The control of flowering by vernalization

Candice C Sheldon, E Jean Finnegan, Dean T Rouse, Million Tadege, David J Bagnall, Chris A Helliwell, W James Peacock and Elizabeth S Dennis*

The process by which vernalization, the exposure of a germinating seed or a juvenile plant to a prolonged period of low temperature, promotes flowering in the adult plant has remained a mystery for many years. The recent isolation of one of the key genes involved in vernalization, *FLOWERING LOCUS C,* has now provided an insight into the molecular mechanism involved, including the role of DNA methylation.

Addresses
Commonwealth Scientific and Industrial Research Organisation, Plant Industry, GPO Box 1600, Canberra, Australian Capital Territory 2601, Australia
*e-mail: e.dennis@pi.csiro.au

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Abbreviations

Col	Columbia
efs	*early flowering in short days*
FLC	*FLOWERING LOCUS C*
FRI	*FRIGIDA*
GA	gibberellin
L*er*	Landsberg *erecta*
LFY	*LEAFY*
METI	*METHYLTRANSFERASEI*
QTL	quantitative trait locus
VRN1	*VERNALIZATION1*

Introduction

The transition from vegetative growth to flowering is controlled by both environmental and developmental signals. *Arabidopsis thaliana* has been widely used as a model plant in the study of the molecular mechanisms that govern this process. Ecotypes of *Arabidopsis* are found over wide geographic and climatic ranges. Ecotypes from high latitudes or from alpine regions often take more than three months to flower when grown under controlled conditions, but flower much more rapidly (after approximately three weeks) when their germinating seeds have been exposed to a prolonged cold-treatment, a process known as vernalization. Ecotypes originating closer to the equator, and the commonly used laboratory ecotypes (e.g. Landsberg *erecta* [L*er*] and Columbia [Col]), flower rapidly without exposure to cold. The promotion of flowering by low temperature, combined with the onset of long days, ensures that flowering occurs in the spring, providing the maximal opportunity for seed set.

Genetic analyses of late- and early-flowering *Arabidopsis* ecotypes identified two major loci determining flowering time: *FRIGIDA* (*FRI*) on chromosome 4 and *FLOWERING LOCUS C* (*FLC*) on chromosome 5 [1–5]. Dominant alleles of these genes act synergistically to cause late-flowering; the late-flowering phenotype can be fully suppressed by vernalization. In *Brassica* species, vernalization-responsive flowering time loci segregate as two major quantitative trait loci (QTLs) that are collinear with the regions of the *Arabidopsis* genome in which *FRI* and *FLC* are located [6], suggesting that the same genes are important in both genera. Multiple genes that confer insensitivity to vernalization have been mapped in wheat and barley but it is not known whether they are homologues of *FRI* or *FLC* [7].

In this review we discuss progress made during the past two years in understanding the molecular basis of the promotion of flowering by vernalization.

Vernalization may be mediated through changes in DNA methylation

Vernalization has a number of unique features that can be accounted for by the hypothesis that it causes the activation, by demethylation, of gene(s) that are essential in the promotion of flowering [8]. The observation that prolonged growth at low temperatures results in reduced genomic DNA methylation is consistent with this hypothesis [8,9]. Genome-wide demethylation, induced either by treatment with 5-azacytidine or by a *METHYLTRANSFERASEI* (*METI*) antisense construct, promotes flowering in vernalization-responsive *Arabidopsis* ecotypes and mutants, but not in non-vernalization-responsive lines [8,9]. 5-azacytidine also largely replaces vernalization in winter wheat [10].

Unlike the day-length trigger to flowering, in which the leaf perceives the stimulus and produces a signal that is transmitted to the apex, exposure to low temperature is perceived by actively dividing cells in the apex itself [11,12]. Growth at low temperatures may disrupt maintenance methylation, the process by which patterns of DNA methylation are transmitted to newly synthesized DNA in dividing cells [13], perhaps through decreasing the activity of a cold-sensitive DNA methyltransferase. Methylation at sites in 'vernalization genes' would be diluted by successive cycles of DNA replication, accounting for the requirement for cell-division for the vernalization response, and the observed correlation between the duration of the cold treatment and the extent to which flowering is promoted [14,15,16••]. As vernalization often occurs at the germinating seed stage with flowering occurring weeks later, the vernalization signal must be transmitted through a number of cycles of cell division; it is not, however, transmitted to progeny [12,17]. It could be that changes in the methylation patterns of specific genes, which are established during growth in the cold, are maintained through mitotic cell divisions, but reset in progeny.

FLC is a key regulator of flowering time and the response to vernalization

The recent isolation of the *FLC* gene has provided a major advance in uncovering the molecular mechanisms involved in the control of flowering time and the response to vernalization [18••,19••]. The data show that *FLC* acts as a repressor of flowering and that the level of *FLC* expression correlates with the time to flowering. Late-flowering ecotypes and the over-expression mutant *flc-11* (previously called *flf-1*) have a high level of expression, whereas early-flowering ecotypes and the loss-of-function mutant *flc-13* (*flf-3*) have little or no activity [16••,18••]. The early-flowering ecotypes L*er* and C24 have been characterized genetically as possessing recessive (i.e. inactive) *FLC* alleles, whereas all other ecotypes have dominant (i.e. active) alleles [4,5]. The predicted translation products of the C24, L*er* and Col *FLC* alleles are, however, identical, suggesting that the observed differences in allelic activity are caused by differences in *FLC* gene regulation [16••], and not to changes in the gene product. Dominant *FRI* alleles function to upregulate *FLC* expression [18••,19••], accounting for the observed genetic interaction between *FRI* and *FLC*. The early-flowering L*er* and Col ecotypes have been hypothesized to contain inactive *FRI* alleles [20], which would not upregulate *FLC*; these ecotypes do have a low level of *FLC* transcript.

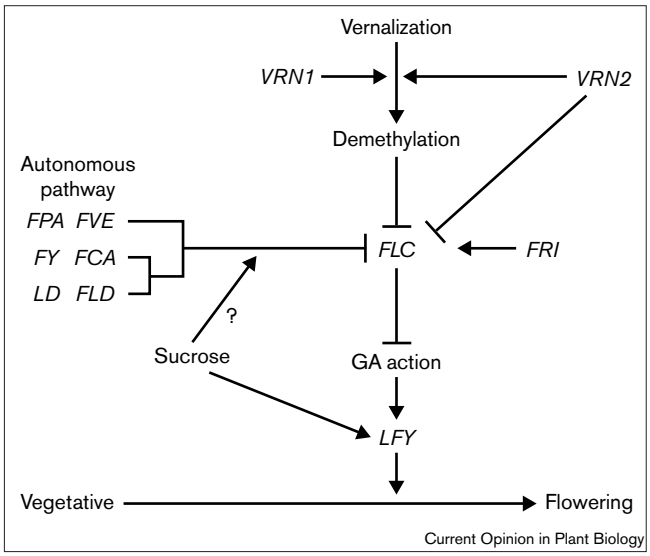
In addition to *FRI* and *FLC*, about 80 loci that influence flowering time have been identified by mutational approaches [21]. These mutants have been mapped to several flowering pathways [22,23] (Figure 1). *FRI* and *FLC* are both active in the autonomous pathway, along with *FCA*, *FVE*, *FPA*, *LUMINIDEPENDENS (LD)*, *FLOWERING LOCUS D (FLD)* and *FY*; the mutation of any of these genes causes a late-flowering phenotype. Mutants of these genes can be further divided into three subgroups (as shown in Figure 1) on the basis of their epistasis relationships [24]. Many of the genes in the autonomous pathway have been shown to regulate *FLC* expression [16••,18••,19••] (Figure 1), placing *FLC* as one of the later genes in the pathway.

Levels of both *FLC* mRNA and protein are downregulated by exposure of the germinating seed to prolonged periods at low temperatures [16••,18••,19••], showing that the *FLC* gene is integrally involved in mediating the response to vernalization. The extent of downregulation is proportional to the duration of the cold-treatment, as is the promotion of flowering [16••]. The downregulation of *FLC* activity persists throughout the development of the vernalized plant, the change in gene expression being transmitted through many mitotic divisions. The high level of *FLC* expression is reset in the progeny of a vernalized plant [16••].

Consistent with the idea that vernalization acts to alter expression of critical genes through changes in methylation status, plants containing a *MET1* antisense construct with only 15% of the wild-type level of genomic methylation are early-flowering [9] and have a reduced level of *FLC* transcript [18••]. Demethylation is generally associated with activation of gene expression rather than downregulation. Thus, the downregulation of *FLC* suggests that methylation may block expression of a repressor of *FLC*, or perhaps the binding of a repressor to the *FLC* promoter.

Far-red light promotes flowering in ecotypes and mutants that have a strong vernalization response [25]. However, plants grown under far-red-rich light show little alteration in *FLC* expression level compared with those grown in far-red-poor light (CC Sheldon, ES Dennis, unpublished data), suggesting that this effect is not mediated through *FLC*. *FLC* expression is also not affected by growth in different photoperiods [16••,19••], and the *flc-13* mutant maintains a response to photoperiod [16••]. Thus, *FLC* appears not to be essential for the photoperiod flowering pathway. However, *flc* loss-of-function mutants have a shortened circadian period, and a QTL for circadian period is closely linked to the *FLC* locus [26].

Figure 1



In *Arabidopsis*, an ability to respond to vernalization is established by an elevated level of *FLC* transcript [16••]. In the late-flowering ecotypes, this is caused by the upregulation of *FLC* by dominant alleles of *FRI* [18••,19••]. In the late-flowering mutants of the autonomous pathway, elevated *FLC* levels are caused by the loss-of-function of one of the genes that normally act to downregulate *FLC* expression. Vernalization causes a decrease in genomic methylation [9], and plants with a reduced level of DNA methylation have reduced expression of the *FLC* gene [18••]. It is not known whether *FLC* expression is directly regulated by demethylation or whether it is mediated through a regulator of *FLC*. The downregulation of *FLC* expression by vernalization is mediated through the effects of the *VRN1* and *VRN2* genes [16••]. *VRN2* also acts as a repressor of *FLC* in the unvernalized plant [18••]. *FLC* may act to regulate flowering time by blocking GA action at the apex, affecting either GA biosynthesis or signal transduction [18••]. It is likely that *LFY* acts downstream of the autonomous pathway and therefore downstream of *FLC*. Sucrose and GA both promote flowering and both synergistically upregulate expression from the *LFY* promoter [38]. Sucrose may also act in the autonomous pathway to downregulate *FLC* expression.

Genes that mediate the vernalization response

All mutants and ecotypes that have an elevated level of *FLC* are late-flowering and are able to respond to vernalization, both by a decrease in their time to flowering and by a decrease in the level of *FLC* transcript [16••]. This finding has two implications. First, it suggests that an elevated level of *FLC* is essential for the ability of a plant to respond to vernalization. This is supported by the lack of vernalization response of the *flc-13* null mutant [16••]. Second, it indicates that the late-flowering phenotype of these mutants is caused by an upregulated level of *FLC* transcript. This was confirmed by the shortening of the time to flowering of the late-flowering *fca-1* mutant by an *FLC* antisense construct [16••].

Other *Arabidopsis* genes that modify the vernalization response have been identified. The pleiotropic early-flowering mutant *efs* (*early flowering in short days*) encodes a repressor of flowering that acts in the autonomous pathway to flowering [27•]. Double mutants between *efs* and the vernalization-responsive late-flowering mutants *fca* and *fve* flower at about the same time as the *efs* mutant and show no promotion of flowering following a vernalization treatment. The loss of a vernalization response in these double mutants may simply indicate that *efs* mutants flower as early as is possible. Alternatively, like *FLC*, *EFS* may link the autonomous and vernalization-dependent pathways to flowering [27•]; perhaps *EFS* regulates *FLC* activity either transcriptionally or posttranscriptionally by interacting directly with FLC.

Mutants showing a reduced response to vernalization have been isolated in an *fca* mutant background; these have been placed in three complementation groups: *VERNALIZATION1* (*VRN1*), *VRN2* and *VRN3* [28]. Both *fca vrn1* and *fca vrn2* show a reduced response to vernalization, with a corresponding decrease in the downregulation of *FLC* in vernalized *fca vrn1* and *fca vrn2* plants [16••,28]. The double mutant *fca vrn2* flowered later than *fca* and had an increased level of *FLC* transcript compared with the *fca* mutant, suggesting that *VRN2* is a repressor of *FLC* [16••,18••]. In contrast, whereas *fca vrn1* flowered no later than *fca*, the *vrn1* mutation alone delayed flowering. This late-flowering phenotype was not associated with elevated *FLC* levels, indicating that *VRN1* may also promote flowering via another pathway [16••].

Genes that act downstream of *FLC* in the autonomous and vernalization-dependent pathways

All of the genes discussed so far act to regulate *FLC*, and so far no genes have been identified that are directly regulated by *FLC* (see Update). *FLC* belongs to the MADS-box family of transcription factors and is likely to regulate the transcription of other genes, acting either as a homodimer or as a heterodimer with other MADS-box proteins.

One clue as to the type of gene that *FLC* may regulate comes from the observation that the late-flowering *flc-11* mutant has a reduced response to applied gibberellin (GA) compared to the wild-type or to other late-flowering mutants. This suggests that *FLC* may block GA action in the apex [18••] either by regulating the expression of GA-biosynthetic genes or by affecting signal transduction genes. Exogenous GA does not change *FLC* transcript levels [18••], suggesting that GA acts downstream of *FLC*. Consistent with this, the *vrn1* mutant did not affect the promotion of flowering by application of GA [28].

Strengthening the evidence for a link between *FLC* and GA action is the observation that plants that constitutively overexpress *FLC* display phenotypes that are consistent with reduced GA activity [18••]. The *fve* mutant (which has an elevated level of *FLC*) has reduced internode length compared with wild-type plants [29], suggesting a deficiency in GA biosynthesis or response. The loss-of-function *flc-13* mutant displays phenotypes that are associated with an enhancement of GA response or plants treated with exogenous GAs, such as early germination, elongated hypocotyls and petioles (DJ Bagnall, CC Sheldon, unpublished data), and early flowering [18••].

GA is an important flowering promoter in *Arabidopsis*. The GA-insensitive *Arabidopsis* mutant ga1-3, which has low levels of endogenous GA [30], does not flower when grown in short days and its flowering is delayed under long-day conditions [31,32]. Despite these correlations, the involvement of GA in the vernalization response remains a controversial topic. An increase in the activity of the GA biosynthesis enzyme *ent*-kaurenoic acid hydroxylase was observed in apices of *Thlaspi arvense*, a close relative of *Arabidopsis*, after a vernalization treatment [33], suggesting that non-vernalized plants are blocked in GA biosynthesis in the apex and that this block is released by vernalization. However, a GA-deficient mutant *ga1-3* retains the capacity to respond to vernalization when grown in a 10-hour light period [32], but not in shorter photoperiods [31,32]. When *ga1-3* was combined with *fca*, plants were later flowering but vernalization was unaffected, suggesting that *GA1* is not required for vernalization [34].

Autonomous pathway mutants show decreased expression of the meristem identity gene *LEAFY (LFY)* [35•,36], and a 35S::*LFY* construct partially suppresses the late-flowering phenotype of these mutants [36,37•], suggesting that *LFY* may act downstream of this pathway, and presumably downstream of *FLC*. Blázquez *et al.* [38] have recently shown that the *LFY* promoter is upregulated synergistically by GA (see Update) and sucrose. Sucrose has been implicated as having a role in the autonomous pathway because mutants that are impaired in starch mobilization (and therefore have low sucrose availability) have a late-flowering phenotype in short day conditions that is completely suppressed by vernalization [39]. Vernalization reduces *FLC* levels, presumably bypassing the need for sucrose, suggesting that sucrose may act in the autonomous pathway to decrease *FLC* expression. Consistent with this possibility,

the late-flowering phenotype of *fca* can be partially suppressed by the presence of sucrose in the growth medium [40]. In addition, Roldán *et al.* [41•] recently demonstrated that a range of *Arabidopsis* mutants and ecotypes can be induced to flower early, even when grown in total darkness, if grown with their apices in contact with a sugar-containing medium.

Conclusions

The isolation of the *FLC* gene and the demonstration of its role in vernalization has provided some understanding of the molecular basis of vernalization. The observation that *FLC* is downregulated by both vernalization and demethylation supports a role for methylation in vernalization responses. The mechanism by which *FLC* is downregulated by either vernalization or demethylation remains unknown, as does the identity of the genes regulated by *FLC* (see Update). The power of molecular genetics together with knowledge of the *Arabidopsis* genome sequence should allow the rapid isolation of mutants and genes that define these processes. The role of GA in vernalization, and whether *FLC* contributes to controlling it, is under investigation. Finally, the mechanism that operates in one of the most remarkable features of vernalization, that is the epigenetic resetting of the vernalization signal, remains a major unsolved mystery.

Update

Recently, a number of other genes that may be involved in the control of flowering by vernalization have been described. These include genes that are likely to act downstream of *FLC*, including *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*; also known as *AGAMOUS-LIKE 20* [*AGL20*]) and *FT* [42•,43]. *The FLAVIN-BINDING, KELCH REPEAT, F BOX 1* (*FKF1*) gene, which is disrupted in a vernalization-responsive late-flowering mutant, *fkf1*, has been shown to encode a protein with a probable role in targeting proteins for degradation, suggesting that posttranscriptional control of gene expression takes place during vernalization [44•]. In relation to GA induction of flowering, the regulation of *LFY* by GA has been further characterized and a GA-responsive promoter element identified [45•].

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