

Vernalization: the flower school

*“The crowds of flowers come out of a sudden,
from nobody knows where,
and dance upon the grass in wild glee.
Mother, I really think the flowers go to school underground.”*

– from Rabindranath Tagore’s *The Flower School*

1. The crowds of flowers come out of a sudden

The flowers of a given plant species generally appear suddenly and *en masse* at a specific time of year. Such synchrony promotes outbreeding, the ultimate purpose of sexual reproduction. Classical studies in plant physiology elucidated many of the environmental cues that signal plant species to flower at a specific time of season. By far, the most important is the change in “daylength” (or more accurately, “nightlength”) that occurs over the course of the year. Plants are able to measure changes in photoperiod by means of a unique plant photoreceptor called phytochrome. Many species, however, also take advantage of other environmental cues to induce flowering. Indeed, in the case of the model plant *Arabidopsis thaliana*, genetic and physiological analyses have revealed that there are at least four, partially overlapping pathways that promote flowering (Reeves and Coupland 2001): this essay, however, is concerned with just one – the vernalization-dependent pathway.

Vernalization refers to the acceleration of flowering that occurs in many plant species, especially those native to high latitudes, following the extended exposure of their imbibed seeds or young seedlings or buds to low temperature (e.g. 1 month at 4°C). Vernalization is not all-or-none, but a slow, quantitative process in which increasing periods of low temperature cause progressively earlier flowering until a saturation point is reached. In the case of *Arabidopsis*, there is tremendous natural variation between ecotypes in the extent to which their time to flowering is shortened by vernalization. For example, a standard vernalization treatment decreased the time to floral initiation from 186 days to 49 days in a naturally late-flowering *Arabidopsis* accession from North Carolina, USA, but only from 54 days to 52 days in an early-flowering accession from Köln, Germany (Nordborg and Bergelson 1999).

Although it might be expected that the vernalization responsiveness of various *Arabidopsis* ecotypes would increase with the latitude of their natural habitat, this is not the case. In all likelihood, this is because humans have, in the recent evolutionary past, unintentionally introduced *Arabidopsis* ecotypes to new and distant locales, thereby obfuscating its natural, global patterns of adaptation. A case in point is afforded by the ecotype “Kashmir”, the only Indian representative in the *Arabidopsis* Resource Centers of both Europe and North America. Molecular comparisons indicate that “Kashmir” is probably a descendant of a contaminating Scottish ecotype that hitchhiked to India in a shipment of grain within the last 150 years (Van der Zwan *et al* 2000). Regardless of how disturbed the natural pattern of vernalization responsiveness has become, the variations in vernalization between *Arabidopsis* ecotypes as well as vernalization mutants, have been enormously valuable in allowing biologists to address the fundamental question of how vernalization works.

2. From nobody knows where

The initiation of flowering remains one of the great, unsolved mysteries of plant physiology. In discussing this question, it is necessary to distinguish between a plant's perception of a floral induction signal and the actual developmental phase change of its vegetative shoot apical meristem into a floral meristem. In the case of photoperiod-dependent flowering, perception and response are spatially separated: It is the leaves that detect the change in daylength, but it is the shoot apical meristem that responds developmentally. Clearly, there is a floral stimulus that moves from the induced leaf to the apical meristem. This "florigen" signal is able to pass through a graft from an induced plant to a non-induced one. Despite decades of research, "florigen" remains unidentified.

In the case of vernalization, perception and response are separated temporally. Vernalization prepares the plant to flower, but does not itself evoke flowering, which commonly occurs only after an extended period of growth at warmer temperatures. The perception of and response to low temperature is localized in the meristematic cells of embryos, growing points and buds. The changes induced in meristems by vernalization are conserved through many generations of cell division even at temperatures much higher than those required for the cold-induction of flowering. Vernalization is required in each generation for winter annuals and biennials, and each year for perennials. Thus, meiosis or some aspect of reproductive growth resets the requirement for vernalization. One model for vernalization is that extended cold, perhaps by altering the sensitivity of the shoot apical meristem to flowering signals produced by the leaves, increases the competency of the shoot apical meristem to flower.

Unlike the vegetative shoot, the reproductive shoots (or flowers) are determinate – they stop growing upon reaching a certain size. In higher plants, a complex gene network regulates the developmental phase change called flowering. Some of the genes responsible for the formation of a floral meristem from a primordium have been identified, but a discussion of these floral meristem identity genes is beyond the scope of this essay (see review by Irish 1999). In short, the details of the link(s) between the floral induction pathways and the regulation of floral meristem identity genes are not well understood.

3. And dance upon the grass with wild glee

If flowers appear to "dance" it is because they are often borne terminally on tall, thin reproductive shoots. Presumably, the exaggerated height of the reproductive shoots makes them more noticeable to passing pollinators or, in the case of wind-pollinated plants, more exposed to the wind that carries their pollen aloft. Members of the Brassicaceae, or mustard family, which includes *Arabidopsis*, commonly exhibit some of the more impressive examples of reproductive shoot elongation in nature. *Arabidopsis* spends most of its vegetative life in an inconspicuous "rosette" form that is characterized by extremely short internodes. With the onset of floral initiation, however, the internodes begin to elongate in a dramatic fashion, a process called bolting. The plant growth hormone gibberellic acid (GA) is the major factor underlying this rapid elongation of the floral stalk in the Brassicaceae and other plants. Vernalization not only increases GA biosynthesis in the vegetative rosette, but also elevates the GA sensitivity of the shoot. As expected, cell wall-modifying enzymes and water transport proteins, both of which are important for cell elongation, are up-regulated during bolting (Hanzawa *et al* 1997; Oka *et al* 2001).

In some plant species, the application of GA can substitute for vernalization, but a similar role for GA in the vernalization of *Arabidopsis* seems unlikely. Although GA has been found to accelerate the time to flowering in non-vernalized *Arabidopsis* grown under short days (Wilson *et al* 1992), the promotion of flowering by GA does not mimic vernalization precisely. For example, the time to flowering in late-flowering *Arabidopsis* mutants is reduced similarly by GA regardless of whether they are vernalization-sensitive or not (Chandler and Dean 1994). Moreover, an *Arabidopsis* mutant (*ga 1-3*) that is severely defective in the synthesis of *ent*-kaurene (a precursor of GA) responds normally to vernalization (Michaels and Amasino 1999a). Thus, although GA may promote flowering generally, it does not appear to have a direct role in the vernalization response in *Arabidopsis*.

4. The flowers go to school underground

Plants that are vernalized as seeds or young seedlings do not flower immediately upon being raised to higher temperatures, but often weeks later. There is, therefore, a clear temporal separation between the perception of cold temperature and the switch from vegetative to reproductive growth. Somehow the shoot apical meristem manages to “remember” the lessons that it learned in its cold underground classroom, even after multiple cell divisions and many weeks passage in time. In some species this “memory” of vernalization can be maintained for up to 330 days (Lang 1965). What is the nature of these lessons? Recently, the door to this secret classroom has opened a crack and allowed researchers their first glimpses of how this process takes place in *Arabidopsis*.

In *Arabidopsis*, two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) play a central role in vernalization. In naturally occurring late-flowering ecotypes, the *FRI* gene acts to increase *FLC* levels, whereas vernalization acts to reduce *FLC* expression. (Michaels and Amasino 1999b; Sheldon *et al* 1999; Johanson *et al* 2000). Allelic variation at the *FRI* locus is a major determinant of natural variation in flowering time. Dominant alleles of *FRI* confer late flowering, which is reversed to earliness by vernalization. Johanson *et al* (2000) have recently cloned *FRI* and analysed the molecular basis of the allelic variation. Most early-flowering ecotypes carry *FRI* alleles that contain one of two different deletions that disrupt the open reading frame of the *FRI* gene. This suggests that loss-of-function mutations at *FRI* have provided the basis for the evolution of many early-flowering *Arabidopsis* ecotypes from ancestral late-flowering types.

FLC is a second gene that plays a major role in vernalization as well as in other floral inductive pathways. An early flowering phenotype results when *FLC*, which encodes for a novel MADS domain protein, is rendered dysfunctional by mutation (Michaels and Amasino 1999b). *FLC* acts as a strong floral repressor by negatively regulating the genes that promote the transition of the shoot apical meristem to a floral meristem. Vernalization promotes flowering by reducing *FLC* mRNA levels. The extent of the reduction is proportional to the duration of vernalization and is closely correlated with flowering time. Michaels and Amasino (2000) have proposed a “rheostat model” of flowering time, in which increasing levels of *FLC* are associated with the conversion of a species or ecotype from an annual growth habit to a biennial one.

The reduction in *FLC* transcript levels by vernalization is mitotically stable and occurs in all tissues. *FLC* activity is restored in each generation, as is the requirement for a low-temperature exposure for the acceleration of flowering. The level of *FLC* transcript determines the extent of the vernalization response in the promotion of flowering, and there is a quantitative relationship between the duration of cold treatment and the extent of down-regulation of *FLC* activity. A surprising discovery, therefore, was that the complete loss of *FLC* function does not eliminate the effect of vernalization. Thus, vernalization is able to promote flowering via *FLC*-dependent and *FLC*-independent mechanisms (Michaels and Amasino 2001).

Mutants have been isolated that reduce the vernalization responsiveness of late-flowering *Arabidopsis* mutants (Chandler *et al* 1996). Some of these vernalization mutants (*vrn*) are unable to reduce *FLC* mRNA in response to low temperature, suggesting that they encode regulators of *FLC* expression (Sheldon *et al* 2000). Gendall *et al* (2001) have recently shown that one of the genes (*VRN2*) encodes a nuclear-localized zinc finger protein that is a structural homologue of *Suppressor of zeste 12*, a Polycomb group (*PcG*) gene of enormous importance in the development of the fruit fly *Drosophila melanogaster*. In *Drosophila*, *PcG* proteins generally act by remodelling chromatin structure and mediating the silencing of homeotic genes. *VRN2* does not appear to be required for the vernalization-induced decrease in *FLC* mRNA, but is essential for the stable repression of *FLC* later in development (Gendall *et al* 2001).

The maintenance of vernalization through multiple cell divisions is reminiscent of epigenetic phenomena. Changes in epigenetic states are often correlated with developmentally imposed alterations in genomic DNA methylation and local chromatin structure (Meyer 2000; Habu *et al* 2001). There is some evidence that DNA methylation may play a role in preventing early flowering in *Arabidopsis* ecotypes (Burn *et al* 1993). Much like vernalized plants, *Arabidopsis* seedlings that have been treated with the demethylating compound 5-azacytidine flower more quickly. Late-flowering mutants that are insensitive to vernalization do not respond to 5-azacytidine treatments. Burn *et al* (1993)

found that *Arabidopsis* plants, either vernalized or 5-azacytidine-treated, had reduced levels of 5-methylcytosine in their DNA compared to non-vernalized plants. Moreover, normal flowering time was found to be reset in the progeny of plants induced to flower early with 5-azacytidine, paralleling the lack of inheritance of the vernalized condition. Based on these findings, Burn *et al* (1993) proposed that vernalization, through general demethylating effects, may release the block to flowering initiation, thereby allowing the plant to flower early. Cold-induced demethylation probably occurs during cell division by preventing the maintenance methylation that follows in the wake of DNA replication. A failure of methylation during cold exposure may lead to the demethylation of cytosines on the newly synthesized DNA strand. After a second round of replication, this would result in the double-stranded demethylation of DNA in one daughter cell.

Because 5-azacytidine may have unintended side effects, Finnegan *et al* (1998) approached the question of the role of demethylation in vernalization by a different approach. They found that *Arabidopsis* plants that had reduced levels of DNA methylation because of their transformation by a methyltransferase (*MET1*) antisense gene, flowered earlier than untransformed control plants, and without the need for a cold treatment. Moreover, the promotion of flowering was directly proportional to the decrease in methylation observed in the *MET1* antisense lines.

Not all of the results of Finnegan *et al* (1998), however, were consistent with the hypothesis that vernalization stems from a general demethylation of DNA. First, although growth at vernalizing temperatures was associated with some reduction of DNA methylation, this demethylation was transient and normal methylation levels were restored when the seedlings were transferred to warm temperatures. Second, unlike the case with vernalization, the early-flowering phenotype was inherited in sexual progeny, even when the antisense transgene was lost by segregation. Thus, the demethylation caused by a *MET1* antisense gene does not mimic all aspects of vernalization. Perhaps the use of methyltransferase-deficient mutants represents too coarse an approach for dissecting out the critical gene and, indeed it may be only one gene, whose demethylation is critical for vernalization to occur. Alternatively, there are emerging examples of epigenetic changes that do not involve alterations in DNA methylation (Amedeo *et al* 2000).

In summary, while vernalization continues to defy full explanation, plant scientists have, in this first decade of the *Arabidopsis* revolution, made enormous strides in identifying some of the key players in the vernalization process. No doubt more discoveries are in the offing.

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PETER V MINORSKY
*Department of Natural Sciences,
Mercy College,
Dobbs Ferry,
NY 10522, USA,
(Email, pminorsky@mercy.edu)*