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HISTORICAL PERSPECTIVE ESSAY

Vernalization, Competence, and the Epigenetic Memory of Winter

Vernalization is the process by which prolonged exposure to cold temperatures promotes flowering. Over the past century, this process has been studied extensively at the physiological level. Recent studies have provided some insight into the molecular basis of vernalization. The rich history of vernalization research has been discussed in detail in many reviews (Chouard, 1960; Lang, 1965; Bernier et al., 1981). I will briefly summarize some highlights and classic experiments that I would like to relate to recent molecular advances.

HISTORY OF VERNALIZATION RESEARCH

The first papers describing exposure to cold as the specific climatic aspect of winter that was necessary for flowering in some species were published in the latter half of the 19th century. However, the work of Gassner (1918) is usually cited as the first report that a wide range of plant species require cold exposure to flower (Chouard, 1960; Lang, 1965).

There are several ways to classify the vernalization responsiveness of plants. One is whether a requirement for exposure to the prolonged cold of winter to flower influences the plant's life history. Monocarpic species senesce after flowering and setting seed. Monocarpic plants that require vernalization to flower thus typically require two seasons to complete the life cycle and are usually classified as biennials or winter annuals. The term biennial is often used for plants that have an obligate requirement for cold exposure to flower, and the term winter annual is often used for plants with a quantitative cold requirement (Lang, 1965; Figure 1A). Monocarpic species that flower in one growing season without a vernalizing cold treatment are often called summer annuals. Many polycarpic species (i.e., perennials) also require a vernalizing cold treatment to enable flowering.

The distinction between summer annuals and winter annuals or biennials is not always absolute. It is possible that genetically identical plants could behave as summer annuals in one location and as winter annuals in a different location with a different climate. Furthermore, these classifications do not imply fundamental differences in the mechanisms that control flowering. In Arabidopsis, for example, single-gene changes can convert plants without a vernalization requirement into plants that have either a quantitative or obligate requirement or vice versa; therefore, the relevant molecular differences between plants in various categories can be minor.

Many winter annuals and biennials become established in the fall, taking advantage of the cool and moist conditions

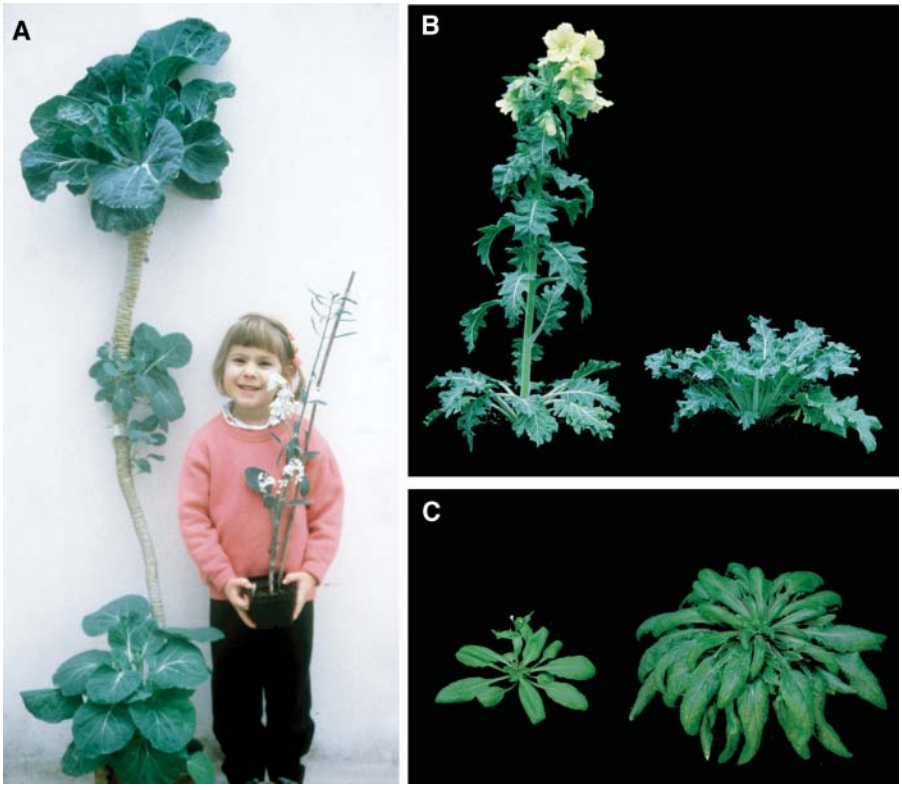

Figure 1. Examples of Plants Requiring Vernalization.

(A) A biennial cabbage (*Brassica oleracea*) variety with an obligate vernalization requirement that had been growing for five years without cold exposure. The small plant in my daughter's hands is a summer-annual variety of *B. oleracea* that flowers rapidly without vernalization.

(B) and **(C)** Summer annual and vernalization-requiring types of henbane **(B)** and Arabidopsis **(C)**. In both examples, a single-dominant gene is responsible for the vernalization-requiring habit. All plants were grown in long days (inductive photoperiods) without vernalization. The rapid-flowering summer annuals (which have initiated flowering) are at left and the winter-annual types at right. (*Henbane images courtesy of Jan Zeevaart*.)

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optimal for their growth. The vernalization requirement of such plants prevents flowering until spring has actually arrived. Weather is often variable, so for a vernalization requirement to work as intended, plants must not only sense cold exposure but also have a mechanism to measure the duration of cold exposure. For example, if a plant is exposed to a short period of cold in the fall season, followed by a return of warm temperatures later that fall or in early winter, it is important for the plant not to perceive the brief exposure to cold and the following warm weather as spring. One mechanism to determine that spring has in fact arrived is to measure the duration of cold and to permit flowering only after a period of cold that is sufficient to ensure that winter has passed. Sensing the increasing daylengths in the spring can also play a role. In many perennial species, the release of buds from dormancy only after perception of a sufficient duration of cold exposure is, like vernalization, designed to measure the duration of a winter season. Processes that require prolonged exposure to cold, such as vernalization and the cold-induced release of bud dormancy, stand in contrast with cold acclimation—a process designed to respond to cold as rapidly as possible (Thomashow, 2001).

Within a given species, there can be variation in the extent to which vernalization affects flowering time. In some species there are varieties that require vernalization and others that do not, such as winter and spring varieties of cereals (e.g., winter wheat and spring wheat). In fact, the term vernalization comes from studies of flowering in cereals. The infamous Russian geneticist Trofim Lysenko, who studied the effect of cold on flowering, coined the term jarovization to describe what we now call vernalization. Spring cereals are called jarovoe in Russian (derived from Jar, the god of spring), and cold exposure causes a winter cereal to behave like a jarovoe (i.e., to flower rapidly). Jarovization was translated from Russian into vernalization; vernal is derived from the Latin word for spring, vernum (Chouard, 1960).

A useful definition of vernalization is provided in Chouard's review (1960, p. 193): ''the acquisition or acceleration of the ability to flower by a chilling treatment.'' Two types of experiments demonstrate that this acquisition or acceleration is occurring at the shoot apex. One is to locally chill only certain parts of the plant. Another is to graft shoot tips: In most species, if a vernalized shoot tip is grafted to nonvernalized stock, it will flower, but a nonvernalized shoot tip grafted to a vernalized stock will not flower.

As noted in the above definition, cold exposure does not necessarily cause flowering but rather renders the plant competent to do so. A classic demonstration of this comes from the work of Lang and Melchers (reviewed in Lang, 1965) using biennial *Hyoscyamus niger* (henbane). Biennial henbane requires vernalization followed by inductive photoperiods to flower. If vernalized henbane plants are grown in noninductive photoperiods, they continue to grow vegetatively. However, if such plants are later shifted to inductive photoperiods, they flower. This shows that the vernalized plants are able to remember their prior vernalization; that is, they had acquired competence to flower but did not actually do so until the photoperiod requirement was met. Thus, vernalization establishes a cellular memory that is stable through mitotic cell divisions. The length of this memory of winter varies among plant species; in some species it is much shorter than in henbane.

I think it is reasonable to refer to the vernalization-induced, mitotically stable acquisition of the competence to flower as an epigenetic switch because it is a change that can be propagated through cell divisions in the absence of the inducing signal. However, there is disagreement over the proper use of the term epigenetic (Wu and Morris, 2001), and some might argue that the term epigenetic should be used only for changes that persist from one generation to the next. This of course does not happen in the case of vernalization; if it did, a biennial would only be a biennial for one generation. One of Lysenko's false claims was that the vernalized state was heritable; that is, a vernalized plant would transmit the rapid-flowering trait to the next generation. This fit the Marxist ideology that the environment of the members of a Marxist society could produce heritable changes in attitude, and, thus, if the proper environment was provided, future generations would consist of improved citizens. Lysenko's efforts to obtain or fabricate results that supported a political ideology and, with the assistance of Stalin's regime, to force others to accept his views had disastrous consequences for Russian genetics (Caspari and Marshak, 1965). It is regrettable that the misuse of science to justify policies based upon ideology is still occurring (for recent examples, see Union of Concerned Scientists, 2004).

GENETIC STUDIES

As noted above, there are species in which there are both summer-annual and vernalization-requiring (biennial or winter-annual) types. In such species, the number of genes responsible for the biennial/winter-annual versus summer-annual habit has been studied. The first study of this type was the demonstration by Correns in 1904 that the biennial habit in henbane (Figure 1B) was conferred by a single dominant gene (discussed in Lang, 1986). Other examples include beet, in which the biennial habit is conferred by a single recessive locus (Abegg, 1936), and diploid wheat and certain other cereals, in which two loci, one dominant and one recessive, distinguish the winter versus spring types (Tranquilli and Dubcovsky, 2000).

Fortunately, *Arabidopsis thaliana* is another species in which there are both rapid-flowering accessions that do not require vernalization and vernalization-requiring accessions that behave as winter annuals (Figure 1C). The rapid-flowering accessions are popular for lab use because they flower and complete their life cycle quickly and, thus, genetic studies progress rapidly. Klaus Napp-Zinn first showed that in crosses of certain winter-annual to rapid-flowering Arabidopsis types, the vernalization-responsive, delayed flowering of the winter annuals is due, in large part, to a dominant gene that he named *FRIGIDA* (*FRI*), although other loci could contribute (reviewed in Napp-Zinn, 1987). Most rapid-flowering accessions are homozygous for

a recessive *fri* allele that confers early flowering. Subsequent studies demonstrated that *FRI* is required for the winter-annual habit in many additional accessions (Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994; Koornneef et al., 1994). In addition, a dominant allele of another gene, *FLOWERING LOCUS C* (*FLC*), is necessary for *FRI* to confer a winter-annual habit (Koornneef et al., 1994; Lee et al., 1994). Thus, in Arabidopsis, *FRI* and *FLC* act together to block the ability of a nonvernalized shoot apex to flower; that is, a requirement for vernalization results from the synergistic interaction of dominant alleles of *FRI* and *FLC*.

WHAT WE HAVE LEARNED FROM GENE IDENTIFICATION

The molecular characterization of *FLC* provided a clue as to how vernalization affects competence to flower in Arabidopsis (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* encodes a MADS domain protein that acts as a potent repressor of flowering. Expression of *FLC* alone (i.e., without *FRI*) from a heterologous promoter is sufficient to block flowering. The role of *FRI* is to elevate the expression of *FLC* to levels that block flowering. Vernalization promotes flowering by repressing *FLC* expression.

The repression of *FLC* by vernalization does not occur via *FRI* regulation; rather, vernalization overrides the effect of *FRI* by repressing *FLC* via a pathway acting in parallel to the activation of *FLC* by *FRI*. An additional pathway that negatively regulates *FLC* is the autonomous floral promotion pathway. Autonomous-pathway mutants in a *fri* null mutant background (i.e., in a summer-annual parental background) behave as winter annuals because mutations in autonomous-pathway genes cause elevated *FLC* expression similar to dominant alleles of *FRI* (Michaels and Amasino, 2001). Vernalization effectively promotes flowering by repressing *FLC* in a *fri* autonomous-pathway double mutant, indicating that the vernalization-mediated repression of *FLC* also occurs independently of the autonomous pathway. An outline of the pathways that regulate flowering in Arabidopsis is presented in Figure 2A.

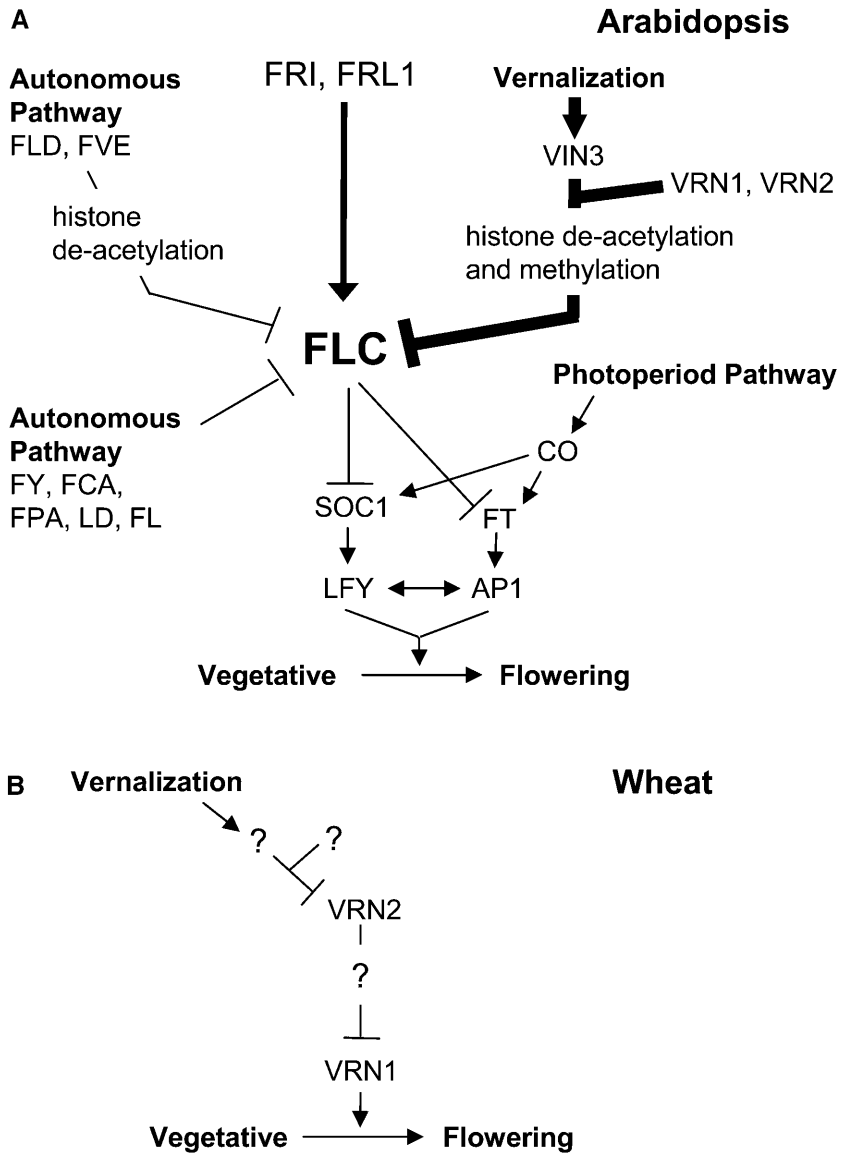

Figure 2. Flowering Pathways.

(A) Diagram of flowering pathways in Arabidopsis. This is a simplified model that does not contain all of the genes involved in flowering time control in Arabidopsis. The thickness of the lines indicates the hierarchy of *FLC* regulation: *FRI* overrides the repressive effect of the autonomous pathway and vernalization overrides the effect of *FRI*.

(B) A model of recent advances in the control of flowering by vernalization in wheat. As discussed in the text, wheat VRN1 and VRN2 are not homologous to Arabidopsis. Wheat VRN1 is a MADS domain protein that is most similar to AP1 in Arabidopsis; wheat VRN2 has no known homolog in Arabidopsis.

The vernalization-mediated repression of *FLC* is epigenetic in the sense discussed above: The repressed state of *FLC* is maintained after vernalized plants are returned to warm growing conditions. Thus, in Arabidopsis, vernalization provides competence to flower by repressing the expression of a flowering repressor. As expected,

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FLC expression is on again in the next generation. This resetting of the epigenetic switch during passage to the next generation is reminiscent of genomic imprinting in animals (e.g., de la Casa-Esperon and Sapienza, 2003). But the unique aspect of this switch is that the on-to-off direction of the switch is set by perception of the environment, whereas the off-to-on direction is set by passage to the next generation.

Recent work has provided an outline of the mechanism by which vernalization represses *FLC*. Screens for Arabidopsis mutants that can no longer respond to vernalization have revealed three genes involved in this process: *VERNALIZATION1* (*VRN1*; Levy et al., 2002), *VERNALIZATION2* (*VRN2*; Gendall et al., 2001), and *VERNALIZATION INSENSITIVE3* (*VIN3*; Sung and Amasino, 2004). VRN1 belongs to a class of plant-specific DNA binding proteins, VRN2 is a relative of the polycomb-group protein SUPPRESSOR OF ZESTE-12, and VIN3 contains a PHD domain. In animals and yeast, proteins related to *VRN2* and *VIN3* are involved in chromatin-remodeling complexes. Such complexes often catalyze the covalent modification of specific histone residues. The spectrum of histone modifications and their effects on gene expression are referred to as the histone code (Jenuwein and Allis, 2001; Iizuka and Smith, 2003; Lachner et al., 2003). Examination of *FLC* chromatin has revealed vernalization-mediated changes. During and after vernalization, the levels of certain modifications associated with active genes are reduced, such as acetylation of histone 3 (H3) at Lys 9 and 14 (K9 and K14; Sung and Amasino, 2004). By contrast, the level of two other modifications, methylation of H3K9 and H3K27, are increased by vernalization (Bastow et al., 2004; Sung and Amasino, 2004). Elevated H3K9 and H3K27 methylation is typically associated with the formation of stable heterochromatin. Thus, the vernalization-mediated formation of heterochromatin at *FLC* appears to account, at least in part, for the epigenetic nature of the vernalized state. It is important to note that deciphering the histone code in eukaryotes and identifying the vernalization-mediated changes in *FLC* chromatin are works in progress; there are many potential histone modifications for which *FLC* chromatin has not been evaluated, and new modifications of eukaryotic chromatin and their effect on gene expression continue to be discovered.

As discussed above, deacetylation is one modification of *FLC* chromatin that occurs during vernalization. Two components of the autonomous pathway, FVE and FLOWERING LOCUS D (FLD), are also involved in deacetylation of *FLC* chromatin (He et al., 2003; Ausin et al., 2004). *fld* and *fve* mutants exhibit a normal vernalization response, and, thus, FVE/FLD-mediated deacetylation is not required for vernalization.

Some types of Arabidopsis are rapid flowering despite the presence of an active *FRI* allele because their allele of *FLC* is not upregulated by *FRI* (Gazzani et al., 2003; Michaels et al., 2003). Such *FRI*-resistant *FLC* alleles can result from the insertion of a transposable element in an *FLC* intron (Michaels et al., 2003). The mechanism by which insertions of transposable elements reduce expression of two different *FLC* alleles appears to be via an alteration of chromatin structure mediated by silencing RNAs directed against the transposable element (J. Liu, Y. He, R. Amasino, and X. Chen, unpublished data). Specifically, the transposable element creates an island of a chromatin modification (H3K9 methylation) characteristic of vernalization-induced heterochromatin at *FLC*. This is an example of the prescient idea of Barbara McClintock that insertion of a transposable element can result in ''the transposition of heterochromatin'' (McClintock, 1950, p. 354). Thus, transposable element insertions may create novel *FLC* alleles that can convert a winter annual into a rapid-flowering type, and the presence of these alleles in natural populations indicates that the resulting change in flowering behavior may have had adaptive value.

The cloning of *FRI* revealed that the recessive alleles found in many rapid-flowering types of Arabidopsis are loss-of-function mutations. Therefore, many of the widely used rapid-flowering types, such as Columbia, have been derived from ancestral winter-annual types (Johanson et al., 2000; Gazzani et al., 2003). There are relatives of *FRI* in Arabidopsis, and at least one of them, *FRI-LIKE1* (*FRL1*), is required for *FRI* to upregulate *FLC* (Michaels et al., 2004). Genetic evidence is consistent with FRI and FRL1 acting in a complex (Michaels et al., 2004).

How *FRI* and *FRL1* elevate *FLC* mRNA levels is not known, and the sequence does not provide any clue—*FRI* and *FRL1* encode plant-specific proteins (Johanson et al., 2000; Michaels et al., 2004). The presence of *FRI* leads to an increased level of another chromatin modification at *FLC*, H3K4 trimethylation (Y. He and R. Amasino, unpublished data). However, increased H3K4 trimethylation at *FLC* is also found in autonomous-pathway mutants in a *fri* null background. Thus, *FRI* activity is not directly required for this *FLC* chromatin modification, although it is possible that *FRI* prevents an autonomous-pathway component from preventing H3K4 trimethylation of *FLC* chromatin. Unlike the repressive K9 and K27 methylations associated with vernalization, H3K4 trimethylation is associated with gene activation (Santos-Rosa et al., 2002). After vernalization, the increased H3K4 trimethylation of *FLC* in *FRI*-containing lines or in autonomous-pathway mutants is reduced, consistent with the vernalization-mediated repressed state of *FLC* overriding the ability of *FRI* or autonomous-pathway mutations to cause activation of *FLC* (Figure 2). One model to account for this hierarchy of regulation is that the heterochromatin-like state of *FLC* that results from vernalization blocks the access of activators involved in H3K4 trimethylation to *FLC*.

COMPARATIVE STUDIES

In wheat, two genes for which allelic variation accounts for the spring versus winter habit have recently been identified. These genes are called *VRN1* and *VRN2*, but there is no relationship to the Arabidopsis genes with the same name. Wheat *VRN1* encodes a MADS domain protein that promotes flowering (Yan et al., 2003). In many winter varieties of wheat, *VRN1* is induced by cold exposure (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al.,

2003). *VRN2* is a repressor of *VRN1*, and *VRN2* expression is repressed by vernalization (Yan et al., 2004; Figure 2B). Wheat *VRN2* encodes a protein with a putative zinc-finger domain, and there are no homologs of *VRN2* in Arabidopsis or rice (Yan et al., 2004). Spring varieties have an allele of *VRN1* that is not repressed by *VRN2.* Therefore, cold exposure is not required for expression of the spring-type *VRN1* allele.

The closest relative of VRN1 in Arabidopsis is the MADS domain protein APETALA1 (AP1), a protein that promotes the formation of flowers (Mandel and Yanofsky, 1995). Thus, in both Arabidopsis and wheat, these relatives appear to play the same role—promoting flowering. However, unlike the situation in wheat, there are no examples in Arabidopsis of allelic variation at *AP1* causing a difference in the vernalization requirement.

Although they are not related at the amino acid level, wheat *VRN2* and Arabidopsis *FLC* play similar roles: Both repress genes involved in the promotion of flowering and both are repressed by vernalization (Figure 2). In winter wheat, *VRN2* represses *VRN1* (Figure 2B). In Arabidopsis, *FLC* represses the floral promoters *SOC1* and *FT*, which are two genes that are also positively regulated by the photoperiod pathway (Figure 2A). *SOC1* and *FT* activate *LEAFY* and *AP1*—genes that promote floral meristem identity. Thus, *FLC* indirectly represses floral meristem-identity genes. Whether wheat *VRN1*, which is similar to Arabidopsis *AP1*, is acting as a floral meristem-identity gene or as a more upstream regulator of flowering like *SOC1* is not known, nor is it known whether *VRN2* directly or indirectly represses *VRN1*.

Did the vernalization response evolve independently in the crucifers and cereals? As discussed above, evidence for this is that genes unrelated at the sequence level (*FLC* and *VRN2*) play similar roles as vernalization-repressed repressors in wheat and Arabidopsis and that allelic variation in an *AP1*-like gene (*VRN1*) plays a role in the vernalization requirement in wheat but not in Arabidopsis. Indeed, if major groups of flowering plants evolved in a warm climate in which a vernalization response was not needed, the vernalization response would have had to evolve independently as different groups of plants radiated into regions with a winter season. Homologs of *FLC* have not been found in wheat or other cereals, and the similar roles of Arabidopsis *FLC* and wheat *VRN2* may be an example of convergent evolution.

In comparative studies, it is important to acknowledge how little is known at a molecular level about the vernalization process in any species. In fact, if the vernalization pathway is strictly defined as the system that senses prolonged cold and transduces the prolonged cold signal to a downstream target, components of the vernalization pathway have not yet been found in any species. By this definition, only targets of the vernalization pathway are known. In Arabidopsis, the most upstream target of the vernalization pathway found to date is *VIN3*, and *FLC* is a downstream target of *VIN3* (Sung and Amasino, 2004; Figure 2A). In wheat, the most upstream target of the vernalization pathway found to date is *VRN2* (Yan et al., 2004; Figure 2A). Thus, Arabidopsis and wheat may have different upstream targets of the vernalization pathway, but it is possible that in cereals a *VIN3*-like target is upstream of *VRN2* just as *VIN3* is upstream of *FLC* in Arabidopsis. Furthermore, because we do not know anything about the system that senses prolonged cold and transduces the prolonged cold signal to a downstream target, we cannot address whether this system is conserved among flowering plants.

FUTURE PROSPECTS

One intriguing area is the mechanism by which plants measure the duration of cold during vernalization. For example, how can a plant distinguish a few days of cold (which typically has no effect on flowering) from several weeks of cold? One link to cold measurement is the unique expression pattern of *VIN3* in Arabidopsis (Sung and Amasino, 2004). *VIN3* is only expressed after several weeks of cold exposure. Furthermore, *VIN3* is only expressed in the cold; *VIN3* mRNA rapidly becomes undetectable after plants are returned to warm conditions (i.e., *VIN3* does not undergo a stable, epigenetic switch of gene expression like *FLC*).

Currently, we do not know any details of how the duration of cold is measured. What is the cold sensor? In cold-sensing neurons, the cold sensors are cold-responsive calcium channels that transduce the cold signal via altered calcium flux (Story et al., 2003). However, cold-responsive calcium channels might not be expected to be involved in measuring the duration of cold because cells typically readjust to ion fluxes (although a cold-responsive channel in plants might be involved in rapid cold responses such as cold acclimation). The classic models of vernalization postulate that during prolonged cold, the levels of some factor(s) slowly decline or increase until a threshold is reached that is then transduced into the acquisition of competence to flower. The duration of cold exposure that is required to reach this threshold determines how long the plant must experience winter to flower in the spring. In such a threshold model, the cold sensor could simply be an enzyme that is more or less active than a balancing enzymatic activity in the cold (i.e., enzymes with antagonistic activities that have different Q_{10} values). For example, a kinase that does not lose activity as the temperature is lowered as rapidly as does a phosphatase that acts on the same substrate would lead to the accumulation of a phosphorylated product in the cold. One challenge for the future is to understand the nature of the measurement of cold duration at a molecular level.

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