not vernalize, this loss may well explain their absence from its genome sequence.

Although the *FLC* repressor is not recognizable in the rice genome, clear homologs of its upstream regulators *FCA*, *FY*, and *LD* are present in both rice and maize (D. Laurie, personal communication) (95). Determination of the function of the autonomous genes in cereals will be important for understanding conservation of their function. A role in flowering control is made likely as floral integrator genes such as *FT*, *AGL20*, and *LFY* have obvious homologs in rice, some of which have a demonstrated role in flowering control (25). In addition, several major QTLs contributing to photoperiodic sensitivity have been cloned in rice and found to specify homologs of the genes controlling this trait in *Arabidopsis* (25), despite the inductive photoperiod being long days in *Arabidopsis* and short days in rice.

FUTURE QUESTIONS

Molecular genetic analysis in *Arabidopsis* has defined the pathways and basic mechanisms involved in the vernalization requirement and maintenance of the vernalization response. How cold temperature signals the initial changes in vernalization is still unknown but is likely to produce some surprises over the next few years. A key question to be addressed is whether the initial mechanism of temperature perception is common to all cold-induced processes in plants.

Changes involved in the cellular memory of vernalization show parallels with the stabilization of developmental changes during animal development caused by homeotic regulators (20, 65). It will be interesting to determine how far these commonalities extend given the independent evolution of plant and animal multicellularity (53) and whether they are similar to other epigenetically regulated processes in plants (49, 101). The pathways conferring the vernalization requirement in *Arabidopsis* function through regulation of at least one common target, *FLC*. It will be important to establish how the up-regulation of *FLC* conferred by FRI interfaces with control from the VRN proteins. In *Drosophila*, the trithorax-group and Polycomb-group proteins mediate antagonistic functions on chromatin structure of *Hox* genes (65). This may provide a paradigm for antagonism between *FRI* and *VRN* genes at *FLC*. Furthermore, how do the autonomous pathway proteins function within this context? Is their role in regulating *FLC* directly at the posttranscriptional level or do they function indirectly, again via alteration of chromatin structure?

Availability of whole-genome microarrays will facilitate identification of the complete set of vernalization targets in *Arabidopsis*. Knowledge of additional targets should help the comparative analysis of vernalization between *Arabidopsis*, *Brassicas*, and cereals. Whether *FRI*, *FLC* and the *VRN* genes have functional equivalents outside of *Arabidopsis* or distinct factors mediate these functions

remains to be seen. A clearer understanding of these issues will enable us to address how many times a vernalization response has arisen during the evolution of angiosperms. Dissection of the molecular basis of the vernalization requirement and response will undoubtedly contribute to our overall understanding of epigenetic regulation and open up the possibility of manipulating this trait in a range of plant species.

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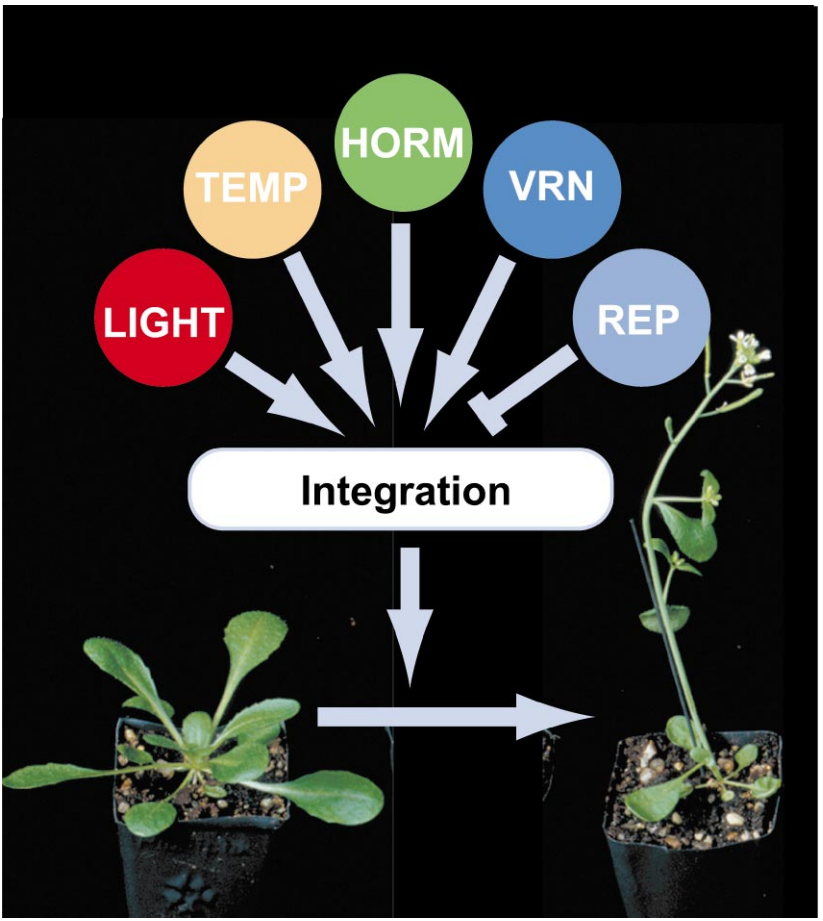

Figure 1 Plants integrate multiple cues during the switch to flowering. In Arabidopsis input signals influencing the transition are light (*photoperiod* and *quality*), ambient temperature, phytohormones and long periods of cold temperature (*vernalization*). Additionally, floral repressors antagonize the activity of promotive cues.

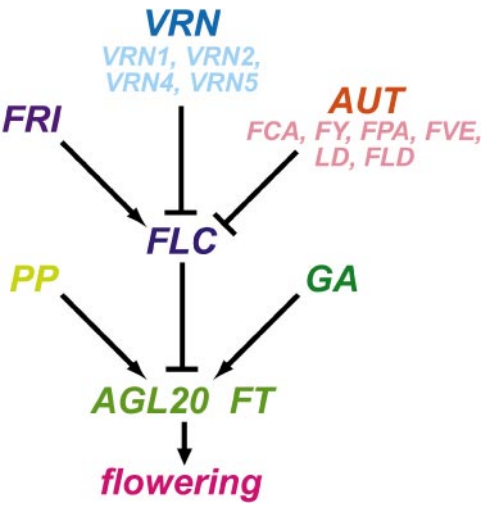

Figure 2 Genetic model for vernalization requirement and response in *Arabidopsis thaliana*. Abbreviations: *VRN*, vernalization pathway; *AUT*, autonomous pathway; *FRI*, *FRIGIDA*; *FLC*, *FLOWERING LOCUS C*; *PP*, photoperiodic pathway; *GA*, gibberellic acid pathway.

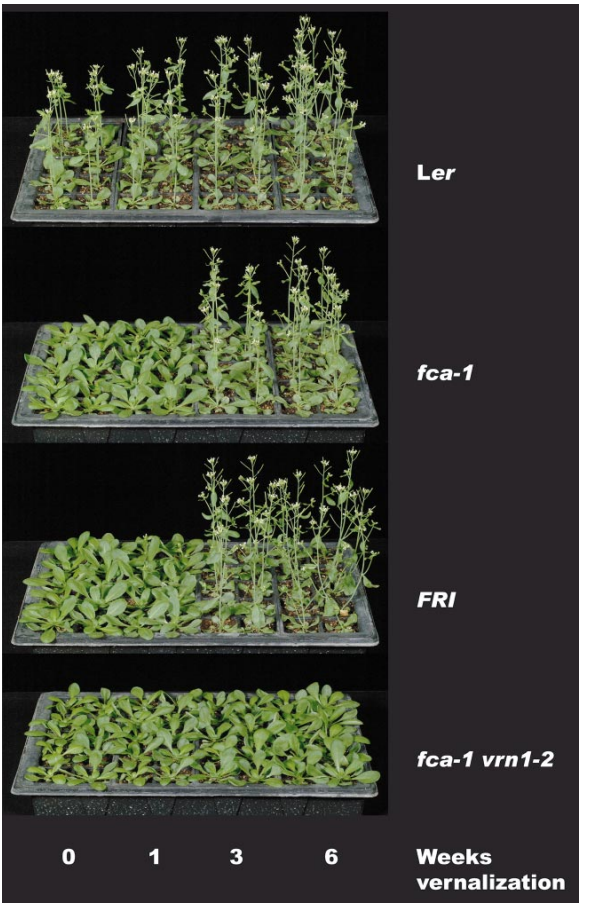

Figure 3 Flowering time responses to vernalization. *Arabidopsis* plants exposed to 0, 1, 3, or 6 weeks at 4°C. Vernalization promotes flowering in L*er*, *fca-1* and *FRI* but not *fca-1vrn1-2*.

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