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Vernalization and epigenetics: how plants remember winter

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One of the remarkable aspects of the promotion of flowering by vernalization is that plants have evolved the ability to measure a complete winter season of cold and to 'remember' this prior cold exposure in the spring. Recent work in *Arabidopsis* demonstrates the molecular basis of this memory of winter: vernalization causes changes in the chromatin structure of a flowering repressor gene, *FLOWERING LOCUS C* (*FLC*), that switch this gene into a repressed state that is mitotically stable. A key component of the vernalization pathway, VERNALIZATION INSENSITIVE3 (VIN3), which is a PHD-domain-containing protein, is induced only after a prolonged period of cold. VIN3 is involved in initiating the modification of *FLC* chromatin structure. The stable silencing of *FLC* also requires the DNA-binding protein VERNALIZATION1 (VRN1) and the polycomb-group protein VRN2.

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Abbreviations

CBF1	C-repeat-/DRE-Binding Factor1
COR	*COLD REGULATED*
FLC	*FLOWERING LOCUS C*
FRI	*FRIGIDA*
HOS1	*HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1*
HP1	HETEROCHROMATIN PROTEIN1
PHD	PLANT HOMEODOMAIN
PRC2	polycomb repressor complex2
Su(z)12	SUPPRESSOR OF ZESTE-12
VIN3	*VERNALIZATION INSENSITIVE3*
VRN1	*VERNALIZATION1*

Introduction

Plants have evolved the ability to alter their developmental program in response to environmental stimuli. A major switch in the developmental program is the transition to flowering. In many plant species, the timing of this transition is determined by seasonal changes that are sensed by the plant. Photoperiod and temperature are two of the main environmental cues that plants monitor to determine the correct time to flower.

Vernalization is a term that describes the promotion of flowering after exposure to cold. Specifically, vernalization results in 'the acquisition or acceleration of the ability to flower by a chilling treatment' [1]; after vernalization, plants do not necessarily initiate flowering but acquire the competence to do so. In many plant species, vernalization requires long-term exposure to the low temperatures of a typical winter. This is a useful adaptation because many vernalization-requiring species have a winter-annual or biennial habit; the plants begin growing in one season but flower in the spring of the second growing season. The term vernalization is derived from the Latin word *vernus*, meaning 'of the spring'. In vernalization-requiring species, it is crucial that the plants are not 'tricked' into flowering in the late autumn by transient exposure to cold followed by warm conditions, thus the requirement for prolonged cold. The flowering of many vernalization-requiring species is also promoted by long photoperiods, and this photoperiod requirement provides another level of assurance that flowering does not occur in late autumn when the days are short.

The physiology of vernalization has been studied extensively since the defining work of Gustav Gassner in the early 20th century (discussed in [2]). Studies involving grafting and localized cooling have shown that the apical meristem is the site of cold perception during vernalization, and that vernalization causes the meristem to become competent to flower [2–4]. Once meristems have been exposed to prolonged cold, they 'remember' that they have been vernalized, and this memory is mitotically stable. One of the classic experiments demonstrated the existence of this memory by vernalizing biennial *Hyoscyamus niger* and subsequently growing the vernalized plants in non-inductive photoperiods (discussed in [2]). The vernalized *H. niger* plants were able to remember the vernalization for long periods of time, and were subsequently able to flower when exposed to inductive photoperiods. Another classic study that both identified the site of vernalization and demonstrated the memory effect involved the *in-vitro* regeneration of plants from various tissues of vernalized *Lunaria biennis* [3,4]. Only tissues that contained dividing cells (including root meristems) regenerated into vernalized plants. Thus dividing cells (or perhaps cells in which DNA replication is occurring) are a prerequisite for vernalization, and the vernalized state is maintained through tissue culture. This type of experiment has also been replicated in *Arabidopsis* [5]. The mitotically stable cellular memory illustrates the

epigenetic nature of vernalization. It is vital, of course, that this memory is lost in the next generation so that the vernalization requirement is re-established.

These classical studies of vernalization raise some interesting questions. How can plants measure long-term cold exposure? For example, why does a week of cold not result in vernalization when four weeks does? What is the basis of the mitotically stable cellular memory of vernalization. Recent genetic and physiological studies of the vernalization pathway in *Arabidopsis*, and the identification of the some of the genes that are involved in this pathway, have provided a framework for addressing these intriguing questions.

Genetics of vernalization

Many species include both summer-annual types, which flower rapidly without vernalization, and biennial or winter-annual types. The number of genes that are responsible for determining whether a plant in such a species has the biennial or the annual habit can be determined readily. One of the first studies of this type was Correns' demonstration in 1904 that the biennial habit was conferred by a single dominant gene in *H. niger* (discussed in [2]). Although most commonly used laboratory strains of *Arabidopsis* are rapid-flowering summer annuals, many accessions of *Arabidopsis* are winter annuals (Figure 1). Napp-Zinn [6] first showed that, in certain winter annual to summer annual crosses, the winter-annual habit in *Arabidopsis* is conferred by a single dominant gene, which he named *FRIGIDA* (*FRI*). Subsequent studies by several groups have shown that *FRI* confers the winter-annual habit in many accessions [7,8]. Studies of natural variation have also shown that a dominant allele of another gene, *FLOWERING LOCUS C* (*FLC*), is necessary for *FRI* to confer a winter-annual habit [9,10].

The cloning of *FLC* [11,12] provided the first insight into the molecular nature of vernalization in *Arabidopsis*. *FLC*
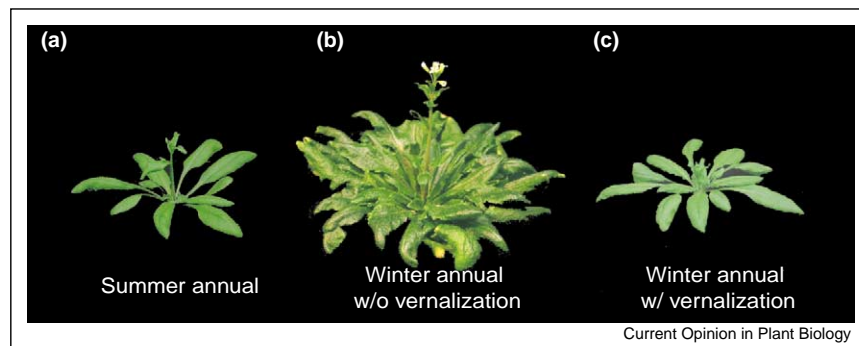
is a repressor of flowering, and the presence of a dominant allele of *FRI* elevates *FLC* expression to a level that inhibits flowering [11,12]. Vernalization overcomes the effect of *FRI* by repressing *FLC* expression, and this repression is stably maintained after a return to warm growth conditions [11,12]. Thus, the epigenetic repression of *FLC* is a key feature of vernalization. *FLC* is expressed predominantly in mitotically active regions [13], such as the shoot and root apical meristems, which are the sites of cold perception and the tissues that achieve the vernalized state. It should be noted that although most of the promotion of flowering by vernalization in *Arabidopsis* is due to *FLC* repression, there is clearly a component of flowering promotion that is *FLC* independent [14]. The targets of the *FLC*-independent component of vernalization are not known.

The cloning of *FRI* [15] demonstrated that the recessive alleles of *fri* that are found in summer annuals are often loss-of-function mutations. Therefore, the summer-annual types of *Arabidopsis* have been derived from winter annuals by the loss of *FRI*. Lesions in *FRI* have arisen independently several times [16•]; presumably, these *fri* mutations result in an adaptation to a particular niche. Recently, it has also been shown that certain summer-annual types contain an active *FRI* allele but also contain an allele of *FLC* that is not upregulated by *FRI* [16•,17•]. Thus, there are at least two routes by which winter-annual types of *Arabidopsis* have become summer annuals.
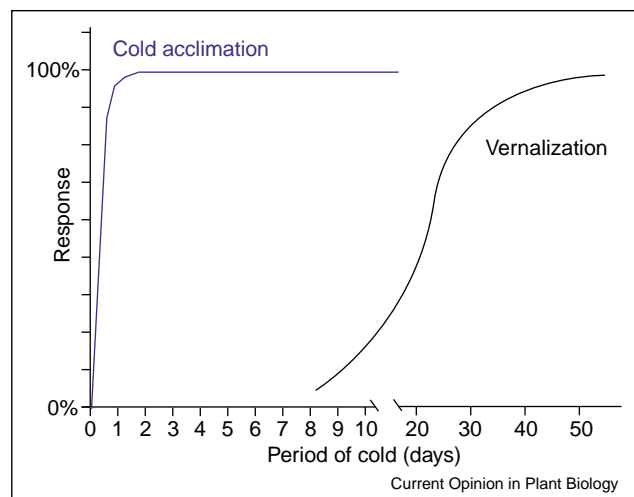
Regulation of gene expression by cold

The requirement of winter annuals and biennials for vernalization means that they will be exposed to freezing temperatures during winter and must be freezing tolerant. The process of preparing to withstand cold is known as cold acclimation [18]. Both cold acclimation and vernalization must occur in cold but non-freezing temperatures because metabolic activity is required for these processes;

Flowering behavior of summer- and winter-annual types of *Arabidopsis thaliana*. **(a)** Summer-annual types of *Arabidopsis* flower rapidly without vernalization treatment. In contrast, winter-annual types, which have functional *FRI* and *FLC* alleles, **(b)** flower very late without vernalization and **(c)** flower rapidly, like a summer annual, if vernalized.

Figure 2

Typical time course of the cold acclimation and vernalization responses in *Arabidopsis thaliana*. The acquisition of cold tolerance occurs within days whereas vernalization requires several weeks of cold exposure.

freezing temperatures would suspend metabolic activity. In contrast to vernalization, cold acclimation can be achieved within a relatively short time period ([19,20]; Figure 2). The rapid establishment of cold acclimation is advantageous because plants need to be rapidly protected from freezing, even if the cold spell is only temporary (as is often the case in late autumn). On the other hand, the requirement for a longer period of cold for vernalization ensures that plants only respond to a complete winter and not to temperature fluctuations during the autumn. The premature induction of flowering in late autumn just before the onset of winter would be disastrous; thus, in temperate climates, alleles of genes in the vernalization pathway that favor a requirement for a long cold exposure are obviously selected for.

In cold acclimation, a suite of genes are induced by cold exposure; for example, C-repeat-/DRE-Binding Factor1 (CBF1), a transcription factor that activates many cold-regulated genes (*COR*) that are responsible for freezing tolerance, appears within several hours of the start of cold treatment [19]. In contrast, the vernalization-mediated repression of *FLC* requires 30–40 days of continuous cold for a maximal response [20]. The different induction kinetics of the cold acclimation and the vernalization responses indicate that different mechanisms are involved. The overexpression of *CBF1* induces *COR* genes but does not affect *FLC* expression, providing additional evidence that the regulation of genes during cold acclimation and vernalization involves distinct mechanisms [21]. Furthermore, none of the mutations that affect the vernalization pathway compromise cold acclimation [21]. However, a lesion in *HIGH EXPRESSION OF*

OSMOTICALLY RESPONSIVE GENES1 (*HOS1*) causes elevated *CBF* expression as well as early flowering and reduced *FLC* expression, suggesting that some early components of cold signaling might be shared, at least in part, by the cold acclimation and vernalization pathways [22]. The *hos1* phenotype indicates that *HOS1* is a negative regulator of cold signaling [22]. *HOS1* encodes a RING-finger protein, and such proteins are usually associated with the ubiquitin protein-degradation pathway. It is not yet known how protein degradation is related to early events in cold signaling.

Mechanism of vernalization
It has been proposed that the pathways that lead to cold acclimation involve sensing changes in membrane fluidity, Ca^{2+} fluxes and cascades of phosphorylation [18,23]. Applications of chemicals that affect membrane fluidity, Ca^{2+} fluxes or protein phosphorylation are often sufficient to repress or induce some cold-acclimation genes [23,24]. None of these treatments has been shown to have an effect on vernalization, but such experiments are technically difficult because applying such treatments for long periods of time would be stressful to the plants. Nevertheless, we would not expect that sensing changes in membrane fluidity forms part of the long-term cold sensing in vernalization because plants rapidly adjust their membrane composition after exposure to cold so as to maintain the proper fluidity for cellular activity. Furthermore, a major difference between cold acclimation and vernalization is that vernalization results in a stable epigenetic switch (i.e. a memory of the past winter's cold), whereas cold acclimation does not remain stable for long periods upon return to warm conditions.
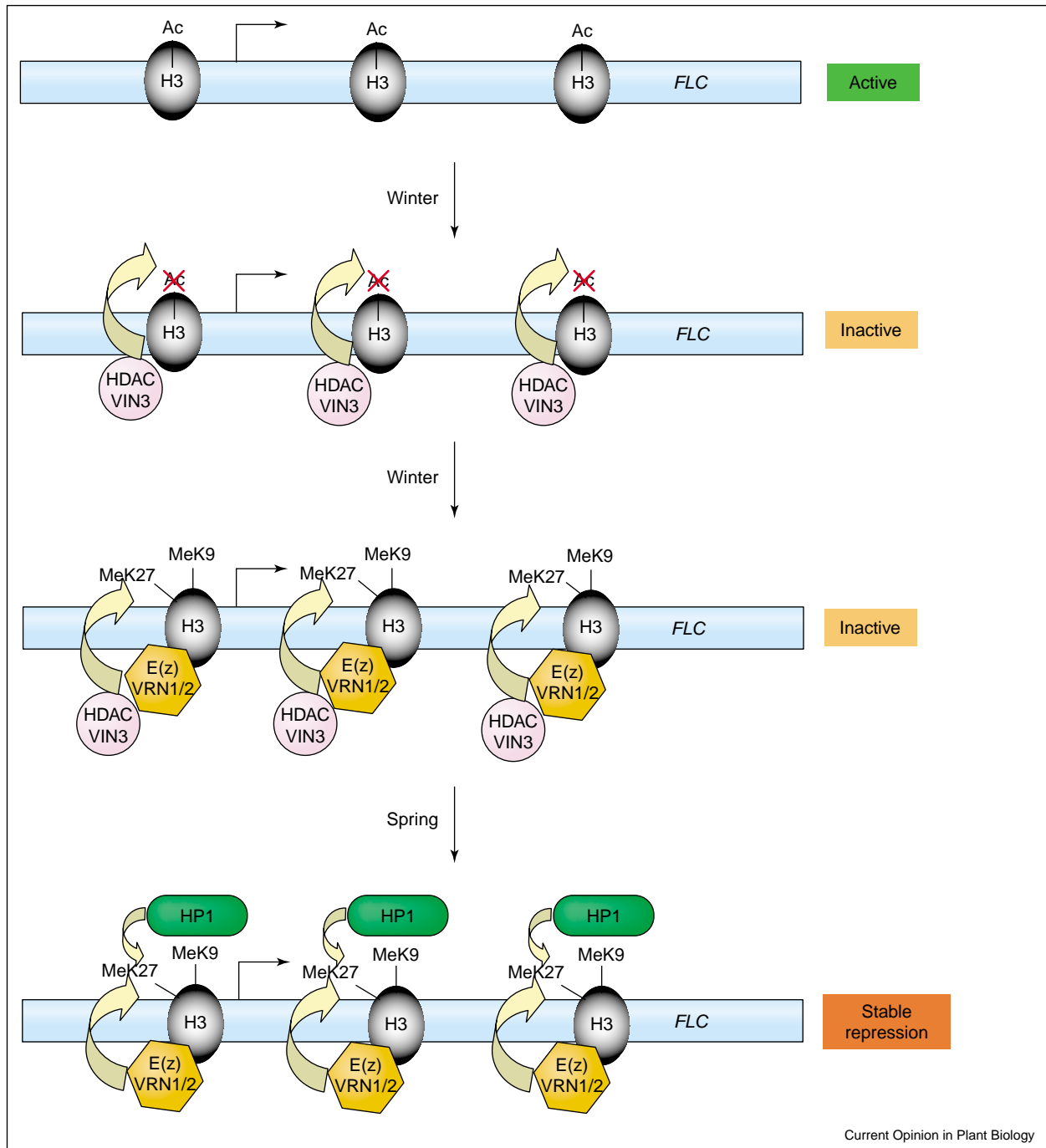
Without a nervous system and brain to provide memory, plants must rely on a cellular memory to remember seasonal change. Cellular memory has a crucial role in development and differentiation in many organisms. Tissue-specific and developmental-stage-specific gene expression is often achieved through histone modifications, the so-called histone code [25•]. Localized heterochromatin formation caused by a series of histone modifications often accounts for the epigenetic regulation of genes in many situations [26,27]. Recent results indicate that the cellular memory of vernalization results from an altered *FLC* chromatin structure.

Screens for mutants that remain late flowering after a long cold treatment have been used to identify genes in the vernalization pathway. Two *Arabidopsis* genes, *VERNALIZATION1* (*VRN1*) and *VRN2*, have been identified this way. The study of *vrn1* and *vrn2* mutants has revealed an interesting feature of the vernalization mechanism [28,29••]. *FLC* is repressed during vernalization in the same way in these mutants and in wildtype *Arabidopsis* plants. However, the repressed state of *FLC* is not stably maintained in *vrn1* and *vrn2* mutants upon return to warm

conditions. Thus, *VRN1* and *VRN2* are responsible for the stable maintenance of the vernalized state but not for its initial establishment. Lesions in *VRN2* also affect the chromatin structure of *FLC* [28], suggesting that the remodeling of *FLC* chromatin is part of the vernalization process in *Arabidopsis*. The expression of *VRN1* or *VRN2* is

Figure 3

Hypothetical model of the vernalization-mediated, epigenetic silencing of *FLC*. During winter, cold-induced expression of *VIN3* is necessary for a histone deacetylase (HDAC) complex to de-acetylate H3 in *FLC* chromatin. De-acetylation in turn creates an environment in which a VRN1-/VRN2-containing complex can methylate H3 at Lys9 as well as at Lys27. By analogy with mammalian and *Drosophila* complexes, the histone-methylating activity of the VRN1/VRN2 complex may be provided by an ENHANCER OF ZESTE [E(z)] homolog [33]. In the spring, *VIN3* is no longer expressed and the maintenance of *FLC* repression requires the continued presence of VRN1 and VRN2, and perhaps other proteins such as HP1. HP1 binds to dimethylated H3 Lys9 [32**,34**] and is thought to be involved in the silencing of genes in plant euchromatin [35*].

not regulated by vernalization, and both of these genes are expressed more broadly than is *FLC*. This raises the question of how these rather ubiquitously and constitutively expressed genes repress *FLC* only after a vernalizing cold treatment.

The identification of *VERNALIZATION INSENSITIVE3* (*VIN3*) provides an answer to this question [30••]. In *vin3* mutants, the repression of *FLC* under extended cold conditions never occurs, indicating that *VIN3* is responsible for the initial repression of *FLC* during cold exposure. Furthermore, the expression of *VIN3* is only induced by a long period of cold, and as *VIN3* is induced, *FLC* is repressed (Figure 3a). The induction of *VIN3* by cold is transient; *VIN3* mRNA becomes undetectable upon return to warm conditions. The induction of *VIN3* occurs predominantly in the shoot and root apical meristems, the sites of cold perception and *FLC* repression during vernalization. This behavior is consistent with a role for *VIN3* as a vernalization-specific regulator.

VIN3 encodes a PLANT HOMEODOMAIN (PHD)-finger-containing protein. PHD-finger motifs are thought to be involved in protein–protein interactions and are often found in various components of chromatin-remodeling complexes [31]. *VRN1* encodes a Myb-related DNA-binding protein, whereas *VRN2* encodes a polycomb group protein that is similar to the *Drosophila* SUPPRESSOR OF ZESTE-12 (Su[z]12). In mammalian systems, the Su(z)12 homolog is a component of PRC2 (polycomb repressor complex2), which has histone methyltransferase activity [32••]. PRC2 also contains Enhancer of Zeste [E(z)] and there are at least three homologs of E(z) in *Arabidopsis* [33]. This class of polycomb group genes causes stable gene repression by promoting a series of histone modifications [27]. Thus, it is possible that *VRN1*, *VRN2* and *VIN3* participate in *FLC* chromatin remodeling. Indeed, chromatin immunoprecipitation (ChIP) assays using the *vin3*, *vrn2* and *vrn1* mutants revealed that vernalization results in a series of *FLC* chromatin modifications ([30••]; Figure 3b).

During vernalization, the acetylation levels of specific regions of *FLC* chromatin decrease, and this is followed by an increase in methylation of Histone H3 at Lys9 and Lys27. The evidence for this temporal order of changes comes from studies of the mutants. In *vin3*, none of the vernalization-mediated histone modifications are observed, suggesting that during vernalization, *VIN3* is an establishing factor for these chromatin modifications. In *vrn2* and *vrn1* mutants, hypoacetylation (and *FLC* repression) is observed during vernalization, but the hypoacetylation and *FLC* repression are not maintained upon return to a warm temperature. Furthermore, none of the histone methylations are observed in *vrn2* mutants, and only methylation on Histone H3 at Lys27 is observed in *vrn1*. These results suggest a model in which *VIN3* is

involved in the initial repression of *FLC* through hypoacetylation. The hypoacetylated state of *FLC* chromatin creates a favorable condition for subsequent histone modifications that involve *VRN1* and *VRN2*. In animals, methylation of Histone H3 at Lys9 is thought to promote stable heterochromatin formation by recruiting HETEROCHROMATIN PROTEIN1 (HP1) [32••,34••]. The involvement of HP1 in repression in plant euchromatic gene also has been recently reported [35•]. Thus, vernalization triggers a series of histone modifications, ultimately resulting in a mitotically stable repressive heterochromatin state that serves as a mechanism for remembering winter.

Conclusions and perspectives
Much of the current knowledge on the mechanism of vernalization has come from studies of the model plant *Arabidopsis*. Such studies first demonstrated that vernalization promoted flowering through the epigenetic repression of the flowering repressor *FLC*. Subsequent studies have revealed that the mechanism of *FLC* repression involves a series of modifications of *FLC* chromatin that ultimately result in a stable repressed state. The extent, if any, to which vernalization mechanisms are conserved among plant species remains to be determined. Recent work in a vernalization-requiring type of wheat indicates that the genetically identified targets of the vernalization pathway in wheat are not related to *FLC* [36•], but the basic mechanisms that sense prolonged cold could be conserved.

Now that we have the framework to explain how prolonged cold represses *FLC*, the most 'upstream' question that can be addressed is how does prolonged cold induces the expression of *VIN3*? It will also be interesting to explore the nature of the VIN3- and VRN1-/VRN2-containing protein complexes to address the details of the biochemical mechanism of this cellular memory in plants.

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wheat, which confers a requirement for vernalization. (The wheat *VRN1*
has no molecular relatedness to the *Arabidopsis VRN1* or to *FRI* and *FLC*.)
Natural variation exists in both *VRN1* in wheat and *FRI* and *FLC* in
Arabidopsis, and this variation accounts for the difference between
vernalization-requiring and summer-annual types. Thus different types
of genes account for natural variation in the vernalization-requirement in
wheat and *Arabidopsis*.