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

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**Review**

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

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.

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, 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
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 the photoperiodic- or gibberellin-promotive floral pathways. This pathway was named because of its lack of involvement in either FLC, 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 (PSE1), 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 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
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

### Table 1. Floral promotive genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein function</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Floral pathway integrators</strong></td>
<td></td>
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<tr>
<td>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1/AGAMOUS-LIKE20 (SOC1/AGL20)</td>
<td>MADS-box transcription factor</td>
<td>Samach et al., 2000; Lee et al., 2000</td>
</tr>
<tr>
<td>FT</td>
<td>Putative kinase inhibitor</td>
<td>Kardailsky et al., 1999; Kobayashi et al., 1999</td>
</tr>
<tr>
<td>LEAFY (LFY)</td>
<td>Plant specific transcription factor</td>
<td>Weigel et al., 1992; Nilsson et al., 1998</td>
</tr>
<tr>
<td><strong>Photoperiodic pathway</strong></td>
<td></td>
<td></td>
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<tr>
<td>CONSTANS (CO)</td>
<td>B-box transcription factor</td>
<td>Putterill et al., 1995</td>
</tr>
<tr>
<td><strong>Light-quality pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHYTOCHROME AND FLOWERING TIME1 (PFT1)</td>
<td>Nuclear protein</td>
<td>Cerdan and Chory, 2003</td>
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<td><strong>Autonomous pathway</strong></td>
<td></td>
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<tr>
<td>FCA</td>
<td>RNA-binding protein</td>
<td>Macknight et al., 1997</td>
</tr>
<tr>
<td>FY</td>
<td>Polyadenylation factor</td>
<td>Simpson et al., 2003</td>
</tr>
<tr>
<td>FPA</td>
<td>RNA-binding protein</td>
<td>Schomburg et al., 2001</td>
</tr>
<tr>
<td>FLOWERING LOCUS K (FLK)</td>
<td>RNA-binding protein</td>
<td>Lim et al., 2004</td>
</tr>
<tr>
<td>FVE</td>
<td>MS14</td>
<td>Ausin et al., 2004</td>
</tr>
<tr>
<td>FLOWERING LOCUS D (FLD)</td>
<td>HDAC-associated protein</td>
<td>He et al., 2003</td>
</tr>
<tr>
<td>LUMINIDEPENDENS (LD)</td>
<td>Homeodomain protein</td>
<td>Lee et al., 1994a</td>
</tr>
<tr>
<td><strong>Vernalization pathway</strong></td>
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<td></td>
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<tr>
<td>VERNALIZATION INSENSITIVE3 (VIN3)</td>
<td>Protein with fibronectin repeats and PHD domain</td>
<td>Sung and Amasino, 2004</td>
</tr>
<tr>
<td>VERNALIZATION1 (VRN1)</td>
<td>B3 domain DNA-binding protein</td>
<td>Levy et al., 2002</td>
</tr>
<tr>
<td>VERNALIZATION2 (VRN2)</td>
<td>Sut(z)12-like polycomb protein</td>
<td>Gendall et al., 2001</td>
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</table>

### Table 2. Floral repressive genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein function</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Activators of FLC</strong></td>
<td></td>
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<tr>
<td>FRIGIDA (FRI)</td>
<td>Novel protein</td>
<td>Johanson et al., 2000</td>
</tr>
<tr>
<td>FRIGIDA-LIKE1 (FRL1)</td>
<td>Novel protein related to FRIGIDA</td>
<td>Michaels et al., 2004</td>
</tr>
<tr>
<td>FRIGIDA-LIKE2 (FRL2)</td>
<td>Novel protein related to FRIGIDA</td>
<td>Michaels et al., 2004</td>
</tr>
<tr>
<td>VERNALIZATION INDEPENDENCE3 (VIP3)</td>
<td>Novel WD-repeat protein</td>
<td>Zhang et al., 2003</td>
</tr>
<tr>
<td>VERNALIZATION INDEPENDENCE4 (VIP4)</td>
<td>Protein with homology to the yeast transcriptional activator, Leolp</td>
<td>Zhang and van Nocker, 2002</td>
</tr>
<tr>
<td>EARLY IN SHORT DAYS4 (ESD4)</td>
<td>Nuclear protease regulating SUMOylation</td>
<td>Murta et al., 2003</td>
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<tr>
<td>AERIAL ROSETTE1 (ART1)</td>
<td>Not yet identified</td>
<td>Poduska et al., 2003</td>
</tr>
<tr>
<td>PHOTOPERIOD INSENSITIVE1 (PIE1)</td>
<td>SWI/SNF-helicase-like protein</td>
<td>Noh and Amasino, 2003</td>
</tr>
<tr>
<td><strong>Floral repressors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLOWERING LOCUS C (FLC)</td>
<td>MADS-box transcription factor</td>
<td>Michaels and Amasino, 1999; Sheldon et al., 1999</td>
</tr>
<tr>
<td>FLOWERING LOCUS M (FLM)</td>
<td>MADS-box transcription factor</td>
<td>Scortecci et al., 2001</td>
</tr>
<tr>
<td>SHORT VEGETATIVE PHASE (SVP)</td>
<td>MADS-box transcription factor</td>
<td>Hartmann et al., 2000</td>
</tr>
<tr>
<td>TARGET OF EAR1/2 (TOE1/2)</td>
<td>AP2-like transcription factor</td>
<td>Aukerman and Sakai, 2003</td>
</tr>
<tr>
<td>SCHNARCHZAPFEN/SCHLAFMUTZE (SNZ/SMZ)</td>
<td>AP2-like transcription factor</td>
<td>Schmid et al., 2004</td>
</tr>
<tr>
<td>TFL1</td>
<td>Putative kinase inhibitor</td>
<td>Bradley et al., 1997</td>
</tr>
<tr>
<td>TFL2/LHP1</td>
<td>Heterochromatin protein1 (HP1)-like protein</td>
<td>Gaudin et al., 2001; Kotake et al., 2003</td>
</tr>
<tr>
<td>EMBRYONIC FLOWER1</td>
<td>Novel protein</td>
<td>Aubert et al., 2001</td>
</tr>
<tr>
<td>EMBRYONIC FLOWER2</td>
<td>Sut(z)12-like polycomb protein</td>
<td>Yoshida et al., 2001</td>
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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) (Shio 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 deacetylases (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 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.

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.
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 Pc gene 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 FTT, 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 Ler, 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 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
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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References


