Control of *Arabidopsis* flowering: the chill before the bloom

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Summary

The timing of the floral transition has significant consequences for reproductive success in plants. Plants gauge both environmental and endogenous signals before switching to reproductive development. Many temperate species only flower after they have experienced a prolonged period of cold, a process known as vernalization, which aligns flowering with the favourable conditions of spring. Considerable progress has been made in understanding the molecular basis of vernalization in *Arabidopsis*. A central player in this process is *FLC*, which blocks flowering by inhibiting genes required to switch the meristem from vegetative to floral development. Recent data shows that many regulators of *FLC* alter chromatin structure or are involved in RNA processing.

Introduction

The switch to flowering is a major developmental transition in the plant life cycle (Simpson and Dean, 2002). Plants initially undergo a period of vegetative development, characterised by the iterative production of leaves from the shoot meristem (Poethig, 1990). Later in development, the meristem undergoes a change in fate and enters reproductive development, producing flowers and differentiating the germ line. Plant species exhibit great variability in flowering-time, and the timing of this floral switch is controlled by multiple environmental and endogenous cues (Battey, 2000; Izawa et al., 2003; Simpson and Dean, 2002). This enables plants to align their life history with favourable environmental conditions.

Genetic analysis of *Arabidopsis thaliana* has identified numerous pathways that control the timing of the floral transition (Fig. 1, Table 1 and Table 2). Downstream of many of the floral pathways are a set of floral pathway integrator genes (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Lee et al., 2000; Moon et al., 2003a; Hepworth et al., 2002; Nilsson et al., 1998; Blazquez et al., 2000) (see Fig. 1 and Table 1). It is the activation of these floral pathway integrator genes that triggers the floral transition. In turn, the integrators activate a set of genes known as floral meristem identity (FMI) genes, which encode proteins that promote floral development, not only by positively regulating genes required for flower development, but also by repressing *AGAMOUS-LIKE 24* (*AGL24*), a promoter of inflorescence fate (Yu et al., 2004).

The multiple pathways that regulate the floral pathway integrators in *Arabidopsis* are classified as promotion, enabling and resetting pathways (Boss et al., 2004). Those that promote the floral transition are currently defined as the photoperiod, gibberellin, ambient-temperature and light-quality pathways (Fig. 1 and Table 1). Many angiosperms flower in response to the changing length of the day and night as the year progresses – this is called photoperiodism. Long day photoperiods promote flowering in *Arabidopsis* by activating the B-box transcription factor CONSTANS (CO), which is required for the upregulation of the floral integrator genes (Putterill et al., 1995; Suarez-Lopez et al., 2001; Samach et al., 2000; Blazquez and Weigel, 2000). *CO* mRNA exhibits rhythmic, diurnal expression controlled by the circadian clock (Suarez-Lopez et al., 2001). This rhythm is reinforced through different photoreceptors acting on CO protein stability (Valverde et al., 2004). Phytochromes and cryptochromes are two groups of photoreceptors: PHYTOCHROME B (PHYB) promotes degradation of CO protein, whereas PHYTOCHROME A (PHYA), CRYPTOCHROME1 (CRY1) and CRYPTOCHROME2 (CRY2) stabilise it. These antagonistic activities result in the accumulation of CO only in the evening (Valverde et al., 2004; Yanovsky and Kay, 2002). The hormone gibberellin promotes *Arabidopsis* flowering by upregulating the expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; also known as *AGL20*) (Wilson et al., 1992; Moon et al., 2003a) and *LEAFY* (*LFY*) (Blazquez et al., 1998; Blazquez and Weigel, 2000). The light-quality and ambient-temperature pathways appear to control *FT* expression, and activation of this integrator by the light-quality pathway requires the nuclear protein PHYTOCHROME AND FLOWERING TIME1 (PFT1) (Blázquez et al., 2003; Cerdan and Chory, 2003; Halliday et al., 2003). After flowering, a series of genes in the resetting pathway appear to be required to reset the expression states of floral genes during formation of the gametes or during embryo development. Perturbation of their function results in ectopic expression of floral genes and premature flowering (Kinoshita et al., 2001; Moon et al., 2003b).

In contrast to the promotion pathways, enabling pathways determine the activity of repressors of the floral pathway integrators (Fig. 1, Table 2). The expression of the floral repressor *FLC* is regulated by several independent pathways. *FLC* is upregulated by a number of genes, including *FRIGIDA* (*FRI*), and is downregulated by prolonged periods of cold, a process known as vernalization. In temperate environments, the long period of cold temperature experienced over the winter can promote flowering, aligning reproductive development with spring and summer conditions. Once acquired, the vernalized state is ‘remembered’ by the plant during subsequent growth, suggestive of an epigenetic basis. Several proteins, classified as

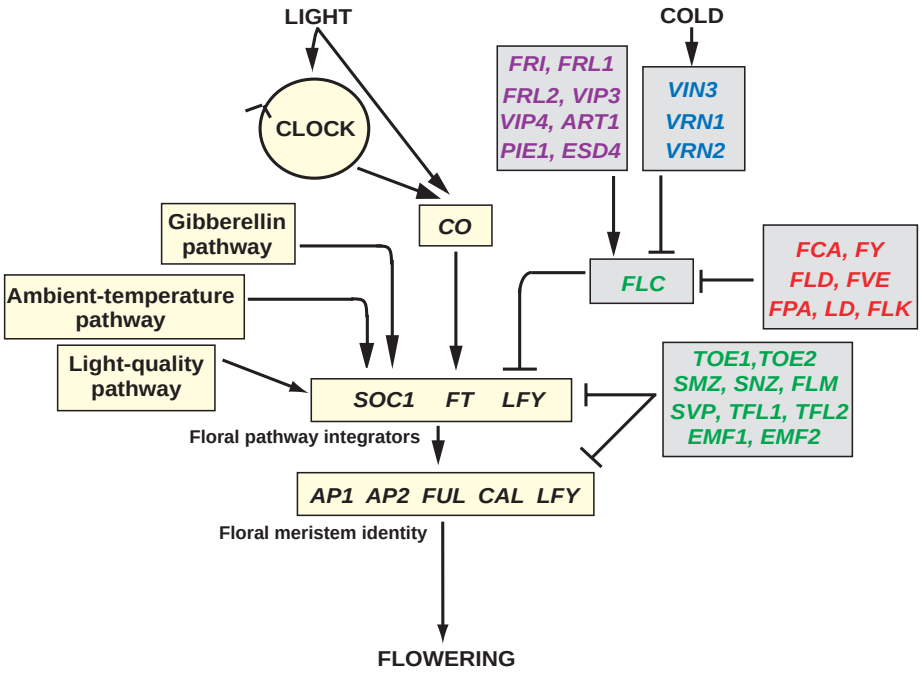


Fig. 1. Pathways controlling flowering-time in *Arabidopsis*. The flowering-time pathways control the expression of the floral pathway integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *FT* and *LEAFY* (*LFY*). These genes encode proteins that activate the floral meristem identity (FMI) genes *APETALA1* (*AP1*), *APETALA2* (*AP2*), *FRUITFULL* (*FUL*), *CAULIFLOWER* (*CAL*) and *LFY*, which convert the vegetative meristem to a floral fate. Recent expression data has indicated that *FUL* may also act as a floral integrator (Schmid et al., 2004). The photoperiod, gibberellin, light-quality and ambient-temperature pathways activate floral pathway integrators. The CONSTANS (CO) transcription factor functions in the photoperiod pathway; long-day photoperiods promote flowering by circadian clock (CLOCK) dependent and independent mechanisms, which control the activity of CO. Activation of flowering is antagonised by the floral repressors encoded by (shown in green) *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS M* (*FLM*), *TERMINAL FLOWER1* (*TFL1*), *TERMINAL FLOWER2* (*TFL2*), *SHORT VEGETATIVE PHASE* (*SVP*), *TARGET OF EAT1* (*TOE1*), *TARGET OF EAT2* (*TOE2*), *SCHNARCHZAPFEN* (*SNZ*), *SCHLAFMUTZE* (*SMZ*) and *EMBRYONIC FLOWER1/2* (*EMF1*, *EMF2*). *TFL1* may also be downstream of *CO*, as it is induced after CO activation (Simon et al., 1996). *FLC* expression is controlled by a number of different pathways. The genes shown in purple, *FRIGIDA* (*FRI*), *FRIGIDA-LIKE1* (*FRL1*), *FRIGIDA-LIKE2* (*FRL2*), *PHOTOPERIOD INSENSITIVE EARLY FLOWERING1* (*PIE1*), *AERIAL ROSETTE1* (*ART1*), *EARLY UNDER SHORT DAYS4* (*ESD4*), *VERNALIZATION INDEPENDENCE3* (*VIP3*) and *VERNALIZATION INDEPENDENCE4* (*VIP4*), encode proteins that promote *FLC* expression and delay flowering. *FLC* expression is downregulated in response to prolonged cold by proteins encoded by the genes (shown in blue) *VERNALIZATION INSENSITIVE3* (*VIN3*), *VERNALIZATION1* (*VRN1*) and *VERNALIZATION2* (*VRN2*), and also by proteins encoded by the genes of the autonomous pathway (red): *FCA*, *FY*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), *FVE*, *FLOWERING LOCUS K* (*FLK*) and *FPA*. The distinction between potential transcriptional and post-transcriptional functions of genes of the autonomous pathway is not made here, but is shown more clearly in Fig. 3.

the autonomous promotion pathway, act in parallel to vernalization and also repress *FLC* (Table 1). The autonomous pathway was named because of its lack of involvement in either the photoperiodic- or gibberellin-promotive floral pathways. This review focuses on recent work addressing the control of *FLC* expression in response to the prolonged periods of cold experienced during vernalization. Chromatin regulation and RNA processing have emerged as key mechanisms that modulate expression of the floral repressor *FLC*. We will speculate on how the different pathways controlling *FLC* expression may be integrated at the molecular level.

The floral repressor, FLC

FLC is a MADS-box transcriptional repressor, expressed predominantly in shoot and root apices and vasculature, that quantitatively represses flowering by repressing the floral pathway integrators (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001). The mechanism by which it does this is not well understood, although a MADS-box binding site within the promoter of *SOC1* is required (Hepworth et al., 2002).

Natural *Arabidopsis* accessions vary in their requirement for a vernalization treatment before flowering. Accessions are isogenic *Arabidopsis* backgrounds collected from a single location and maintained in a seed bank. Rapid cycling accessions, such as the laboratory strains Columbia and Landsberg *erecta*, flower early without a vernalization treatment. By contrast, many wild accessions flower much later, unless they receive a vernalization treatment; these are termed winter annual backgrounds (Fig. 2). Allelic variation at *FLC* contributes to natural variation in vernalization requirement, with weak alleles leading to a rapid-cycling habit (Fig. 2) (Michaels et al., 2003; Gazzani et al., 2003). Interestingly, the phenotypes of plants with naturally occurring weak *FLC* alleles appear to be caused by changes in the regulation of expression rather than alterations of protein function. The rapid-cycling Landsberg *erecta* and Da accessions contain *FLC* alleles with independent transposon insertions within the large *FLC* intron 1 (Michaels et al., 2003; Gazzani et al., 2003). Sequences important for *FLC* regulation and expression have been mapped to this intron, which may account for the effects of these insertions (Sheldon et al., 2002; He et al., 2003).

There are five close homologues of *FLC* in the *Arabidopsis* genome, and these are called *MADS AFFECTING FLOWERING1* (*MAF1*) to *MAF5* (Ratcliffe et al., 2003; Ratcliffe et al., 2001), with *MAF1* also referred to as *FLM* (Scortecci et al., 2001) or *AGL27* (Alvarez-Buylla et al., 2000). FLM is a floral repressor; however, it does not appear to be involved with the vernalization pathway (Scortecci et al., 2001). MAF2 is also a floral repressor and *maf2* mutants show

Table 1. Floral promotive genes

Gene name	Protein function	Reference
Floral pathway integrators		
SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1/AGAMOUS-LIKE20 (*SOC1/AGL20*)	MADS-box transcription factor	Samach et al., 2000; Lee et al., 2000
FT	Putative kinase inhibitor	Kardailsky et al., 1999; Kobayashi et al., 1999
LEAFY (*LFY*)	Plant specific transcription factor	Weigel et al., 1992; Nilsson et al., 1998
Photoperiodic pathway		
CONSTANS (*CO*)	B-box transcription factor	Putterill et al., 1995
Light-quality pathway		
PHYTOCHROME AND FLOWERING TIME1 (*PFT1*)	Nuclear protein	Cerdan and Chory, 2003
Autonomous pathway		
FCA	RNA-binding protein	Macknight et al., 1997
FY	Polyadenylation factor	Simpson et al., 2003
FPA	RNA-binding protein	Schomburg et al., 2001
FLOWERING LOCUS K (*FLK*)	RNA-binding protein	Lim et al., 2004
FVE	MSI4	Ausin et al., 2004
FLOWERING LOCUS D (*FLD*)	HDAC-associated protein	He et al., 2003
LUMINIDEPENDENS (*LD*)	Homeodomain protein	Lee et al., 1994a
Vernalization pathway		
VERNALIZATION INSENSITIVE3 (*VIN3*)	Protein with fibronectin repeats and PHD domain	Sung and Amasino, 2004
VERNALIZATION1 (*VRN1*)	B3 domain DNA-binding protein	Levy et al., 2002
VERNALIZATION2 (*VRN2*)	Su(z)12-like polycomb protein	Gendall et al., 2001

Table 2. Floral repressive genes

Gene	Protein function	Reference
Activators of *FLC*		
FRIGIDA (*FRI*)	Novel protein	Johanson et al., 2000
FRIGIDA-LIKE1 (*FRL1*)	Novel protein related to FRIGIDA	Michaels et al., 2004
FRIGIDA-LIKE2 (*FRL2*)	Novel protein related to FRIGIDA	Michaels et al., 2004
VERNALIZATION INDEPENDENCE3 (*VIP3*)	Novel WD-repeat protein	Zhang et al., 2003
VERNALIZATION INDEPENDENCE4 (*VIP4*)	Protein with homology to the yeast transcriptional activator, Leo1p	Zhang and van Nocker, 2002
EARLY IN SHORT DAYS4 (*ESD4*)	Nuclear protease regulating SUMOylation	Murtas et al., 2003
AERIAL ROSETTE1 (*ART1*)	Not yet identified	Poduska et al., 2003
PHOTOPERIOD INSENSITIVE1 (*PIE1*)	SWI/SNF-helicase-like protein	Noh and Amasino, 2003
Floral repressors		
FLOWERING LOCUS C (*FLC*)	MADS-box transcription factor	Michaels and Amasino, 1999; Sheldon et al., 1999
FLOWERING LOCUS M (*FLM*)	MADS-box transcription factor	Scortecci et al., 2001
SHORT VEGETATIVE PHASE (*SVP*)	MADS-box transcription factor	Hartmann et al., 2000
TARGET OF EAT1/2 (*TOE1/2*)	AP2-like transcription factor	Aukerman and Sakai, 2003
SCHNARCHZAPFEN/SCHLAFMUTZE (*SNZ/SMZ*)	AP2-like transcription factor	Schmid et al., 2004
TFL1	Putative kinase inhibitor	Bradley et al., 1997
TFL2/LHP1	Heterochromatin protein1 (HP1)-like protein	Gaudin et al., 2001; Kotake et al., 2003
EMBRYONIC FLOWER1	Novel protein	Aubert et al., 2001
EMBRYONIC FLOWER2	Su(z)12-like polycomb protein	Yoshida et al., 2001

a pronounced vernalization response when subjected to short periods of cold that would not affect wild-type plants (Ratcliffe et al., 2003). *MAF3* and *MAF4* may act as floral repressors; however, the expression of *MAF5* is increased by vernalization, so *MAF5* may play an opposite role to *FLC* during vernalization (Ratcliffe et al., 2003).

Activation of *FLC*

A key activator of *FLC* expression is FRI (Fig. 1, Table 1). Pioneering genetic analysis performed by Klaus Napp-Zinn (University of Cologne) in the 1950s identified allelic variation at *FRI* as the major determinant of flowering-time variation between rapid-cycling and winter annual accessions. Active *FRI* alleles confer late flowering and a vernalization requirement for early-flowering (Napp-Zinn, 1955; Napp-Zinn, 1957). It is striking, given that so many genes regulate *FLC*, that the winter annual habit can be mapped as a single gene trait to *FRI*. FRI represses flowering by upregulating *FLC* RNA levels (Michaels and Amasino, 1999; Sheldon et al., 2000) and, consistent with this, loss of *FLC* function eliminates the ability of *FRI* to delay flowering (Michaels and Amasino, 2001) (Fig. 2). Map-based cloning of *FRI* revealed that it encodes a novel protein with coiled-coil domains, but gave no indication as to the mechanism by which it upregulates *FLC* (Johanson et al., 2000). Analysis of natural *Arabidopsis* accessions identified at least nine independent loss-of-function mutations in *FRI* (Gazzani et al., 2003; Johanson et al., 2000; Le Corre et al., 2002). Hence, evolution of the rapid-cycling growth habit in

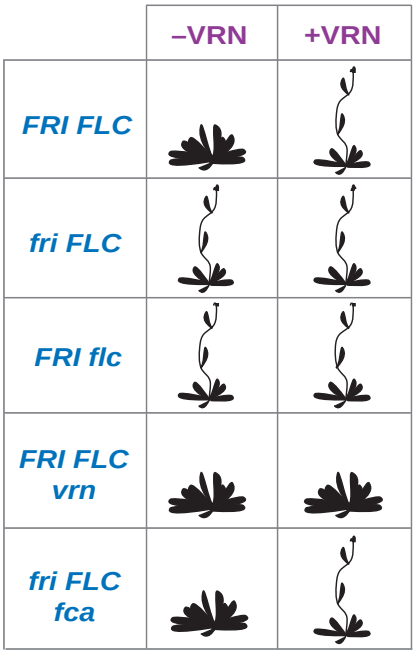


Fig. 2. Schematic representations of *Arabidopsis* plants summarizing the genetic control of vernalization requirement and response. The flowering phenotype of *Arabidopsis* is represented as either a rapid cycler (e.g. top right), which produces a flowering inflorescence, or as a winter annual accession (e.g. top left), which continues to produce rosette leaves. Rapid-cycling accessions do not require a vernalization treatment to flower early and are commonly used as laboratory backgrounds. By contrast, the majority of *Arabidopsis* accessions are winter annuals, which flower late unless they have been exposed to a prior vernalization treatment. Typically, 6 weeks of growth at 4°C produces a saturated vernalization response in *Arabidopsis*. Growth habit is indicated either with (+VRN) or without (–VRN) a vernalization treatment. When both *FRI* and *FLC* are active, the plant is vernalization responsive, as is found in many winter annual accessions. Mutations in either *fri* or *flc* can lead to rapid cycling. A vernalization-responsive *FRI FLC* accession is rendered insensitive to vernalization by a *vrn* mutation. Finally, a rapid-cycling *fri FLC* genotype becomes a winter annual background in the presence of an autonomous pathway mutation such as *fca*.

some strains of *Arabidopsis* may have evolved multiple times through the loss of *FRI*. Genetic analysis of natural variation in flowering time has also identified *AERIAL ROSETTE1* (*ART1*) from the extremely late-flowering accession Sy-0; ART1 acts synergistically with *FRI* to upregulate *FLC* (Poduska et al., 2003).

Recently, an increasing number of *FLC* activators have been identified by the analysis of early-flowering mutants. Two FRIGIDA-LIKE (FRL) genes, *FRL1* and *FRL2*, are required for the upregulation of *FLC* expression by FRI (Michaels et al., 2004). Although FRI, FRL1 and FRL2 are related at the amino acid sequence level, they appear not to be functionally redundant (Michaels et al., 2004). The VERNALIZATION INDEPENDENCE (VIP) genes are also required for high *FLC* expression (Zhang et al., 2003; Zhang and van Nocker, 2002). The VIP4 protein exhibits homology with the yeast Leo1p protein, a component of the Paf complex, which is required for chromatin modification and transcriptional activation (Zhang et al., 2002; Porter et al., 2002). The VIP3 protein encodes WD repeats that typically mediate protein-protein interactions (Zhang et al., 2003). Hence, the VIP proteins may represent a complex that is required for *FLC* transcription and chromatin regulation. *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1* (*PIE1*) also provides a link to chromatin regulation, as it encodes a protein that activates *FLC* and has homology to ATP-dependent chromatin-remodelling proteins of the ISWI and SWI2/SNF2 family (Noh and Amasino, 2003). A more tenuous link to chromatin regulation may be *EARLY IN SHORT DAYS 4* (*ESD4*), which encodes a nuclear protease that upregulates *FLC* and is required for the regulation of SUMOylation (SMALL UBIQUITIN-RELATED MODIFIER) in *Arabidopsis* (Murtas et al., 2003; Reeves et al., 2002). SUMOylation is a recently discovered modification of histones and may be a part of the 'histone code' (see Box 1) (Shiio and Eisenman, 2003). Many other proteins, however, are also SUMOylated, so ESD4 may function to regulate the levels, activity or compartmentalization of an *FLC* regulator. Interestingly, mutations in *VIP3*, *PIE1* and *ESD4* suppress the high *FLC* expression caused by either dominant *FRI* alleles or mutations in the autonomous pathway (Zhang et al., 2003). By contrast, *FRL1* and *FRL2* are specifically required for the activation of *FLC* via *FRI* (Michaels et al., 2004).

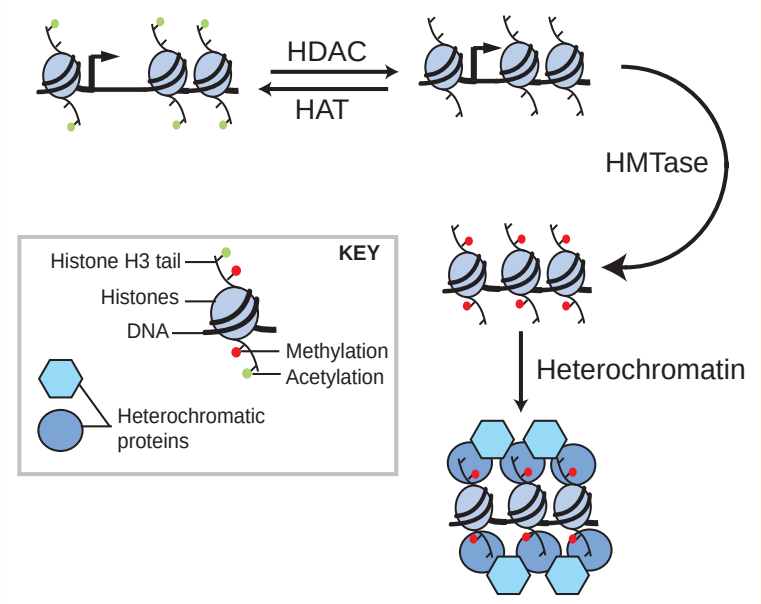
Repression of *FLC* by vernalization

FLC mRNA levels are downregulated by vernalization. In nature, winter provides the necessary cold and results in the alignment of flowering with the favourable conditions of spring. At the molecular level, *FLC* regulation by the cold shows similarities with many of the physiological properties of vernalization (Chouard, 1960; Lang, 1965). The vernalization response is strongly quantitative, with increasing durations of cold leading to progressively accelerated flowering once the plants return to ambient temperatures (Chouard, 1960; Lang, 1965). The downregulation of *FLC* RNA is also a quantitative process, with longer periods of cold exposure leading to progressively lower *FLC* mRNA expression (Michaels and Amasino, 1999; Sheldon et al., 2000). For annual plants (those that germinate and flower within one year), the vernalization response is saturated after several weeks of cold and, once established, the vernalized state is stable though subsequent growth at ambient temperatures, although it is reset after meiosis (Chouard, 1960; Lang, 1965). Similarly, repression of *FLC* levels is achieved after several weeks of cold and is then maintained at low levels throughout subsequent development, whilst being reset in the next generation (Sheldon et al., 2000). *FLC* repression must therefore be 'remembered' through mitotic proliferation until flowering occurs. Furthermore, grafting experiments reveal that the site of cold perception during vernalization is the shoot apex and this is a region of *FLC* expression (Wellensiek, 1962; Wellensiek, 1964; Sung et al., 2004).

The maintenance of *FLC* repression following vernalization indicates that this gene is epigenetically silenced. Epigenetic silencing of genes is mediated by numerous covalent modifications of both the DNA and histones (Box 1) (Fischle et al., 2003; Bird, 2002). Early work on the control of vernalization focused on the role of DNA cytosine methylation (Finnegan et al., 1998). However, recent data has demonstrated

Box 1. The histone code

The N-terminal tails of histones H3 and H4 undergo extensive post-translational modifications, including acetylation, phosphorylation, methylation, ubiquitination, SUMOylation and ADP-ribosylation (Wolffe, 1998; Lachner et al., 2003). These modifications have diverse consequences for gene expression and chromosomal organisation. Importantly, histone tail modifications can be inherited through cell division and thus they facilitate epigenetic control. Once acquired, several of these marks are bound by further proteins, which leads to the reinforcement of expression states or chromatin structure. Methylation and acetylation are two intensively studied modifications with well-defined roles in the control of gene expression states.



The N-terminal tail of histone H3 has four lysine residues – K4, K9, K27 and K36 – that are capable of being methylated by histone methyltransferases (HMTases) (Wolffe, 1998; Lachner et al., 2003). There is also diversity in whether particular lysines acquire mono-, di- or tri-methylation, and the relative importance of these marks may differ between organisms (Jackson et al., 2004). Typically, methylation at K9 and K27 is associated with gene repression, whereas methylation at K4 is associated with gene activation (Wolffe, 1998; Lachner et al., 2003). Methylation of K9 and K27 leads to the binding of the chromodomain proteins HP1 and POLYCOMB, respectively (indicated as 'heterochromatic proteins' in the figure), which then results in mitotically stable, heterochromatic gene silencing (Bannister et al., 2001; Cao et al., 2002). Acetylation of histone H3 and H4 is generally present in active chromatin. In the case of H3, K9 acetylation and methylation are mutually exclusive marks, and deacetylation must occur first as a prelude to methylation and silencing (Wolffe, 1998; Lachner et al., 2003). The acetylation state of histone tails is determined by the relative activities of histone acetyltransferase (HAT) and histone decatylases (HDAC) acting at a particular locus. Recently, histone SUMOylation has also been demonstrated to mediate gene repression. SUMO is a small peptide related to ubiquitin, which when attached to H4 leads to gene silencing (Shiio and Eisenman, 2003).

a more important role for histone modifications at *FLC* chromatin during vernalization (Sung and Amasino, 2004; Bastow et al., 2004). Specific residues of histone H3 tails are modified by acetylation and methylation, and changes in these modifications serve as part of a 'histone-code' specifying active or repressed gene activity states (Fischle et al., 2003). Vernalization increases histone H3 deacetylation in the 5′-region of *FLC* very early after exposure to the cold, a modification typically associated with gene repression (Sung and Amasino, 2004). Vernalization also induces increased methylation of histone H3 lysine residues 9 and 27, modifications associated with repressed gene states (Sung and Amasino, 2004; Bastow et al., 2004). In animal systems, deacetylation is typically a prelude to acquisition of histone methylation (Fischle et al., 2003). Furthermore, histone methylation marks can act as signals to recruit further mediators of gene silencing (Orlando, 2003). Interestingly, the histone marks observed at the *FLC* locus appear to be localised to specific regions of the gene (Bastow et al., 2004), at the 5′ end of the gene and within intron 1, co-localising with sequences already known to be involved in the regulation of *FLC* by vernalization (Sheldon et al., 2002; He et al., 2003).

Genetic screens for mutants compromised in vernalization have identified trans-factors that mediate repression of *FLC* in response to the cold (Chandler et al., 1996; Sung and Amasino, 2004). The earliest acting gene is *VERNALIZATION INSENSITIVE 3* (*VIN3*), which encodes a protein with a plant homeodomain (PHD) and fibronectrin type III repeats (Sung and Amasino, 2004). PHD domains have been found in proteins associated with chromatin-remodelling complexes and can bind phosphoinositides, whereas fibronectin repeats are often involved in protein-protein interactions (Sung and Amasino, 2004). In *vin3* mutants, the vernalization-mediated decrease in histone acetylation and increase in H3 K9 and K27 methylation does not occur, and thus *FLC* is not repressed by vernalization (Sung and Amasino, 2004). Intriguingly, *VIN3* expression increases with cold, and only significantly accumulates after a period of cold sufficient to trigger vernalization (Sung and Amasino, 2004). The *VIN3* expression domain also overlaps with that of *FLC* (Sung and Amasino, 2004). Hence, upregulation of *VIN3* expression is an early step during the vernalization-signalling pathway. Understanding how prolonged cold induces expression of *VIN3* is a key question for future research.

A second class of gene involved in the vernalization response is represented by the genes *VERNALIZATION1* (*VRN1*) and *VERNALIZATION2* (*VRN2*) (Chandler et al., 1996; Gendall et al., 2001; Levy et al., 2002). The *vrn1* and *vrn2* mutants are distinct from *vin3* in that initial repression of *FLC* expression by the cold still occurs (Gendall et al., 2001; Levy et al., 2002). However, when *vrn1* and *vrn2* mutants return to ambient temperatures, *FLC* repression is not maintained and *FLC* RNA levels progressively increase (Chandler et al., 1996; Gendall et al., 2001; Levy et al., 2002). Unlike *VIN3*, expression of *VRN1* and *VRN2* is not upregulated by cold, and hence VIN3 may provide a cold-induced activity

that recruits them to *FLC* (Sung and Amasino, 2004; Gendall et al., 2001; Levy et al., 2002). Furthermore, VRN1 and VRN2 are not required for the VIN3-mediated *FLC* deacetylation early in vernalization (Sung and Amasino, 2004). The VRN2 protein shows homology to the *Drosophila* Polycomb protein Suppressor of Zeste 12 (Su(z)12) (Gendall et al., 2001). The Polycomb-Group (PcG) proteins function to maintain epigenetic gene activity states throughout *Drosophila* embryogenesis and cell proliferation (Orlando, 2003). Su(z)12 acts in a PcG complex, PRC2, with histone methyltransferase activity directed against histone H3 lysines 27 and 9 (Kuzmichev et al., 2002; Muller et al., 2002). Hence, VRN2 is likely to mediate stable repression of *FLC* activity by a PcG-like mechanism. Indeed, in *vrn2* mutants, increased histone H3 methylation of lysines 27 and 9 does not occur at *FLC* during vernalization (Sung and Amasino, 2004; Bastow et al., 2004). This indicates that elements of the 'histone-code' involved in developmental gene regulation are highly conserved between plants and animals. By contrast, the VRN1 protein is plant-specific and carries two B3 domains, which mediate non-sequence specific DNA binding in vitro (Levy et al., 2002). Unlike VRN2, VRN1 is required only for increases in histone H3 lysine 9 methylation, and not for methylation of lysine 27 (Sung and Amasino, 2004; Bastow et al., 2004). This suggests that VRN1 may function either downstream or independently of VRN2 during *FLC* repression. Overexpression of VRN1 revealed a vernalization-independent function for VRN1, mediated predominantly through the floral pathway integrator *FT*, and demonstrated that VRN1 requires vernalization-specific factors to target *FLC* (Levy et al., 2002).

Repression of *FLC* by autonomous pathway genes

The autonomous pathway acts in parallel to vernalization to repress *FLC* expression (Koornneef et al., 1991; Simpson and Dean, 2002). In the absence of *FRI*, this pathway is the major regulator of *FLC* levels and therefore confers a vernalization requirement (Koornneef et al., 1991). Mutants in the autonomous pathway are late-flowering because of elevated levels of *FLC* mRNA, and this late-flowering is vernalization responsive (Koornneef et al., 1991; Sheldon et al., 2000; Michaels and Amasino, 2001) (Fig. 1 and Table 1). Although all members of this pathway act to limit *FLC* expression, genetic analysis has revealed that they have distinct functions. Two epistasis groups – *FCA*, *FY* and *FPA*, *FVE* – have been found using double mutants, although the significance of this is not yet fully understood (Koornneef et al., 1998). The *ld* and *fld* mutations are strongly suppressed by the *FLC* allele in L*er*, the background in which the other mutations were isolated, so epistasis analysis of these genes has not yet been performed (Lee et al., 1994b; Sanda and Amasino, 1996).

HDACs in the flowering response

FLD encodes a protein with homology to a human protein that functions in the histone deacetylase 1,2 (HDAC1/2) co-repressor complex (He et al., 2003). Histone deacetylation mediated by this complex is commonly associated with gene repression (He et al., 2003). The FLD protein carries an N-terminal SWIRM domain, such as that found in chromatin remodelling enzymes, in addition to a polyamine oxidase domain (He et al., 2003). In *fld* mutants, the 5′-end of *FLC* displays hyperacetylation of histone H4 (He et al., 2003), indicating that FLD is required to deacetylate *FLC* chromatin and thereby repress its expression. Intriguingly, removal of a 295-base pair region of *FLC* intron 1 prevents this regulation and results in high *FLC* expression, independent of FLD activity (He et al., 2003). Thus, this *FLC* intronic region may contain cis sequences required for recruitment of a HDAC complex. Currently, the identity of the HDAC that functions with FLD is unknown.

The *Arabidopsis* genome encodes four HDAC1/2 homologs but late-flowering mutations in these genes have yet to be identified (Pandey et al., 2002). However, an antisense construct designed to target multiple HDACs does result in delayed flowering, which may be due to a failure to repress *FLC* (Tian and Chen, 2001). Analysis of histone H4 acetylation status in the other autonomous mutants revealed a similar hyperacetylation phenotype only in *fve* (He et al., 2003). *FVE* encodes the nuclear WD-repeat protein, MSI4 (Ausin et al., 2004). There are five MSI-related proteins in *Arabidopsis*, which display homology to the mammalian Retinoblastoma Associated Protein46 (RbAp46) and RbAp48 proteins (Ausin et al., 2004). MSI-like proteins are typically found in complexes involved in chromatin assembly and histone modification, and FVE was demonstrated to co-immunoprecipitate with plant Rb (Retinoblastoma protein) (Ausin et al., 2004). In other systems, Rb functions in histone deacetylase complexes, which again is consistent with the histone hyperacetylation of *FLC* observed in *fve* and *fld* mutants (Ausin et al., 2004; He et al., 2003). In addition to a histone H4 hyperacetylation phenotype, analysis in *fve* mutants also revealed hyperacetylation of histone H3, indicating that both histones are deacetylated by this pathway (Ausin et al., 2004). Hence, FVE and FLD are likely to act together in a HDAC complex to repress *FLC* expression (Ausin et al., 2004; He et al., 2003). It will be important to determine if this HDAC complex is specifically targeted to *FLC* or whether it performs broader functions that are covered by redundancy. How this deacetylase activity integrates with the epigenetic modifications directed by vernalization is also an interesting question.

RNA processing

Mutations in the autonomous pathway gene *FCA* display no effect on *FLC* acetylation status (He et al., 2003). Indeed, *FCA* appears to be genetically distinct from *FVE* (Koornneef et al., 1998). *FCA* encodes a plant-specific, nuclear RNA-binding protein (Macknight et al., 1997). In addition to two RNA recognition motif (RRM) domains, FCA possesses a C-terminal WW protein interaction domain (Macknight et al., 1997; Sudol and Hunter, 2000). This domain mediates interaction with another component of the autonomous pathway, FY (Simpson et al., 2003). In contrast to FCA, FY is highly conserved throughout eukaryotes and displays homology to the yeast polyadenylation factor, Pfs2p (Ohnacker et al., 2000; Simpson et al., 2003). Pfs2p carries seven WD repeats and acts as a scaffold protein within the large CPF (cleavage and polyadenylation factor) complex (Ohnacker et al., 2000). The CPF complex is required for 3′-cleavage and polyadenylation of pre-mRNA transcripts, and strong mutations in polyadenylation factors, including *PFS2*, are lethal because of a failure to correctly express RNA polymerase II transcripts (Ohnacker et al., 2000). In addition to these WD repeats, FY possesses a novel C-terminal domain with which FCA interacts. FY may perform a generic function in RNA processing, while

also functioning in regulated polyadenylation through interaction with FCA. *FPA* encodes a second plant-specific RRM domain protein within the autonomous pathway (Schomburg et al., 2001). Although FPA is required for the regulation of *FLC*, the level at which it functions is unknown. Finally, *FLK* is the most recently identified member of the autonomous pathway and encodes a nuclear KH-type RNA-binding protein (Lim et al., 2004). Hence, multiple RNA-binding proteins are required for repression of *FLC* expression by the autonomous pathway. Determining whether this reflects a cascade of post-transcriptional regulators or a complex of RNA-binding factors will require further analysis of proteins of the autonomous pathway.

Currently there is no evidence that FCA/FY, FPA or FLK directly regulates *FLC* mRNA processing. However, *FCA* expression itself is complex and exhibits an autoregulatory mechanism involving polyadenylation site choice (Macknight et al., 1997; Macknight et al., 2002; Quesada et al., 2003). There are four *FCA* transcripts, and intron 3 is a major site of alternative processing. Premature cleavage and polyadenylation within this intron generates the truncated, non-functional *FCA-β* transcript (Macknight et al., 1997; Macknight et al., 2002). FCA negatively autoregulates its own expression by promoting intron 3 polyadenylation (Quesada et al., 2003). This regulation also requires the functional interaction between FCA and FY, demonstrating that these proteins mediate alternative 3′-end processing (Macknight et al., 1997; Macknight et al., 2002; Quesada et al., 2003). Hence, FCA may function as a novel trans-regulator of polyadenylation site choice via interaction with the core 3′-processing factor FY. An intriguing aspect of *FCA* autoregulation is its tissue specificity. Premature polyadenylation is inhibited in meristematic regions relative to non-meristematic regions (Macknight et al., 2002; Quesada et al., 2003). The mechanism by which this occurs is currently unknown but might also have a consequence for the regulation of *FLC*. *FPA* and *FLK* appear not to be required for *FCA* intron 3 regulation (Lim et al., 2004; Quesada et al., 2003). Hence, the proteins of the autonomous pathway appear to have partially redundant activities that repress *FLC* by distinct mechanisms. It is not known whether the chromatin regulation and RNA processing activities of the autonomous pathway are integrated during the control of *FLC* expression, although chromatin modification and 3′-processing interact functionally in yeast (Alen et al., 2002).

Integration of the pathways regulating *FLC* expression

Plants need to monitor their environmental conditions during growth and development, and acquire sufficient resources to complete reproductive development. The *FLC* activators are considered to function early in development to ensure high levels of FLC and floral repression at germination, thus avoiding precocious flowering before resources have accumulated. The repressors of *FLC* expression may be downregulated early in development for the same reason. This appears to be the case for *FCA*, as production of the active *FCA* transcript via a change in polyadenylation site usage increases significantly in meristems 4-5 days after germination (Macknight et al., 2002). Indeed, bypassing this control on *FCA* overrides *FRI* repression of flowering (Quesada et al., 2003). However, the precise temporal expression of many *FLC* activators, and when their functions are required in flowering control, remains to be determined.

The interaction between *FLC* activators and repressors effectively determines whether a plant adopts a winter annual or rapid-cycling habit. It is possible that this interaction is determined by the antagonistic effects of the different pathways on *FLC* chromatin. PIE1 and VIP proteins are *FLC* upregulators that may act to promote active chromatin, whereas FVE and FLD act to deacetylate histones, thus promoting a silent chromatin state. The roles of the multiple RNA-binding proteins (FCA, FPA, FLK), and the polyadenylation factor FY, in repressing *FLC* raises some interesting possibilities. They may function to repress *FLC* directly or by regulating components of the activation pathway. Alternatively, the recent demonstrations of non-coding RNA acting in chromatin regulation means that they may play a role in generating RNA intermediates that feed back to regulate *FLC* chromatin (Volpe et al., 2002; Zilberman et al., 2003).

The onset of winter perturbs the steady-state *FLC* expression by the induction of *VIN3* after several weeks of cold, potentially initiating a chain of epigenetic modifications at the *FLC* locus. An early step in this sequence appears to be histone deacetylation (Fig. 3), and the stable maintenance of *FLC* repression involves the activities of VRN1, VRN2 and histone methylation. In animals, histone methylation recruits further proteins required to maintain gene repression (Orlando, 2003), although the identity of such factors in plants and during vernalization remains unknown. *FLC* expression then remains low during subsequent development and flowering, but at some stage during meiosis, gametogenesis or early embryogenesis, *FLC* expression is reset. The epigenetic modifications at *FLC* established during vernalization, or by the activity of the autonomous pathway, are erased, allowing high *FLC* expression in the young seedlings and determining a requirement for vernalization in each generation. This molecular sequence accounts for flowering in annual plants. Many plants, however, are perennials, that is they live for many years with only a proportion of the apical meristems undergoing the transition to flowering each year. Whether similar mechanisms are involved in controlling flowering in perennials remains to be established.

Conclusions

Multiple mechanisms have evolved to ensure the fine control of *FLC* levels and thus the timing of the transition to flowering. Considerable progress has been made towards elucidating the molecular mechanisms involved, but several important questions remain. Is *VIN3* expression really the cold-induced trigger that initiates the chromatin changes at *FLC*? How do these changes overcome the function of activators such as FRI, and how do genes of the autonomous pathway fit into the molecular picture? Understanding the mechanisms involved in the resetting of *FLC* expression may provide insights into fundamental aspects of epigenetic reprogramming in plants and animals. The power of forward genetics, together with the exploitation of natural variation, will undoubtedly be key to unravelling many of these questions, and will provide answers as to how the different *Arabidopsis* reproductive strategies have been selected.

Recent progress in wheat has also identified key regulators

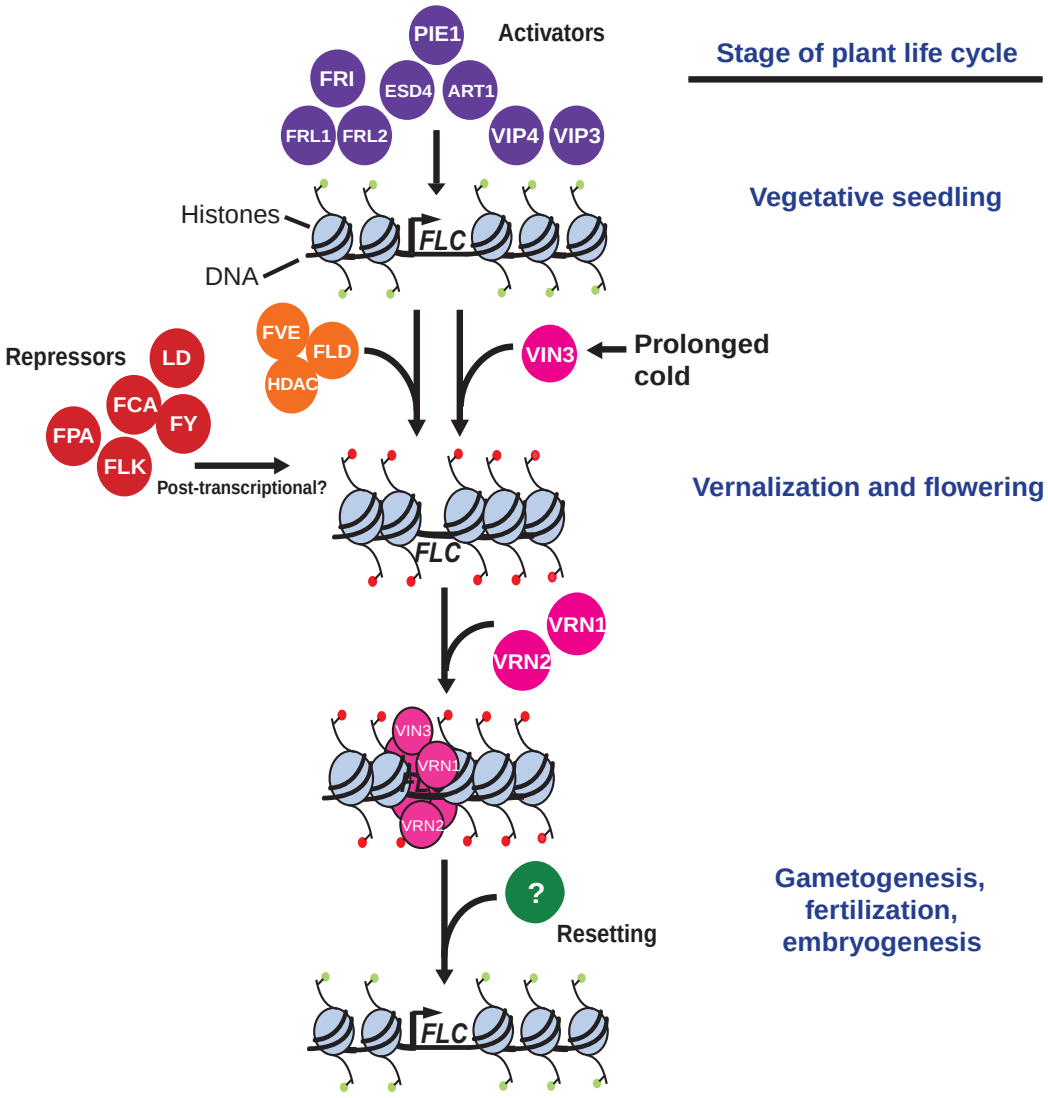


Fig. 3. Model for the regulation of the floral repressor *FLC* throughout the *Arabidopsis* life cycle. During seedling growth, a group of genes encode proteins that function as activators of *FLC* expression (shown in purple); these genes include *FRI*, *FRL1*, *FRL2*, *ESD4*, *ART1*, *PIE1*, *VIP3* and *VIP4*. These proteins may maintain *FLC* chromatin in an active state (indicated by an open structure and the presence of active histone tail modifications shown in green). The autonomous pathway functions antagonistically to the activators to repress *FLC* expression. The RNA-binding proteins FCA, FPA and FLK, and the polyadenylation factor FY, may function post-transcriptionally to achieve this and are shown in red. The FVE/FLD proteins act with a putative histone deacetylase (HDAC; all shown in orange) to promote an inactive *FLC* chromatin state, represented by a closed structure with inactive histone tail modifications (red). *FLC* is also repressed by exposure to long periods of cold (vernalization). The proteins acting in the vernalization pathway are shown in pink. Prolonged cold induces *VIN3* expression, which promotes an inactive *FLC* chromatin state. Subsequently, the VRN1 and VRN2 proteins are recruited to *FLC*, and are required for the methylation of *FLC* histones and the maintenance of silencing. These marks may promote the association of silencing factors with *FLC* chromatin that reinforce its repression. During meiosis, gametogenesis or early embryogenesis, *FLC* repression is overcome, thus resetting its expression in the next generation.

determining the vernalization requirement in cereals (Yan et al., 2003; Trevaskis et al., 2003; Yan et al., 2004). The genes identified are so far distinct from those identified in *Arabidopsis*. Wheat VRN1 functions as a floral promoter and is a MADS-box protein with homology to APETALA1 (Yan et al., 2003). Wheat VRN2 contains a CCT domain (a 43-amino acid region with homology to *Arabidopsis* proteins CO, CO-LIKE and TOC1), and it functions to repress directly or indirectly the expression of wheat *VRN1* (Yan et al., 2004). Vernalization progressively reduces levels of wheat *VRN2* RNA, preventing repression of *VRN1* and promoting flowering. The involvement of distinct proteins in cereals and *Arabidopsis* implies that different pathways have evolved to regulate the vernalization requirement. However, it will be interesting to determine whether chromatin regulation of these targets also mediates the epigenetic memory of winter in wheat. Together, work in cereals and *Arabidopsis* should allow the manipulation of vernalization, a key agricultural trait.

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