The Timing of Flowering[1]

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The initiation of flowering is a critical life-history trait; plants have presumably evolved to flower at a time of year that ensures maximal reproductive success in a given region. Decades of physiological studies have revealed that flowering is initiated in response to both environmental cues and endogenous pathways. Commonly studied environmental cues include changes in temperature and daylength. Endogenous pathways function independently of environmental signals and are related to the developmental state of the plant; such pathways are sometimes referred to as "autonomous" to indicate the lack of environmental influence. The relative contributions of autonomous and environmental inputs to the flowering "decision" vary among, and even within, species. For example, flowering is considered entirely due to autonomous pathways in a variety of tobacco (*Nicotiana tabacum*) that forms a fixed number of nodes before flowering regardless of the environment in which it is grown (McDaniel and Hsu, 1976). Yet, a single-gene change can cause tobacco to require short days to flower (Allard, 1919), which indicates that the underlying biochemical differences between environment-sensing and endogenous pathways can be minimal. Also, endogenous and environmental pathways can interact. For example, some plants pass through a juvenile phase in which they are not responsive to environmental cues that promote flowering (Poethig, 1990); that is, the transition from the juvenile to adult phase is a type of endogenous pathway that is necessary to provide competence for environmental pathways to promote flowering. The recent addition of molecular genetics to the range of approaches used to study the initiation of flowering has provided some molecular insights into these endogenous and environment-sensing pathways and has revealed how inputs from multiple pathways are integrated into the flowering decision.

(Due to the sustained efforts of a multitude of scientists working in many species, we have learned much about the timing of flowering that is worth celebrating. Unfortunately, only a small part of this extensive body of work can be covered in this article because of length and reference limits. Accordingly, we frequently refer readers to recent review articles for more in-depth discussions, and we apologize to our colleagues whose work was not cited due to these constraints.)

PHOTOPERIODISM AND FLORIGEN: AN ANCIENT PATHWAY

The annual fluctuations in daylength that occur over much of the surface of our planet provide a reliable environmental cue regarding the time of year. It is not surprising, therefore, that the pathways that detect and promote flowering in response to photoperiod are among the most ancient and conserved. Physiological experiments first done in the 1930s (Knott, 1934) demonstrated that inductive photoperiods are sensed by leaves. This raised two fundamental questions: how do leaves measure daylength, and what is the nature of the flowering signal (known as florigen) that must travel from the leaves to the shoot apical meristem? After another seven decades of research, we now have relatively clear and satisfying answers to these questions, especially in Arabidopsis (*Arabidopsis thaliana*).

Arabidopsis flowers more rapidly in long days than in short days and is thus a facultative long-day plant. The regulation of the floral promoter CONSTANS (CO) is key in the perception of inductive long days (Turck et al., 2008). The circadian clock regulates *CO* transcription such that peak expression occurs late in the day in long days but after dusk in short days (Suarez-Lopez et al., 2001). CO protein, in turn, is stabilized by light and rapidly degraded in darkness (Valverde et al., 2004). As a result, CO protein can only accumulate during inductive long days. CO is expressed in the vasculature of leaves, and its role in flowering is to activate the expression of *FLOWERING LOCUS T* (*FT*), which encodes a small protein that is florigen (Fig. 1). In both rice (*Oryza sativa*) and Arabidopsis, FT is a strong promoter of flowering that is translocated from the vasculature of leaves to the shoot apical meristem (Corbesier et al., 2007; Tamaki et al., 2007). In the meristem, FT forms a complex with the bZIP transcription factor FD and initiates flowering by activating floral meristem-identity genes such as *APETALA1* and other floral promoters such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Michaels, 2009). Thus, *FT* up-regulation lies at the end of an environment-sensing pathway and initiates flower development. In addition to the pho-

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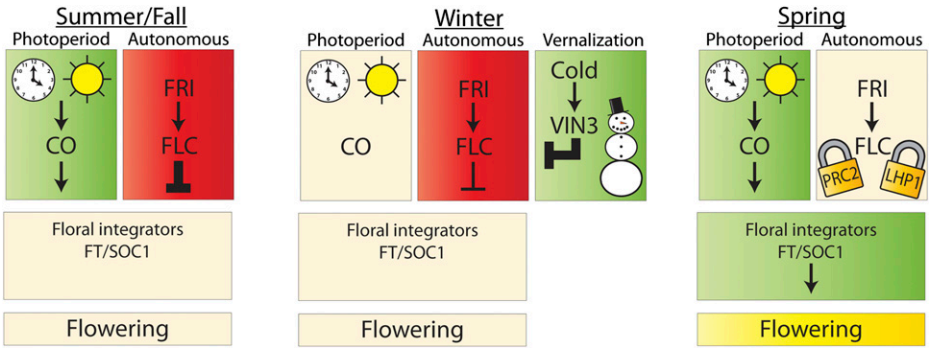

Figure 1. Seasonal regulation of flowering in winter-annual Arabidopsis. The flowering pathways that are active in each season are indicated by green or red boxes; green is promotive and red is repressive. Beige indicates inactive. In the summer/fall establishment phase, FLC prevents flowering by repressing floral integrators that would otherwise be induced by CO in response to long days (left). During the short days of winter, the photoperiod pathway is not active and vernalization leads to the induction of *VIN3* and epigenetic repression of *FLC* (center). By the spring season, *FLC* repression is complete and is maintained by PRC2 and LHP1 (and VRN1; not pictured); in the lengthening days of spring, CO activates floral integrators free of competition from FLC and flowering is initiated (right).

toperiod pathway, *FT* and *SOC1* are also regulated by other flowering pathways (e.g. vernalization; see below) and therefore are referred to as floral integrators.

The coupling of CO and FT appears to be an ancient and evolutionarily adaptable module. Unlike the situation in Arabidopsis, in which long days lead to CO activation and *FT* induction, the rice CO/FT homologs (HEADING DATE1/HEADING DATE3A) have evolved different circuitry that triggers flowering in response to short days (Turck et al., 2008). It will be interesting to explore the possible role of CO and FT in more complex photoperiod response types, such as various species of *Bryophyllum*, which require long days followed by short days for flowering to occur (i.e. plants maintained under constant long or short days do not flower). There is also evidence that the role of CO and FT extends beyond flowering. In poplar (*Populus* spp.) trees, CO and FT are involved in the initiation of photoperiod-dependent dormancy (Turck et al., 2008). There is also intriguing data demonstrating that the use of CO as a daylength indicator may predate flowering plants. *Chlamydomonas reinhardtii* lacks *FT* but does contain a *CO*-like gene (*CrCO*) that is an output of the circadian clock; remarkably, *CrCO* can partially rescue *co* mutants in Arabidopsis (Serrano et al., 2009). Given that CO exists in a relatively large gene family (17 *CO*-like genes in Arabidopsis), it is possible that *CO*-related genes play additional yet-to-be-discovered roles in plant responses to daylength.

VERNALIZATION

Vernalization is defined as the process by which exposure to the cold of winter renders plants competent to flower (Kim et al., 2009). The passage of winter is an environmental cue that, when coupled to photoperiod sensing, provides clear seasonal information that distinguishes the spring and fall seasons. For cold to be a reliable cue for winter, plants need to be able to distinguish the long cold exposure characteristic of winter from short fluctuations in temperature that might occur, for example, in the fall. Thus, it is not surprising that vernalization (and in many species the breaking of bud dormancy) requires exposure to prolonged cold. A vernalization requirement is often found in winter-annual and biennial plants that flower early in the spring; these plants typically become established in the fall, and a vernalization requirement ensures that premature flowering does not occur during the fall establishment phase.

In winter-annual Arabidopsis, the vernalization-responsive block to flowering requires the interaction of two genes, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*; Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). *FLC* is a MADS domain-containing transcription factor that acts as a floral repressor, and *FRI* is a plant-specific gene of unknown biochemical function that is required for high levels of *FLC* expression. FLC inhibits flowering by directly repressing the key promoters of flowering, *FT*, *SOC1*, and *FD* (Michaels, 2009; Fig. 1). Vernalization permits plants to flower rapidly in the lengthening days of spring through repression of *FLC* (Fig. 1). *FRI* and *FLC* were first identified genetically in crosses between winter-annual and rapid-cycling accessions (Napp-Zinn, 1979; Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994; Gazzani et al., 2003; Michaels et al., 2003); winter annuals contain functional alleles of both genes, whereas rapid-cycling accessions contain loss-/reduction-of-function mutations in either *FRI* or *FLC* (Kim et al., 2009). Thus, rapid-cycling accessions evolved from winter annuals by shedding the vernalization requirement conferred by the interaction of *FRI* and *FLC*.

After winter has passed, there is a permanent "memory" of winter in many plant species (i.e. the

vernalized state is stable during subsequent growth and mitotic cell division). Mitotic stability in the absence of the inducing signal (cold) is a classic definition of an epigenetic change of state (Amasino, 2004). In Arabidopsis, the epigenetic nature of the vernalized state results from a series of modifications to *FLC* chromatin that result in mitotically stable repression. Specifically, the levels of two repressive modifications, trimethylation of histone H3 at Lys-9 (H3K9) and Lys-27 (H3K27), increase at *FLC* chromatin during and after cold exposure (Bastow et al., 2004; Sung and Amasino, 2004). H3K27 methylation at *FLC* results from the activity of Polycomb Repressive Complex2 (PRC2), which was first identified in animals and is conserved in eukaryotes (Kim et al., 2009). During cold exposure, *VERNALIZATION INSENSITIVE3* (*VIN3*), a gene encoding a plant-specific component of the PRC2 complex that is essential for *FLC* repression, is induced (Wood et al., 2006; De Lucia et al., 2008). The PRC2 complex in plants and animals is involved in the repression of a large number of genes, but in Arabidopsis, the cold-induced VIN3 is a component specific for the vernalization process; thus, the VIN3-containing version of PRC2 is likely to target a vernalization-specific subset of genes. There is a family of *VIN3*-like genes in Arabidopsis (Kim et al., 2009), and why VIN3 is specifically critical for vernalization-mediated silencing of *FLC* is an intriguing issue to resolve. It is also intriguing that during cold exposure, there is a transient increase in expression of a noncoding RNA complementary to *FLC* known as COOLAIR (Swiezewski et al., 2009), but it remains to be determined what role, if any, this RNA plays in vernalization-mediated *FLC* silencing.

Polycomb repression in animals does not typically involve H3K9 methylation, whereas repression of *FLC* involves both H3K9 and H3K27 methylation. The methylase involved in vernalization-mediated H3K9 methylation has not been identified, but the plant-specific VERNALIZATION1 (VRN1) protein and a plant relative of a protein first identified in animals that binds methylated H3K9 (LIKE HETERCHROMATIN PROTEIN1 [LHP1]) are required to maintain H3K9 methylation and *FLC* repression (Kim et al., 2009). It is interesting that in animals, a Polycomb complex called PRC1 is involved in maintaining Polycomb-mediated repression, but plants do not possess PRC1 components; thus, VRN1 and LHP1 may play a PRC1-like role.

AUTONOMOUS CONTROL OF FLOWERING

In Arabidopsis, two antagonistic autonomous pathways regulate prevernalization levels of *FLC* expression. *FLC* is positively regulated by the FRI pathway and negatively regulated by a group of genes known collectively as the autonomous floral-promotion pathway. In winter annuals, the FRI pathway acts epistatically to the autonomous pathway and activates *FLC* expression to create vernalization-responsive late flowering. In rapid-cycling accessions, which typically lack functional alleles of *FRI*, the autonomous pathway represses *FLC*; thus, recessive autonomous-pathway mutants are late flowering due to high levels of *FLC* expression and are vernalization responsive. It is important to note that vernalization represses *FLC* expression without impacting the expression of *FRI* or autonomous-pathway genes. This, as well as other data, indicates that the effect of *FRI* and the autonomous pathway on *FLC* does not appear to be regulatory per se; rather, they are involved in setting basal levels of *FLC* expression via constitutive activation/repression.

Although the genetic circuitry by which *FRI* and the autonomous pathway control *FLC* expression is well established, our knowledge of molecular mechanism remains limited. The predicted biochemical functions of many autonomous-pathway proteins suggest that *FLC* repression may involve a coupling of RNA-binding/processing and chromatin-remodeling events (Kim et al., 2009; Michaels, 2009). Three proteins, FCA, FPA, and FLOWERING LOCUS K, contain RNA-binding domains, and a fourth, FY, shows homology to RNA-processing factors. In addition, *dicer-like1 dicer-like3* double mutants have elevated levels of *FLC*, suggesting that small RNA processing may play a role in *FLC* repression. Other autonomous-pathway proteins act, or are predicted to act, as histone methyltransferases (e.g. several members of the PRMT family) or histone demethylases (e.g. FLOWERING LOCUS D and RELATIVE OF EARLY FLOWERING6). One of the remaining major challenges in Arabidopsis is to determine how these RNA- and chromatin-related elements function together to set the level of *FLC* expression. One thing that has recently become clear is that the function of many of the so-called autonomous-pathway genes is not restricted to the regulation of flowering time. Although the phenotypes of most autonomous-pathway single mutants are largely limited to delayed flowering, some autonomous-pathway double mutants show strong pleiotropic phenotypes and many autonomous-pathway mutants show defects in gene silencing (Baurle et al., 2007; Veley and Michaels, 2008). Interestingly, these loss-of-gene-silencing phenotypes are correlated with changes in DNA methylation at the affected loci. The fact that DNA methylation is not observed at the *FLC* locus suggests that proteins of the autonomous pathway may participate in multiple repressive pathways.

Genetic screens conducted in *FRI*-containing or autonomous-pathway mutant backgrounds have also identified many genes required for high levels of *FLC* expression (Kim et al., 2009; Michaels, 2009). Perhaps not surprisingly, many of these proteins are associated with activating histone modifications. For example, such screens have identified components of a RNA polymerase II-associated factor 1 complex, which promotes activating H3K4 and H3K36 methylation, as well as other complexes that promote histone 2B

monoubiquitination and deposition of the histone variant H2A.Z. Although the majority of these genes are required for high levels of *FLC* expression in *FRI*-containing or autonomous-pathway mutant backgrounds, it is interesting that some genes, such as *FRI-LIKE1*, *SUPPRESSOR OF FRI4*, and *FRI ESSENTIAL1*, are only required for the up-regulation of *FLC* by FRI (Michaels et al., 2004; Schmitz et al., 2005; Kim et al., 2006; Kim and Michaels, 2006). Thus, in terms of molecular mechanism, the activation of *FLC* in an autonomous-pathway mutant is not exactly the same as the activation of *FLC* by FRI. It will be quite interesting to discover the molecular relationships between the common and pathway-specific components that positively regulate *FLC*.

Whether *FLC*-like genes repress flowering outside of the crucifers is an open question. It is possible that the *FLC*-based pathways described above are crucifer specific; however, other autonomous pathways may be more widespread. There is a microRNA, miR156, involved in the timing of the juvenile-to-adult transition in both maize (*Zea mays*) and Arabidopsis, and expression of this microRNA delays flowering, whereas expression of another microRNA, miR172, promotes flowering in part by relieving *FT* repression (Fornara and Coupland, 2009; for more on miRNAs and phase transitions, see Poethig, 2010). Expression of these microRNAs is in part under developmental control; thus, this system could be considered a more conserved autonomous pathway.

INTEGRATION OF PHOTOPERIOD AND VERNALIZATION

As discussed above, the basic photoperiod pathway appears to be conserved in flowering plants, and as illustrated in Figure 1, in Arabidopsis the circuitry of how the vernalization and photoperiod pathways interact is clear: FLC represses expression of flowering promoters (integrators) until this repression is removed through the silencing of *FLC* by vernalization. However, in cereals, the vernalization pathway is distinct from that in Arabidopsis. In the cereal pathway, there is a flowering repressor, *VRN2*, that, like *FLC*, is turned off during cold exposure. However, FLC is a MADS box protein, whereas VRN2 is a zinc-finger protein that does not have a homolog in the Arabidopsis genome. *FLC* expression is repressed solely by cold, whereas in cereals, *VRN2* expression is repressed by cold, short days, and induction of the meristem-identity gene *VERNALIZATION1* (note: in cereals, *VERNALIZATION1* is a MADS box gene unrelated in amino acid sequence to Arabidopsis *VRN1*).

Despite their differences, there is a common feature of the interface between the vernalization and photoperiod pathways in Arabidopsis and cereals: both FLC and VRN2 repress the key photoperiod pathway gene *FT* (*VRN3* in cereals). This example of convergent evolution in how the pathways interface is perhaps not surprising. In contrast to the ancient photoperiod pathway, vernalization pathways arose after the divergence of major groups of flowering plants, as an adaptation to the new environments created by climate change and continental drift (Amasino, 2010). As vernalization pathways evolved, *FT* presented a prime regulation point for floral repression. It will be interesting to determine how vernalization pathways have been "constructed" in other groups of plants and how often these pathways target *FT* expression.

How plants sense and measure the prolonged cold of winter and transduce this into a vernalization response is not understood. An output of this cold-sensing system in Arabidopsis is the induction of *VIN3* expression and increased expression of the COOLAIR RNA. However, at present, genetic variation (i.e. mutants or natural variation) in the cold-sensing system has not been identified, and there are not any biochemical clues to how this system operates. It will be quite interesting, as well as a challenge, to understand the molecular basis of how plants sense prolonged cold for both vernalization and the breaking of bud dormancy, and whether such a system is conserved or has independently evolved multiple times, as appears to be the case for downstream parts of the vernalization pathway in cereals and Arabidopsis.

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