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Flowering time regulation produces much fruit

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Many of the molecular details regarding the promotion of flowering in response to prolonged exposure to cold temperatures (vernalization) and daylength have recently been elucidated in Arabidopsis. The daylength and vernalization pathway converge in the regulation of floral promoters referred to as floral integrators. In the meristem, vernalization promotes flowering through the epigenetic repression of the floral repressor *FLOWERING LOCUS C*. This allows for the induction of floral integrators by *CONSTANS* under inductive long days. In the vasculature of leaves, CONSTANS protein is produced only in long days where it acts to promote the expression of *FLOWERING LOCUS T* (*FT*). FT protein is then translocated to the meristem where it acts to promote floral induction. Thus a detailed molecular framework for the regulation of flowering time has now been established in Arabidopsis.

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Introduction

Physiological studies, performed largely during the early to mid-20th century, have identified a number of interesting characteristics concerning the promotion of flowering by both daylength (a.k.a photoperiod) and prolonged exposure to cold temperatures. Grafting experiments and experiments in which inductive photoperiods are applied to specific portions of the plant, indicate that photoperiod perception takes place in leaves, a site that is physically separated from the site of flower production (the shoot apical meristem, SAM). These observations led to the suggestion (ca. 1936) that a mobile flower-promoting signal, termed florigen, is produced in the leaves in response to inductive photoperiods and travels to the shoot apical meristem to induce flowering. With regard to vernalization, one of the most interesting properties is that cold-treated plants retain a relatively permanent memory of vernalization. Cuttings of *Lunaria biennis*, for example, taken from cold-treated plants regenerate into flowering plants, whereas cuttings from non-cold-treated plants yield only vegetative plants after regeneration [1]. Thus the memory of vernalization is stable even through the regeneration of plants from tissue culture. Although cells have a mitotically stable memory of vernalization, the vernalized state is not passed on to the next generation, thus each generation of plants must experience winter before flowering.

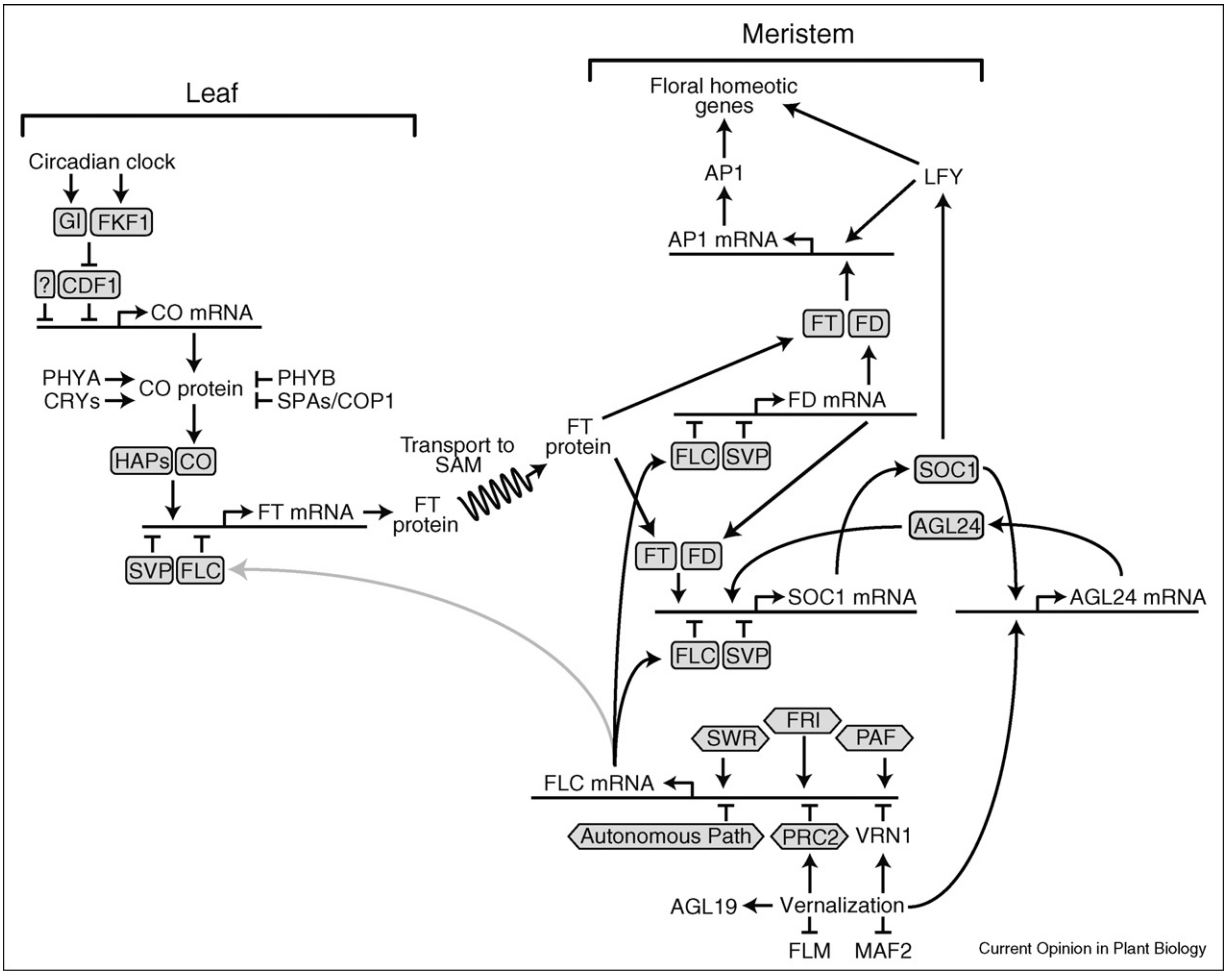
In recent years, a great deal of progress has been made in understanding the molecular mechanisms that regulate flowering time, particularly in Arabidopsis. This is not a comprehensive review, but rather will highlight some of the recent advances in our understanding of flowering time regulation by photoperiod and vernalization, the implications for florigen and the mitotically stable memory of vernalization, and the integration of signals from multiple environment-sensing pathways into a single flowering decision.

CONSTANS is a critical component in the regulation of flowering by daylength

Arabidopsis flowers more rapidly under long days than under short days. The ability to distinguish long days from short days is largely the result of the complex regulation of the B-box containing gene *CONSTANS* (*CO*). *CO* acts as a floral promoter and is regulated at both the mRNA and protein levels (Figure 1). *CO* transcription is regulated by the circadian clock; expression is low early in the day, but increases sharply 8–10 hours after dawn [2,3]. CO protein, in turn, is stabilized by light and degraded in darkness [4]. Because peak *CO* mRNA levels occur late in the day under long days, but after dusk in short days, CO protein is only produced and stabilized under long days. As a result, CO accumulates, and hence promotes flowering, in a long-day specific manner.

The circadian regulation of *CO* mRNA requires a number of proteins, which are themselves regulated by the circadian clock. CYCLING DOF FACTOR1 (CDF1) binds to the *CO* promoter and acts as a negative regulator of *CO* transcription [5]. *CDF1* mRNA is highly expressed in the early part of the day, when CO transcript levels are lowest [5]. The repression of *CO* by CDF1 is removed by the activities of *GIGANTEA (GI)* and *FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1)*, which are expressed late in the day [5–7,8•]. FKF1 contains an F-box and is likely to be a subunit of an SCF ubiquitin ligase. FKF1 and GI have been shown to physically interact with each other and CDF1, suggesting that the FKF1–GI complex is involved in targeting CDF1 for

Figure 1



A simplified model for the regulation of flowering time by photoperiod and vernalization. Interactions depicted by proteins in shaded boxes are thought to be direct. Hexagons depict protein complexes. *FLC* is expressed to highest levels in the shoot apex, but is also expressed in leaves.

degradation [8•]. It should be noted that, although *CDF1* overexpression suppresses *CO* transcription, reduction of function mutants in *CDF1* do not strongly increase *CO* mRNA levels [5]. This suggests that there are likely to be additional repressors of *CO* transcription yet to be identified that act redundantly with CDF1.

When expressed from a constitutive promoter, CO protein accumulates under white, blue, or far-red light, but is degraded in red light or darkness [4]. Multiple photoreceptors have been implicated in the regulation of CO protein; PHYTOCHROME B (PHYB) promotes the degradation of CO early in the day, whereas PHYA, CRYPTOCHROME1 (CRY1), and CRY2 stabilize CO late in the day [4]. The degradation of CO protein is thought to occur via ubiquitination and proteolysis by the 20S proteasome [4] and is likely to involve the SUPPRESSOR OF PHYA-105 (SPA) family of proteins. SPA1 has been shown to physically interact with the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) and in a *spa1 spa3 spa4* triple mutant, CO protein accumulates to higher levels than in wild type, despite the fact that *CO* mRNA levels are unchanged [9,10•,11•,12]. Consistent with this model, *cop1* mutants have also recently been shown to contain elevated levels of CO protein [13]. These data support a model in which a SPA–COP1 complex plays an important role in CO protein degradation.

FLOWERING LOCUS C is the primary target of vernalization

In contrast to rapid-cycling Arabidopsis, many naturally occurring accessions are late flowering unless vernalized and thus behave as winter annuals. The vernalization-responsive block to flowering is created by the interaction of two genes: *FLOWERING LOCUS C (FLC)*, a MADS-

domain-containing transcription factor that acts as a floral repressor [14,15], and *FRIGIDA (FRI)*, a gene of unknown biochemical function that is required for high levels of *FLC* expression [14–16]. Vernalization in turn leads to an increase in repressive histone modifications, such as histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27) methylation, at the *FLC* locus [17,18]. Thus *FLC* appears to be a direct target of vernalization. It should be noted that although *FLC* is the major target of vernalization in Arabidopsis, *flc* null mutants do exhibit a weak vernalization response. This result indicates that there are other targets of vernalization. Recent work has shown that other MADS-domain containing genes, such as *FLOWERING LOCUS M (FLM)/MADS AFFECTING FLOWERING 1 (MAF1), MAF2, AGAMOUS-LIKE 24 (AGL24)*, and *AGL19*, are also regulated by vernalization [19] (Figure 1).

The repression of *FLC* by vernalization involves both an initial repression of *FLC* during the cold and subsequent maintenance of repression after return to warm temperatures. Although these two processes are closely related, the activities of several vernalization-associated genes are more closely associated with either the initial repression of *FLC* or maintenance. For example, mutations in the PHD-domain containing *VERNALIZATION INSENSITIVE 3 (VIN3)* or its homolog *VERNALIZATION 5 (VRN5)/VIN3-LIKE 1 (VIL1)* primarily block the initial repression of *FLC* during cold treatment [18–21]. In contrast, *FLC* is initially repressed by vernalization in *vrn1* and *vrn2* mutants, but the repression is not maintained upon return to warm temperatures [18–21]. It should be noted, however, that the separation between initial and maintenance repression of *FLC* is not clear-cut. For example, *VRN1* and *VRN2* have also been shown to play a role in the initial repression of *FLC* [22]. VIN3 and VRN2 have been shown to participate in a Polycomb Repressor Complex 2 (PRC2)-like complex with other chromatin-remodeling proteins such as CURLY LEAF (CLF), SWINGER (SWN), and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) that are involved in H3K27 methylation [23•]. LIKE HETEROCHROMATIN 1 (LHP1) has been shown to bind to H3K27 methylated histones [24] and *lhp1* mutants are defective in the maintenance of *FLC* suppression after vernalization [25•,26•]. LHP1 shows increased binding to the *FLC* locus following vernalization and thus may play a role in recognizing vernalization induced H3K27 marks and mediate the mitotically stable suppression of *FLC*, possibly via heterochromatinization. A major unanswered question in vernalization is how cold is perceived and how the length of the cold period is measured. *VIN3* is the most upstream component thus far identified in the vernalization pathway. *VIN3* expression, however, is only induced after several weeks of cold treatment (e.g. *VIN3* is itself a target of the vernalization pathway) [18]. Thus the fact that *VIN3* is regulated by vernalization, suggests that there are additional upstream components of the vernalization pathway yet to be identified.

It is interesting to note that, in addition to the repression of *FLC* by vernalization, chromatin remodeling has also been implicated in the positive regulation of *FLC*. Recent work from several laboratories have shown that the upregulation of *FLC* by FRI requires the activity of chromatin-remodeling complexes similar to the RNA Polymerase II Associated Factor 1 (PAF1) and SWR1 complexes from yeast [27–31]. In yeast the PAF1 complex facilitates transcription by recruiting the histone methyltransferases SET1 and SET2. These enzymes are responsible for H3K4 and H3K36 methylation respectively, which are marks associated with transcriptionally active chromatin. The SWR1 complex also plays a role in the regulation of chromatin structure in yeast by inserting histone variant H2A.Z in to chromatin. Mutations in the Arabidopsis orthologs of many of the PAF1 and SWR1 complex components prevent the upregulation of *FLC* by FRI.

Negative regulation of *FLC* by the autonomous floral-promotion pathway

Most rapid-cycling accessions of Arabidopsis contain naturally occurring loss-of-function mutations in *FRI* and therefore have low levels of *FLC* expression and are early flowering [14–16]. Genetic screens in rapid-cycling backgrounds have identified a group of genes that act to constitutively repress *FLC*. These genes are collectively referred to as the 'autonomous' floral-promotion pathway. Autonomous-pathway mutants contain elevated levels of *FLC* and are late-flowering; like winter annuals, however, *FLC* can be epigenetically silenced by vernalization [14,15]. There are seven 'classic' autonomous pathway genes: *FCA*, *FLOWERING LOCUS D* (*FLD*), *FLOWERING LOCUS K* (*FLK*), *FPA*, *FVE*, *FY*, and *LUMINIDEPENDENS* (*LD*). FCA, FPA, FLK, and FY are predicted to have functions relating to RNA binding or RNA metabolism, LD contains a divergent homeodomain, and FVE and FLD are homologs of human histone deacetylase complex (HDAC) components, a retinoblastoma-associated protein and a histone demethylase, respectively [32].

Although many of the molecular details of how these genes act to repress *FLC* expression are still unknown, an intriguing picture is beginning to emerge that links RNA metabolism with chromatin structure and transcription. FLD and FVE are thought to function directly at the *FLC* locus and facilitate the deacetylation of *FLC* chromatin [33]. Interestingly, recent studies have shown that the RNA-binding proteins FCA and FPA are also localized to *FLC* chromatin and both proteins require FLD function for the promotion of flowering [34–36]. Thus both RNA-binding and chromatin-remodeling activities are important at the *FLC* locus. In addition to their roles in the

regulation of flowering time, several recent studies have also demonstrated that autonomous-pathway genes play important roles in gene silencing and other aspects of development as well [34,35,37]. A major unanswered question is the identity of the RNA molecule(s) that FPA, FCA, and FLK might be binding. Small antisense RNA molecules corresponding to the 3′ untranslated region of *FLC* have been identified, however, their significance in the regulation of *FLC* remains unclear [38].

Integration of flowering signals from the photoperiod and vernalization pathways

The photoperiod and vernalization pathways regulate flowering time through the regulation of a group of genes referred to as floral integrators. These genes, which include *FLOWERING LOCUS T (FT)*, the *FT* homolog *TWIN SISTER OF FT (TSF)*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)/AGAMOUS LIKE 20 (AGL20)*, act as strong floral promoters [32] and are antagonistically regulated by CO and FLC (Figure 1).

In non-vernalized winter annuals or AP-mutants, *FLC* is highly expressed and acts to repress the expression of *FT*, *SOC1*, and possibly *TSF*, thereby delaying flowering [39–43]. Several studies have provided data that FLC is likely to bind directly to *FT* and *SOC1* [40,44•]. MADS-domain-containing transcription factors often function in heteromultimeric complexes and recent work suggests that FLC acts in a complex with another MADS protein, SHORT VEGETATIVE PHASE (SVP) [45]. Like FLC, SVP acts as a floral repressor [45]. In addition, SVP physically interacts with FLC and mutations in *svp* largely suppress the late-flowering phenotype caused by FLC [46,47•]. Thus it appears that, similar to the case in floral development, multiple MADS proteins may participate in a single complex.

In rapid-cycling Arabidopsis, or winter annuals/AP-mutants following vernalization, FLC levels are low. It should be noted, however, that full expression of the floral integrators requires not only elimination of the repression by FLC, but also activation by long days through CO. Unlike FLC, CO has not been demonstrated to bind the floral integrators directly. A current model for the biochemical activity of CO is that it acts as part of a Heme Activator Protein (HAP)-like complex [48••,49,50]. In yeast, the HAP complex binds DNA and is composed of HAP2, HAP3, and HAP5 subunits. CO contains domains that exhibit similarity to HAP2, suggesting that CO might replace HAP2 in a HAP complex. This model is supported by the findings that CO interacts with HAP3 and HAP5 in yeast and in plants, and that alteration of HAP expression levels affects flowering time [48••,49,51]. A complicating factor in this investigation, however, is the fact that each of the HAP subunits is encoded by a family of 10–13 genes in Arabidopsis.

Spatial considerations

Physiological and grafting experiments in many species have demonstrated the site of photoperiod sensing (leaves) is physically separated from the site of vernalization perception and flower production (the shoot apex). Consistent with the model that photoperiodic induction leads to the production of a mobile signal, CO and FT expression occurs in the phloem of leaves [52,53]. Further, recent experiments in Arabidopsis and other species indicate that FT is likely to be that signal; FT produced in leaves is translocated to the meristem [54••,55••]. At the meristem, FT physically interacts with bZIP transcription factor FD and the FT/FD complex activates the expression of *SOC1* and the floral meristem identity gene *APETALA1 (AP1)* [42,56–58]. In contrast to CO and FT, FLC is expressed at highest levels in the shoot apex where it represses expression of *SOC1* and *FD* [41,59–61]. It should be noted, however, that FLC is also expressed to a lesser extent in leaves where it acts to repress *FT*.

Conclusions

Thanks to the sustained efforts of many laboratories we now have detailed molecular framework for the regulation of flowering time by photoperiod and vernalization (Figure 1). The action of these pathways is nicely illustrated in the case of FRI-containing winter-annual Arabidopsis. For plants that germinate in the summer or fall, high levels of FLC expression repress the expression of the floral promoters *FT*, *SOC1*, and *FD*, thereby preventing flowering before winter. Vernalization in turn removes this block to flowering by epigenetically silencing *FLC* via repressive histone modifications. The removal of *FLC* then allows for the induction of *FT* and *SOC1* in response to inductive photoperiods. In vasculature of leaves CO protein accumulates under long days and activates transcription of *FT*. FT protein in turn is translocated to the SAM where it acts with FD to promote the expression of *SOC1* and *AP1*, which leads to the induction of floral development. With this knowledge in hand, it will be very interesting to investigate other species to determine the degree to which these mechanisms are conserved in other species. Studies to date in other species suggest that there is likely to be a good deal of conservation in the photoperiod pathway, however, it appears that there may be more divergence in the regulation of flowering time by vernalization [62,63].

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