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ARABIDOPSIS: A RICH HARVEST 10 YEARS AFTER COMPLETION OF THE GENOME SEQUENCE

Seasonal and developmental timing of flowering

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SUMMARY

The coordination of the timing of flowering with seasonal and development cues is a critical life-history trait that has been shaped by evolution to maximize reproductive success. Decades of studying many plant species have revealed several of the fascinating systems that plants have evolved to control flowering time: such as the perception of day length in leaves, which leads to the production of a mobile signal, florigen, that promotes flowering at the shoot apical meristem; the vernalization process in which exposure to prolonged cold results in meristem competence to flower; and the juvenile to adult phase transition. Arabidopsis research has contributed greatly to understanding these systems at a molecular level.

Keywords: flowering, photoperiod, florigen vernalization, juvenile, evolution.

INTRODUCTION

Over the past decade there has been an explosion of literature on the timing of flowering in *Arabidopsis thaliana*. The intent of this article is to provide an overview of how Arabidopsis research has contributed to a molecular understanding of some longstanding questions in flowering research: i.e. this is not a comprehensive review, and I apologize in advance to those whose flowering-time papers I have not had space to cite. For more comprehensive coverage of specific aspects of flowering there are many recent reviews (e.g. Turck *et al.*, 2008; Zeevaart, 2008; Adrian *et al.*, 2009; He, 2009; Kim *et al.*, 2009; Michaels, 2009; Yant *et al.*, 2009).

When to initiate flowering is obviously a critical life-history trait that has been shaped, over evolutionary time, to maximize reproductive success in a range of environments. In a broad sense, there are two types of information that plants use to provide input on when to initiate flowering. One is environmental – for example, the changes in temperature and/or day length that consistently reflect seasonal shifts. The other is endogenous. An example of a system involving endogenous cues is the juvenile to adult transition that affects many aspects of plant development, including competence to flower. Specifically, upon germination some plant species enter a juvenile phase in which they are not competent to flower, despite receiving inductive environmental cues. The juvenile phase can be short or long: many annuals become competent to flower after forming just a few leaves, whereas the juvenile phase of certain perennials can be several decades long.

The genetic and biochemical routes by which sensing such cues influences flowering are often referred to as flowering pathways: for example, the photoperiod pathway is the path to flowering caused by exposure to inductive photoperiods. The endogenous changes that permit, or in some cases cause, flowering are sometimes referred to as autonomous pathways. The term 'autonomous' is typically used to indicate that the pathway appears to be independent of environmental sensing.

PHOTOPERIOD AND FLOWERING

Tournois (1914) and Klebs (1918) published the first reports indicating that day length had a role in flowering. The general concept of the photoperiodic induction of flowering (photoperiodism) and the range of response types among plant species was firmly established by Garner and Allard (1920): for example, short-day plants (SDPs) flower when the night length exceeds a critical length, and long-day plants (LDPs) flower as day length increases. There are other response types as well, such as intermediate-day plants and long-short-day plants. A key finding was the demonstration by Knott (1934) that day length is perceived by leaves. Because flowering occurs in the shoot apical meristem (SAM), leaves must transmit a signal to the SAM. This signal is often referred to as florigen (Chailakhyan, 1936). Since the

mid-1930s, much effort was devoted to identifying florigen: most investigations involved making extracts of induced leaves and attempting to set up bioassays that would respond to florigen. After several decades without success, the identity of florigen was sometimes referred to as the 'Holy Grail' of flowering research (Zeevaart, 2008). As discussed below, the 'Holy Grail' was found from the application of genetics and molecular biology to this question, but it is important to emphasize the question was clearly defined by classical physiological studies (Lang, 1965; Zeevaart, 1976). Work in Arabidopsis provided the final piece of this puzzle.

Before discussing the discovery of florigen and progress in understanding photoperiodism, some background information may be useful. Arabidopsis is a quantitative LDP – it flowers more rapidly in LDs, but will eventually flower in SDs (Gregory and Hussey, 1953). Mutants that affect flowering time are readily recognized: for example, mutants in which the LD-promotion of flowering might be compromised will form many more leaves from the primary SAM than their wild-type siblings when grown in LDs, and flower temporally much later (Figure 1a,b). Conversely, mutants in which the photoperiod pathway might be 'hyperactivated' will flower more rapidly, and with fewer leaves, than the wild type in non-inductive SD photoperiods. It is important to note that in Arabidopsis research, mutants that just change growth rate but not leaf number at flowering are not considered flowering 'timing' mutants; rather, a bona fide flowering-time mutant alters 'development time' such that the primary SAM forms more or fewer leaves than the wild type in given conditions (Koornneef *et al.*, 1991). This developmental criterion is not always applied in flowering studies in other species, particularly in crop species in which days to flower is often the only measure of flowering differences among genotypes (e.g. Buckler *et al.*, 2009).

In some of the classic studies of Arabidopsis flowering, two classes of delayed-flowering mutants were identified (Redei, 1962; Koornneef *et al.*, 1991). Mutants delayed only in LDs, but not in SDs, were proposed to affect a day length-sensing pathway, whereas those that were delayed relative to the wild type in both LDs and SDs were classified as affecting a constitutive promotion pathway (Redei, 1962; Koornneef *et al.*, 1991) that has come to be referred to as an autonomous pathway (e.g. Simpson and Dean, 2002). It was

Figure 1. Genetic differences in Arabidopsis flowering behaviors.
(a) Wild-type Wassilewskija (Ws) accession in long days (LDs), which are inductive for flowering. The primary shoot apical meristem (SAM) forms approximately eight rosette and cauline leaves before converting to flower formation.
(b) A *gigantea* mutant in Ws in LDs. The delay in flowering results in the formation of over 25 leaves before flowering. (Leaves formed later in Arabidopsis development are always larger, regardless of whether the delay is genetic or the result of growth in non-inductive photoperiods. Rapid flowering does not provide an opportunity for larger leaves to form.)
(c) Wild-type Columbia accession in LDs. The primary SAM forms approximately 15 rosette and cauline leaves before converting to flower formation.
(d) Columbia plant, into which an active allele of *FRIGIDA* has been introgressed, grown in LDs without vernalization. The SAM has formed over 60 leaves before flowering.

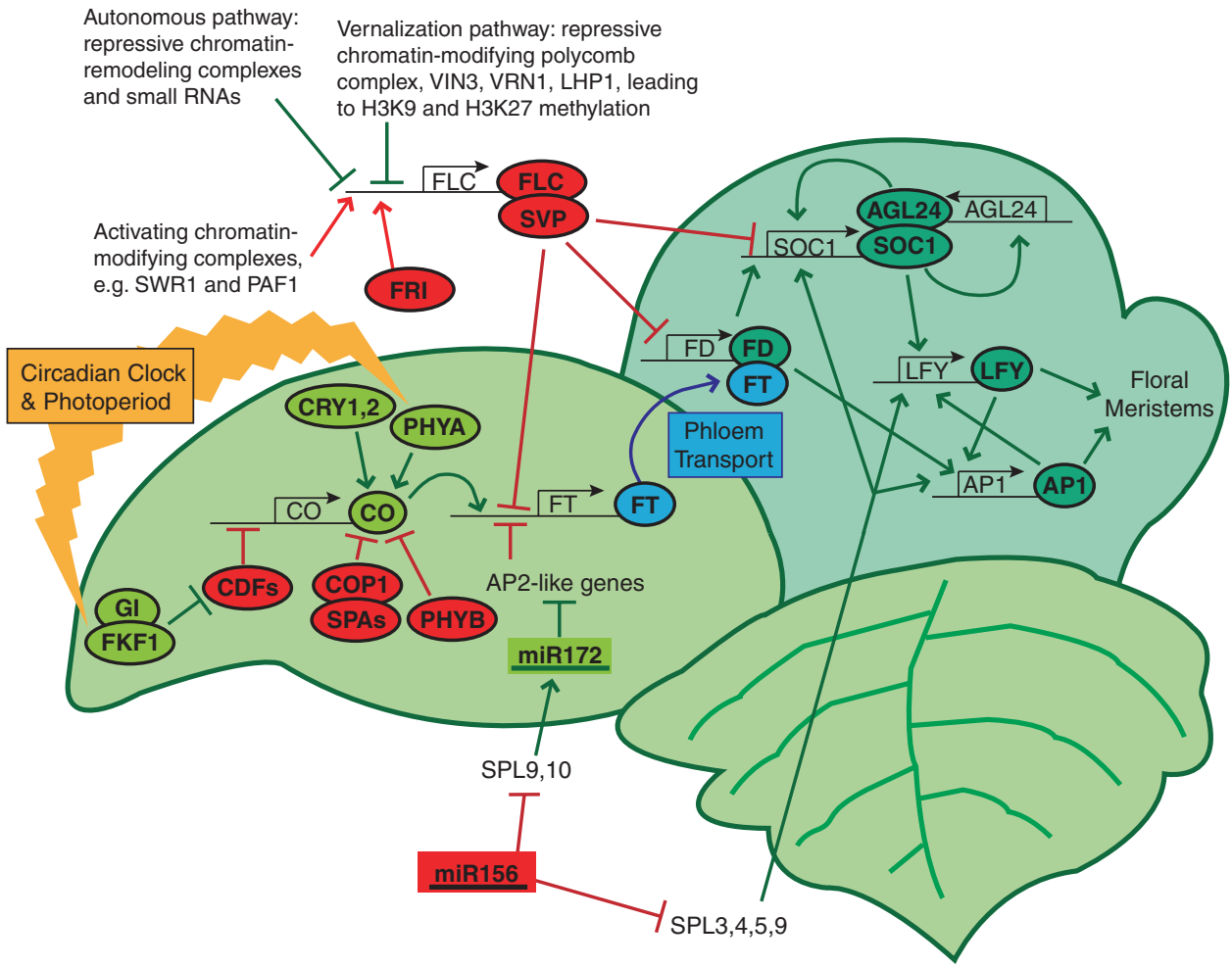

Figure 2. Overview of flowering-time regulation in Arabidopsis.
Genes, proteins (represented as ovals), microRNAs and pathways are described in the text. Solid green or red lines with an arrow represent promotion, and those with a perpendicular bar represent repression. Components that overall promote flowering are shown in green, and those that repress flowering are shown in red.

also proposed, based on the flowering behavior of the mutants, that three of the genes [*CONSTANS* (*CO*), *GIGANTEA* (*GI*) and *FT*[1]] were involved in the production of a flowering promoter in LDs (Koornneef *et al.*, 1991).

IDENTIFICATION OF FLORIGEN

FT is now known to be florigen, and CO and GI are key players in the activation of FT expression. FT is a potent inducer of flowering: it is a small protein with some resemblance to RAF kinase inhibitors (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999). CO is a zinc-finger protein that is likely to act as a transcription factor (Putterill *et al.*, 1995), and GI is a large plant-specific protein involved in circadian clock function (Fowler *et al.*, 1999; Park *et al.*, 1999). *CO* expression is affected by the circadian clock (and thus mutants in clock components like *GI* affect *CO* expression), and CO is necessary for inducing *FT* expression in LDs (Suarez-Lopez *et al.*, 2001; Yanovsky and Kay, 2002) (Figure 2). Indeed, the only role of CO may be to activate expression of *FT* in inductive photoperiods [and to activate an *FT* relative known as *TWIN SISTER OF FT* (*TSF*)] (Wigge *et al.*, 2005; Yamaguchi *et al.*, 2005).

One of the first demonstrations that CO acts in leaves to produce a signal (FT) that moves to the SAM came from the use of various promoters driving *CO* expression. *CO* was effective in promoting flowering when expressed in phloem companion cells of leaves, but SAM expression did not cause flowering (An *et al.*, 2004; Ayre and Turgeon, 2004). On the other hand, *FT* causes flowering when expressed either in leaves or the SAM, and this promotion of flowering does not require CO activity (An *et al.*, 2004). *FT* is normally

[1]Several Arabidopsis 'flowering-time genes' do not have 'full' names, only two or three letter abbreviations (see Koornneef *et al.*, 1991). Over time, FT has come to be called FLOWERING LOCUS T, but others such as FCA, FD, FPA, FVE and FY lack full names.

expressed in the phloem of leaves (Takada and Goto, 2003); however, a combination of elegant genetic and molecular studies indicated that FT acts in the meristem by partnering with bZIP transcription factor FD (Abe *et al.*, 2005; Wigge *et al.*, 2005) (Figure 2). A direct demonstration that FT does in fact move from young leaves to the SAM was demonstrated with FT:GFP and FT:MYC fusion proteins in Arabidopsis, and in rice using an FT ortholog:GFP fusion (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Tamaki *et al.*, 2007). Also, in grafts of *Cucurbita moschata*, movement of an FT ortholog across a graft junction in the phloem system was correlated with flowering (Lin *et al.*, 2007). These studies provide strong evidence that the long-standing mystery of the identity of florigen is solved.

Studies on the photoperiod pathway in LDP Arabidopsis have been complemented by studies in SDP rice: these studies show how similar components such as CO/HEADING DATE 1 and FT/HEADING DATE 3a can be 'wired' to create a photoperiod pathway that responds to SD in rice and to LD in Arabidopsis (for a review see Turck *et al.*, 2008). Moreover, studies in poplar show that a 'CO/FT module' is involved in the induction of dormancy in response to perception of changing day length (Bohlenius *et al.*, 2006). Thus, components of the photoperiod pathway first discovered in Arabidopsis are likely to be widely used in higher plants for a range of developmental events that are under day length control. Furthermore, the recent demonstration of a role for CO in circadian outputs in the green algae *Chlamydomonas reinhardtii* indicates that *CO*-like genes may be ancient regulators of photoperiod-regulated processes that pre-date the appearance of flowering plants (Serrano *et al.*, 2009).

There has been much progress in understanding the interface between *CO* transcription, photoperiod and the circadian clock (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007; Fornara and Coupland, 2009; Imaizumi, 2009) (Figure 2). Proteins referred to as CYCLING DOF FACTORS (CDFs) exhibit circadian cycling, and bind to and repress the *CO* promoter. The abundance of CDFs is controlled by an F-box protein, FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1), which is a blue-light photoreceptor that appears to be involved in the ubiquitin-mediated degradation of CDFs. The clock protein GI physically interacts with and stabilizes FKF1, promoting CDF degradation and subsequent *CO* expression at certain times of the circadian cycle in the proper photoperiod. This interface is likely to be conserved throughout the plant kingdom, and its discovery serves as a general paradigm in eukaryotes for how the clock can be linked to outputs. There has been tremendous progress from Arabidopsis research in understanding the molecular workings of the plant circadian clock that is not discussed above, but this progress has been recently reviewed elsewhere (Harmer, 2009; Imaizumi, 2009; Mas and Yanovsky, 2009; McClung, 2009).

Photoperiod and light quality also affect *FT* expression through stability of the CO protein. CRYPTOCHROME 1 (CRY1) and CRY2, which are blue-light receptors, and PHYTOCHROME A (PHYA), which is a red/far-red receptor, stabilize CO protein at the end of an LD photoperiod, but another red/far-red receptor, PHYTOCHROME B (PHYB), promotes the turnover of CO (Valverde *et al.*, 2004). The involvement in CO turnover of the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1), as well as SUPPRESSOR OF PHYA-105 (SPA) family proteins, which interact with COP1, implicates regulated proteolysis via the proteasome in the control of CO activity (Valverde *et al.*, 2004; Laubinger *et al.*, 2006; Jang *et al.*, 2008; Liu *et al.*, 2008b).

After FT/florigen travels to the SAM, promotion of flowering requires interaction with a meristem-expressed transcription factor of the 'bZIP' family, known as FD (Figure 2). The evidence for this critical FT/FD interaction comes from both protein interaction studies as well as genetic studies showing that loss of *FD* suppresses the effects of overexpressing *FT* (Abe *et al.*, 2005; Wigge *et al.*, 2005). The FT/FD complex activates downstream floral meristem-identity genes such as *APETALA 1* (*AP1*) (Abe *et al.*, 2005; Wigge *et al.*, 2005), and other floral promoters such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Michaels *et al.*, 2005; Yoo *et al.*, 2005): these genes direct certain groups of cells in the flanks of the SAM (primordia) to differentiate into floral meristems. There are other pathways that lead to the increased expression of downstream genes such as *AP1*: for example, *LEAFY* (*LFY*), which itself promotes differentiation of primordia into floral meristems, and can be activated by a gibberellin pathway, also activates *AP1* (Blazquez *et al.*, 1998; Liljegren *et al.*, 1999). Consistent with multiple pathways affecting the formation of flowers in the SAM, double mutants of *lfy* and either *ft* or *fd* have a stronger phenotype than single mutants: in the double mutants, primordia produce leaves instead of flowers (Ruiz-Garcia *et al.*, 1997; Abe *et al.*, 2005; Wigge *et al.*, 2005).

Once flowering commences in an Arabidopsis SAM it is irreversible. This irreversibility seems to be to the result of multiple positive feedback loops involving genes expressed in the SAM activating the expression of each other, such as *LFY* and *AP1*, or *SOC1* and *AGAMOUS-LIKE 24*, which are activators of *LFY* (Liljegren *et al.*, 1999; Michaels *et al.*, 2005; Sablowski, 2007; Lee *et al.*, 2008; Liu *et al.*, 2008a) (Figure 2). Not all plant species exhibit this irreversibility of flowering in an individual meristem: for example, in some species reversion to vegetative growth can occur if exposure to inductive photoperiods is not maintained (Zeevaart, 1985; Tooke *et al.*, 2005).

VERNALIZATION: 'REMEMBERING' WINTER

Vernalization is the process by which prolonged exposure to cold renders plants competent to flower (Chouard, 1960).

As with photoperiodism, aspects of the molecular basis of vernalization were first revealed from studies with Arabidopsis, but there was also a wealth of physiological research that provided a foundation for subsequent genetic and molecular studies (for reviews of physiological studies see Chouard, 1960; Lang, 1965; Bernier *et al.*, 1981).

For most plant species, a requirement for vernalization serves to prevent flowering in the fall season, and then permits flowering the following spring. In temperate climates, for example, many winter annuals and biennials occupy a niche in which they become established during the fall season to enable rapid flowering in spring. Because temperatures often fluctuate widely in the fall, using cold as a reliable environmental cue for flowering requires that plants perceive the duration of cold. For example, it is important that vernalization-requiring plants do not become vernalized by exposure to a short period of cold early in the fall season, because a short cold period followed by warm temperatures later in the fall might result in flowering as winter begins. Thus, exposure to periods of cold that are typical of a winter season in a given region are usually required for effective vernalization of plants well adapted to that region, and, as expected, the duration of cold exposure and the optimal temperature for vernalization vary among species, and within ecotypes of a given species. The photoperiod response often shows a remarkable range of adaptation as well: for example, ecotypes of *Xanthium strumarium*, an SDP, exhibit a wide range in the length of night required for flowering, and this corresponds to the great latitudinal range of this species (Ray and Alexander, 1966; McMillan, 1974).

Unlike photoperiod, which is perceived in leaves, cells of the SAM directly sense cold and become vernalized. This has been demonstrated in a wide range of vernalization-requiring species by two types of experiments: localized chilling and grafting (Chouard, 1960; Lang, 1965; Bernier *et al.*, 1981). Furthermore, in many species the vernalized state is mitotically stable: i.e. once SAM cells have become vernalized they retain a 'memory' of a prior cold exposure and competence to flower. This was first demonstrated by Lang and Melchers working with a biennial variety of henbane (*Hyoscyamus niger*) that has an obligate requirement for both cold exposure followed by inductive photoperiods. Vernalized henbane plants 'remember' a prior cold treatment throughout a long period of growth in non-inductive photoperiods because the vernalized plants flower when shifted to inductive photoperiods (Lang, 1965). Another demonstration that the vernalized state can be mitotically stable is that vernalized plants can be regenerated from vernalized cells. This was first performed in the crucifer *Lunaria biennis* (Wellensiek, 1962), and is also the case in Arabidopsis (Burn *et al.*, 1993b). However, there are also species, including crucifers, in which the vernalized state is not stable (i.e. the plants do not 'remember' winter), and thus a cold-promoted transition to flowering must take place during cold exposure (Bernier *et al.*, 1981; Wang *et al.*, 2009b).

THE VERNALIZATION REQUIREMENT IN ARABIDOPSIS

The physiological studies described above raised the question of how cold exposure renders the SAM competent to undergo the floral transition. Part of the answer to this question in Arabidopsis came from studies of the genetic basis of natural variation in flowering. Typical 'lab strains' of Arabidopsis flower rapidly without a vernalizing cold treatment – in fact, such types were chosen as lab models because the lack of a vernalization requirement results in a more rapid life cycle (Figure 1a,c). However, there are many Arabidopsis accessions that require a vernalizing cold treatment of several weeks to flower rapidly (Figure 1d). Analyses of segregating populations derived from crosses of vernalization-requiring and rapid-flowering types provides a way to assess the number of genes involved in the natural variation for vernalization requirement. This approach was first taken by Napp-Zinn, who found that the vernalization requirement is often caused by a dominant gene that he named *FRIGIDA* (*FRI*) (Napp-Zinn, 1987). Many years after Napp-Zinn's natural variation studies, several groups found dominant alleles of *FRI* associated with a vernalization requirement in a broad range of accessions (Burn *et al.*, 1993a; Lee *et al.*, 1993; Clarke and Dean, 1994; Koornneef *et al.*, 1994).

The molecular cloning of *FRI* demonstrated that dominant alleles of *FRI* encoded a full-length protein, whereas recessive alleles contained mutations that would render the protein non-functional (Johanson *et al.*, 2000). Thus, the ancestral state of *A. thaliana* was vernalization-requiring, and rapid-flowering types arose independently several times from loss-of-function mutations (Johanson *et al.*, 2000). There have been a number of studies examining the possible adaptive value of the presence or absence of an active *FRI* allele in different environments (e.g. McKay *et al.*, 2003; Brock *et al.*, 2009; Scarcelli and Kover, 2009; Wilczek *et al.*, 2009).

Natural variation studies also revealed a second gene, *FLOWERING LOCUS C* (*FLC*), that, with *FRI*, confers a vernalization requirement (Koornneef *et al.*, 1994; Lee *et al.*, 1994). The molecular cloning of *FLC*, which encodes a MADS-box protein, provided insight into the molecular basis of vernalization-mediated competence of the Arabidopsis SAM (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Overexpression of *FLC*, even in the absence of a functional *FRI* allele, prevents flowering, demonstrating that *FLC* is a potent suppressor of flowering. The block to flowering in the fall season in vernalization-requiring strains of Arabidopsis is largely a result of the *FRI*-mediated elevation of *FLC* expression to a level that suppresses flowering. Vernalization turns off *FLC* expression, and

thereby creates an SAM that is competent to undergo the floral transition. Vernalization has no effect on *FRI* expression (Michaels *et al.*, 2004); rather, as discussed below, vernalization appears to render *FLC* insensitive to the activating effects of *FRI*.

FLC suppresses flowering, at least in part, by repressing the expression of the floral activators *SOC1*, *FT* and *FD* (Figure 2): *FLC* has been shown to bind directly to these activators (Hepworth *et al.*, 2002; Helliwell *et al.*, 2006; Searle *et al.*, 2006). There is much redundancy in the genome/proteome of most plant species, and in Arabidopsis there is a clade of *FLC* relatives in which most members have been shown to be flowering repressors (Scortecci *et al.*, 2001; Ratcliffe *et al.*, 2003). As noted above, there is an *FT* relative, *TSF*, that appears to be redundant with *FT*. In most situations, *FLC* seems to be the major repressor of flowering, and *FT* seems to be the major contributor to florigen, but it is likely that other *FLC* relatives contribute to the repression of *SOC1* and *FT*, and that *FLC* and its relatives also directly repress the *FT* relative *TSF* (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005).

MADS-domain proteins often work in multimeric complexes with other MADS-domain proteins. FLC (and perhaps other members of the FLC clade) appears to interact with the MADS-domain protein SHORT VEGETATIVE PHASE (SVP). SVP binds to *FT* and *SOC1* – the same targets as FLC (Lee *et al.*, 2007; Li *et al.*, 2008). The SVP–FLC interaction is important for flowering, as loss of *SVP* partially suppresses the ability of *FLC* to inhibit flowering (Fujiwara *et al.*, 2008; Li *et al.*, 2008). That loss of *SVP* causes only partial suppression of the effect of *FLC* indicates that there may be other redundant *FLC* partners. The level of *FLC* expression, but not that of *SVP*, plays a key role in flowering-time control. *FLC* mRNA levels change in response to environmental cues like vernalizing cold, whereas *SVP* levels do not, and increasing *FLC* alone is sufficient to cause extremely late flowering (Michaels and Amasino, 2001), indicating that the levels of potential FLC binding partners are not limiting.

As noted above, allelic variation at *FRI* is a major determinant of natural variation for the vernalization requirement. *FRI* is a member of a small family of proteins found only in the plant kingdom, and is located in the nucleus (Johanson *et al.*, 2000). In Arabidopsis, two other members of the *FRI* family [*FRI-LIKE 1* (*FRL1*) and *FRL2*] are required for *FLC* to be expressed to levels that create a vernalization requirement (Michaels *et al.*, 2004; Schlappi, 2006). That the *FRI* family is found in all sequenced higher plant genomes, regardless of whether the species has a vernalization response, indicates that members of the family are likely to play roles in aspects of plant growth and development other than flowering. However, *FRI* itself appears to specifically affect *FLC* expression: in microarray studies the major gene that appears to be upregulated by the presence of *FRI* is *FLC* (see http://www.weigelworld.org/resources/microarray/microarray).

Studies of the molecular components required, along with FRI and FRI family members, for active *FLC* expression in the fall have identified a range of genes, most of which, perhaps not surprisingly, encode components of chromatin-modifying complexes involved in covalent modifying histone residues to promote active chromatin. That many of these genes are involved in chromatin modification is inferred from prior studies of homologous genes in other species, and from studies of the changes in the spectrum of modifications at *FLC* chromatin when such genes are mutated. In brief, these studies have revealed the requirement for methylation of histone 3 at lysines 4 and 36 by the Arabidopsis equivalent of the RNA Polymerase-II Associated Factor-1 (PAF1) complex, mono-ubiquitination of histone 2B at lysine 123 by a RAD6-BRE1-type complex, and H2A.Z deposition by a SWR1/SRCAP-type complex. Several recent reviews discuss the components of these complexes (He, 2009; Kim *et al.*, 2009). This research direction has provided much insight into how chromatin modifications are used in developmental contexts in plants. Not surprisingly, *FRI* affects the state of *FLC* chromatin (Doyle and Amasino, 2009; Jiang *et al.*, 2009), but whether or not FRI is involved directly in interactions with chromatin-modifying complexes remains to be determined.

In addition to the FRI family and chromatin-modifying complexes, other components required for active *FLC* expression have been identified, such as the plant-specific zinc-finger proteins FRIGIDA-ESSENTIAL 1 (FES1) (Schmitz *et al.*, 2005) and SERRATE (SE), and the mRNA cap-binding complex (Bezerra *et al.*, 2004). *FES1* appears to be specific to flowering (Schmitz *et al.*, 2005), whereas lesions in *SE* or genes encoding mRNA cap-binding complex subunits have pleiotropic phenotypes (e.g. Prigge and Wagner, 2001; Bezerra *et al.*, 2004), as expected for proteins involved in mRNA processing as well as the production of microRNAs (Gregory *et al.*, 2008; Laubinger *et al.*, 2008). That loss of SE or the mRNA cap-binding complex results in lower levels of *FLC* mRNA could be because *FLC* RNA processing is quite sensitive to a lack of these factors (Bezerra *et al.*, 2004). A recent report indicates that FRI interacts with the mRNA cap-binding complex, and may elevate *FLC* expression through a co-transcriptional mechanism linking FLC transcription with RNA processing (Geraldo *et al.*, 2009).

A COLD-MEDIATED EPIGENETIC SWITCH

As discussed above, the vernalized state is mitotically stable in many plant species, including Arabidopsis. Although the definition of an epigenetic switch is not universally agreed upon, one could consider the vernalization-mediated repression of *FLC* epigenetic in the following sense: *FLC* is repressed in response to a transient signal (cold), and the

'off switch' is maintained through mitotic cell divisions in the absence of the cold signal (e.g. Amasino, 2004).

The mechanism by which vernalization results in the mitotically stable repression of *FLC* has been extensively reviewed (Adrian *et al.*, 2009; Greenup *et al.*, 2009; He, 2009; Kim *et al.*, 2009; Michaels, 2009), and thus only an overview is provided below. First, as with other aspects of flowering, much of the current understanding has genetics at its foundation: in this case screens for mutants in which vernalization is impaired. The first 'vernalization gene' reported was *VERNALIZATION 2* (*VRN2*), which encodes an Arabidopsis homolog of the *Drosophila* protein SUPPRESSOR OF ZESTE 12 (Gendall *et al.*, 2001). SUPPRESSOR OF ZESTE 12 is part of a Polycomb repression complex that modifies chromatin (Muller *et al.*, 2002). In *vrn2* mutants, *FLC* expression decreases during long cold exposure, but the repression of *FLC* is not maintained. Although chromatin modification was a candidate for the epigenetic nature of *FLC* repression, the identification of *VRN2* was the first indication that this was likely to be the case. Subsequently, using chromatin immunoprecipitation (ChIP), it was indeed shown that during and after vernalization the levels of two repressive histone modifications, histone H3 Lys 9 (H3K9) and H3K27 methylation, increase at *FLC* chromatin (Bastow *et al.*, 2004; Sung and Amasino, 2004).

A mutant screen also revealed a gene, *VERNALIZATION-INSENSITIVE 3* (*VIN3*), that is only expressed during the prolonged cold required for vernalization: i.e. *VIN3* has a 'vernalization-specific' expression pattern (Sung and Amasino, 2004). In *vin3* mutants, vernalization-mediated changes in *FLC* chromatin, such as H3 deacetylation, and H3K9 and H3K27 methylation, do not occur (Sung and Amasino, 2004). Although the cold induction of *VIN3* is necessary for the vernalization process, overexpression of *VIN3* is not sufficient to cause vernalization (Sung and Amasino, 2004). VIN3 contains a plant homeodomain (PHD) domain: PHD domains can bind to specific histone modifications and are often found in proteins involved in chromatin modifications in a range of eukaryotes (Mellor, 2006). There is a family of VIN3-like proteins in Arabidopsis, and certain family members can dimerize via interactions of their carboxy-terminal domains (Sung *et al.*, 2006; Greb *et al.*, 2007). Another member of the family that can dimerize with VIN3, VERNALIZATION 5 (VRN5)/VIN3-LIKE 1 (VIL1), is also required for vernalization, and for chromatin changes at *FLC* (Sung *et al.*, 2006; Greb *et al.*, 2007).

VRN2, VIN3 and VRN5/VIL1 appear to be part of a repressive complex that is similar to the Polycomb repression complex 2 (PRC2), which has been described in a range of eukaryotes (Wood *et al.*, 2006; De Lucia *et al.*, 2008). PRC2 adds methyl groups to H3 at K27 residues (H3K27me3) via the enzyme activity of the ENHANCER of ZESTE [E(Z)] subunit (Muller *et al.*, 2002). The complex with VIN3 and VIL1/VRN5 has been shown to contain at least two Arabidopsis E(Z) homologs: CURLY LEAF (CLF) and SWINGER (SWN) (Wood *et al.*, 2006; De Lucia *et al.*, 2008).

The H3K27 methylation activity of PRC2 complexes in animals is enhanced by an association with PHD-domain proteins (Cao *et al.*, 2008; Sarma *et al.*, 2008), and recent work indicates that during vernalization VIN3 and VRN5/VIL1 are likely to serve in that enhancing role in Arabidopsis, with respect to activity towards *FLC* (De Lucia *et al.*, 2008). Thus, the molecular events that initiate *FLC* repression during vernalization include the induction of *VIN3* by cold, and probably the enhancement of the repressive activity of a PRC2-like complex at *FLC* by a dimer of VIN3 with VRN5/VIL1 (De Lucia *et al.*, 2008). After cold exposure, *VIN3* expression ceases (Sung and Amasino, 2004), but the levels of H3K27me3 at *FLC* continue to increase (Finnegan and Dennis, 2007; De Lucia *et al.*, 2008), and the association of VRN5/VIL1 with *FLC* chromatin becomes widespread (De Lucia *et al.*, 2008).

Mitotically stable maintenance of repression requires a mechanism to maintain the repressed state through rounds of DNA replication. A general maintenance mechanism is the establishment of feedback loops in which chromatin-modifying complexes recognize certain modifications, and maintain those modifications through cycles of DNA replication. PHD domains can bind to specific chromatin modifications, and thus PHD-domain proteins could 'read' the histone code (Mellor, 2006). It is tempting to speculate that the PHD domain proteins involved in vernalization, VIN3 and VRN5/VIL1, might enhance Polycomb-mediated repression through interactions with modified histones at the *FLC* locus (De Lucia *et al.*, 2008; Kim *et al.*, 2009). Because VIN3 is transiently expressed only during cold and is required for all vernalization-mediated chromatin modifications, it may be part of the system that, along with VRN5/VIL1 (Sung *et al.*, 2006; Greb *et al.*, 2007), initiates the switch of the *FLC* expression state. *VRN5/VIL1,* which is constitutively expressed, remains associated with *FLC* chromatin after cold exposure, and thus is likely to contribute to maintenance (De Lucia *et al.*, 2008). Of course the chromatin changes at *FLC* are likely to be a progression with no clear demarcation between the initiation and progression of gene repression (e.g. De Lucia *et al.*, 2008; Kim *et al.*, 2009).

Certain chromatin-modifying complexes and chromatin modifications do, however, appear to be primarily involved in maintenance. As discussed above, H3K9 methylation is an additional repressive chromatin mark that accumulates at *FLC* as a result of vernalization-mediated repression, and appears to be involved in maintenance (Bastow *et al.*, 2004; Sung and Amasino, 2004; Sung *et al.*, 2006; Greb *et al.*, 2007). For example, in *vrn1* mutants, *FLC* is repressed during cold and H3K27 methylation increases during cold. However, H3K9 methylation does not increase in *vrn1* mutants, and *FLC* silencing cannot be maintained after plants return to warm conditions (Levy *et al.*, 2002; Bastow *et al.*, 2004;

Sung and Amasino, 2004). VRN1 is a plant-specific protein that contains DNA-binding domains (Levy *et al.*, 2002), and it is likely to be part of a feedback loop that maintains H3K9 methylation. Another likely player in an H3K9 methylation feedback loop is LIKE HETERCHROMATIN PROTEIN 1 (LHP1)/TERMINAL FLOWER 2 (TFL2). A related protein from animals, HP1, binds to chromatin by interacting with methylated H3K9, and is involved in gene silencing (e.g. Maison and Almouzni, 2004). In *lhp1* mutants, *FLC* repression also cannot be maintained after plants resume growth in warm conditions (Mylne *et al.*, 2006; Sung *et al.*, 2006), and H3K9 trimethylation only transiently increases during cold (Sung *et al.*, 2006).

In animals, maintenance of repression that initiates with PRC2 typically requires another Polycomb complexL Polycomb repression complex 1 (PRC1). PRC1 binds to H3*K27*me3 and helps to maintain that histone modification (e.g. Muller *et al.*, 2002). However, the Arabidopsis genome does not contain genes encoding PRC1 components (e.g. Schubert *et al.*, 2005), and, as discussed above, LHP1 and VRN1 may serve in the analogous role for 'locking in' *FLC* repression. It is intriguing that the creation of a stable silenced state at *FLC* appears to require PRC2-mediated H3K27 methylation, as well as H3K9 methylation. LHP1 has been shown to bind H3K27me3 and H3K9me3 *in vitro*, and, in broad genome studies, to be associated with regions of the Arabidopsis genome enriched in H3K27me3, but not with regions enriched in H3K9me3 (Turck *et al.*, 2007). However, at certain regulated loci such as *FLC*, 'crosstalk' between H3K9 and H3K27 methylation that involves LHP1 and VRN1 may be necessary for mitotically stable repression. Although LHP1 and VRN1 are required to maintain H3K9 methylation, these proteins are not methyltransferases, and the actual enzymes that carry out this modification at *FLC* remain to be determined.

A major question is how silencing is targeted to *FLC*. There is no evidence that VIN3 has the ability to recognize a specific DNA sequence, and, as noted above, *VIN3* expression alone is not sufficient to initiate *FLC* silencing. Recently, it was found that the level of *FLC* antisense transcripts increases substantially and rapidly during cold exposure (Swiezewski *et al.*, 2009). Perhaps the increase in *FLC* antisense transcripts is a key initiating event in *FLC* silencing: for example, the increase in antisense transcripts might be necessary for the increased activity of a VIN3-containing Polycomb complex at *FLC*. However, there is not yet direct evidence for a role for the antisense transcripts in vernalization, such as a demonstration that eliminating the production of antisense transcripts abrogates *FLC* silencing.

It is also not known how *FLC* is reset to the 'on state' as it is passed to the next generation. Because germ cells are not set aside in plant development as they are in animals, meristem cells in which *FLC* has been repressed by vernalization produce the next generation in which *FLC* becomes active. Although there have been reports investigating when in development the resetting of *FLC* occurs (Sheldon *et al.*, 2008; Choi *et al.*, 2009), the mechanism by which this occurs has not yet been identified.

A final area of vernalization that is ripe for exploration is the mechanism of long-term cold sensing. At present, nothing is known about the mechanism of cold sensing during vernalization in Arabidopsis, or in any other plant species. In Arabidopsis, the induction of *VIN3* and increased *FLC* antisense transcription are formally outputs of a cold-sensing system, but there have not been any reports of genetic variation in these outputs that might provide an entrée to a better understanding of cold sensing.

VERNALIZATION IN ARABIDOPSIS: A PARADIGM?

As discussed above, because of the ancient nature of the photoperiod pathway, progress in understanding the molecular basis of how photoperiod affects flowering in Arabidopsis has provided a molecular foundation for photoperiod sensing in all flowering plants. This is not likely to be the case for vernalization. Flowering plants diversified a few hundred million years ago (Soltis *et al.*, 2008) during an era, and in regions, in which it is likely that temperate-type winters were not encountered (i.e. when the climate overall was much warmer and the continents had not drifted to their present locations). Subsequently, continental drift, climate change and radiation resulted in new environments in which a vernalization response would have had adaptive value.

That vernalization pathways arose by convergent evolution is supported by studies in cereals (for reviews see Distelfeld *et al.*, 2009; Greenup *et al.*, 2009; Kim *et al.*, 2009). For example, although the vernalization requirement in both Arabidopsis and cereals is to the result of a repressor of flowering that is turned off during cold exposure, the repressors are unrelated proteins. As discussed above, the Arabidopsis repressor FLC is a MADS-domain protein, whereas in cereals the repressor (VRN2) is a zinc-finger protein that has no ortholog in the Arabidopsis genome (Yan *et al.*, 2004). There are other differences in the 'circuitry' of vernalization between Arabidopsis and cereals as well (e.g. Distelfeld *et al.*, 2009; Greenup *et al.*, 2009; Kim *et al.*, 2009). As expected, however, in both Arabidopsis and cereals the vernalization pathway appears to be superimposed onto a more ancient and conserved photoperiod pathway. Thus, Arabidopsis has served as a paradigm for how a vernalization pathway can be constructed, and how such a pathway can interface with other flowering pathways (Figure 2). Other long-term cold responses, such as the breaking of bud dormancy by cold exposure, are also likely to have arisen independently in different groups of plants, and it will be interesting to explore how vernalization and bud dormancy systems evolved in a range of plant groups.

AUTONOMOUS FLOWERING

As noted in the Introduction, the term 'autonomous' is typically used in flowering for pathways that are independent of environmental cues. In many plant species, for example, flowering cannot occur until a transition is made from the juvenile to adult phase (e.g. Poethig, 2003): this transition is an example of an autonomous pathway that results in competence to flower.

In the Arabidopsis flowering literature, a specific autonomous pathway has been operationally defined in genes that when mutated cause delayed flowering in both LDs and SDs (as opposed to the photoperiod pathway, which was defined by mutations that affect flowering only in LDs) (e.g. Simpson and Dean, 2002). Such autonomous-pathway mutants have arisen in screens for delayed flowering in 'lab strains' of Arabidopsis, which, as discussed above, do not have an active allele of *FRI*, and flower rapidly without a vernalizing cold treatment. However, mutations in autonomous-pathway genes in such lab strains create a robust vernalization response similar to that of *FRI*-containing lines (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991; Simpson and Dean, 2002). Furthermore, as described earlier for *FRI*-containing lines, the delayed flowering of these autonomous-pathway mutants is caused by elevated levels of *FLC* expression, and the robust vernalization response is caused by the repression of *FLC* (Michaels and Amasino, 2001).

Several autonomous-pathway genes that fit the above definition, namely *FCA, FLOWERING LOCUS K HOMOLOGY DOMAIN* (*FLK*), *FPA* and *FY,* have been shown to, or are predicted to, encode proteins involved in RNA metabolism (Macknight *et al.*, 1997; Schomburg *et al.*, 2001; Simpson *et al.*, 2003; Lim *et al.*, 2004; Manzano *et al.*, 2009), and it is possible that the autonomous-pathway gene *LUMINIDEPENDENS* (*LD*), which encodes a homeodomain-containing protein, is also involved in RNA metabolism, as homeodomain-containing proteins have been shown to bind RNA (Chan and Struhl, 1997). Other autonomous-pathway genes encode components of chromatin-remodeling complexes (for reviews see He, 2009; Kim *et al.*, 2009; Michaels, 2009). *RELATIVE OF EARLY FLOWERING 6* (*REF6*) encodes a jumonji protein belonging to a certain class of histone demethylases (Noh *et al.*, 2004; Agger *et al.*, 2008). *FLOWERING LOCUS D* (*FLD*) encodes a protein belonging to a different class of histone demethylases that were first found in histone deacetylase (HDAC) complexes in other systems (He *et al.*, 2003; Shi *et al.*, 2004). *FVE* encodes a member of an MSI1-like protein family (Ausin *et al.*, 2004; Kim *et al.*, 2004); MSI1s are conserved WD-repeat proteins found in several chromatin-modifying complexes in eukaryotes, including HDAC complexes (Hennig *et al.*, 2005). Histone acetylation patterns at *FLC* are perturbed in *fve* and *fld* mutants (He *et al.*, 2003; Ausin *et al.*, 2004; Kim *et al.*, 2004).

In a recent series of papers, certain histone-modifying protein arginine methyltransferases (PRMT4a, PRMT4b, AtPRMT5 and AtPRMT10) have also been shown to be autonomous-pathway genes involved in *FLC* repression (Wang *et al.*, 2007; Niu *et al.*, 2008; Schmitz *et al.*, 2008). In *prmt* mutants, *FLC* expression is elevated, and the level of histone arginine methylation at *FLC* chromatin is reduced. Interestingly, PRMT5 also appears to be required for *FLC* silencing during vernalization in an *FRI* backgound, but is not required for vernalization-mediated silencing in the absence of *FRI* (Schmitz *et al.*, 2008).

It is important to note that the autonomous-pathway genes discussed above are involved in more than *FLC* regulation and flowering-time control. One of the first indications of this was the demonstration that a double mutant between a weak *fy* allele and *fpa* was lethal (Koornneef *et al.*, 1998), and the later demonstration that a null allele of *fy* is lethal (Henderson *et al.*, 2005). More recently it has been shown that double mutant combinations of many autonomous-pathway mutants have severe pleiotropic phenotypes (Veley and Michaels, 2008). Furthermore, many autonomous-pathway genes have recently been shown to play a broad role in RNA-mediated gene silencing, and in the expression of certain transposons and their DNA methylation patterns (Baurle *et al.*, 2007; Baurle and Dean, 2008; Veley and Michaels, 2008). Another connection between autonomous-pathway effects on *FLC* expression and RNA-mediated gene silencing is the observation that double mutants of *DICER-LIKE 1* and *DICER-LIKE 3* (*dcl1 dcl3*; dicers are involved in small RNA formation) exhibit *FLC*-dependent delayed flowering that is vernalization responsive: i.e. the *dcl1 dcl3* double mutant has a phenotype similar to typical autonomous-pathway mutants (Schmitz *et al.*, 2007). Thus, small RNAs are likely to be involved in *FLC* repression, perhaps by guiding chromatin-modifying complexes to *FLC* chromatin (e.g. Baurle and Dean, 2008).

In summary, the 'classical' Arabidopsis autonomous pathway does not appear to be a linear pathway, but rather represents a collection of genes that are: (i) broadly involved in repression of gene expression, and (ii) participate in setting the 'basal' levels of *FLC* expression.

Recently, an additional flowering pathway has been described that is 'autonomous' in the sense that it represents a developmental pathway, parts of which are independent of environmental variables (for reviews see Fornara and Coupland, 2009; Poethig, 2009; Yant *et al.*, 2009). Studies of the juvenile to adult phase transition revealed that the role of a particular microRNA, miR156, is to promote the juvenile phase in Arabidopsis and maize, and one consequence of this promotion is to prevent precocious flowering (Wu and Poethig, 2006; Chuck *et al.*, 2007). Conversely, expression of another microRNA, miR172, promotes flowering, at least in part, by repressing APETALA 2-like repressors of FT (Figure 2) (Aukerman and Sakai, 2003; Jung *et al.*, 2007; Mathieu

et al., 2009). The levels of miR156 and miR172 exhibit contrasting age/development-specific expression patterns: miR156 levels decline during development whereas miR172 levels correspondingly increase (e.g. Chuck *et al.*, 2007; Wu *et al.*, 2009). In Arabidopsis, the age/development-specific expression patterns of these microRNAs are independent of day length: i.e. they are under autonomous control (Wang *et al.*, 2009a). Recently, it has been shown that there is a regulatory circuit consisting of these microRNAs and transcription factors of the SQUAMOSA PROMOTER BINDING-LIKE (SPL) class: part of this circuit consists of miR156 repressing expression of certain *SPL* genes that are positive regulators of miR172 expression (Wu *et al.*, 2009). Some miR156-repressed *SPL* genes are also positive regulators of other flowering promoters (Figure 2) (Cardon *et al.*, 1999; Wang *et al.*, 2009a; Yamaguchi *et al.*, 2009). Thus, as development proceeds, the decline of miR156 levels, and the increase in levels of miR172 and certain *SPL*s, leads to the activation of *FT* in leaves, and the increase in *SPL*s in the meristem leads to the activation of several genes that promote the floral transition, including *SOC1*, a *SOC1* homolog *AGAMOUS-LIKE 42* and *FUL* (Wang *et al.*, 2009a), as well as the meristem-identity genes *LFY* and *AP1* (Yamaguchi *et al.*, 2009). In contrast to the particular autonomous pathway that sets basal levels of *FLC* expression and appears to be specific to a subset of flowering plants, the mir156/miR172/SPL system is likely to represent an autonomous flowering pathway that is conserved in flowering plants, as it is present in maize, rice and Arabidopsis (e.g. Poethig, 2009).

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