Published in final edited form as:
Adv Bot Res. 2014 January 1; 72: 1–28. doi:10.1016/B978-0-12-417162-6.00001-8.

Photoperiodic flowering regulation in *Arabidopsis thaliana*

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Abstract

Photoperiod, or the duration of light in a given day, is a critical cue that flowering plants utilize to effectively assess seasonal information and coordinate their reproductive development in synchrony with the external environment. The use of the model plant, *Arabidopsis thaliana*, has greatly improved our understanding of the molecular mechanisms that determine how plants process and utilize photoperiodic information to coordinate a flowering response. This mechanism is typified by the transcriptional activation of *FLOWERING LOCUS T (FT)* gene by the transcription factor CONSTANS (CO) under inductive long-day conditions in *Arabidopsis.* FT protein then moves from the leaves to the shoot apex, where floral meristem development can be initiated. As a point of integration from a variety of environmental factors in the context of a larger system of regulatory pathways that affect flowering, the importance of photoreceptors and the circadian clock in CO regulation throughout the day has been a key feature of the photoperiodic flowering pathway. In addition to these established mechanisms, the recent discovery of a photosynthate derivative trehalose-6-phosphate as an activator of *FT* in leaves has interesting implications for the involvement of photosynthesis in the photoperiodic flowering response that were suggested from previous physiological experiments in flowering induction.

Keywords

Photoperiodism; Flowering; Phenology; Circadian Clock; Florigen

I. INTRODUCTION

Seasonal variation in climate has selected for the ability of organisms to predict future environmental conditions and use this information to complete necessary adjustments to thrive. The tilt of the earth's axis relative to the sun throughout the solar year can lead to radical changes in weather patterns and temperature, especially in non-equatorial regions (Thomas and Vince-Prue, 1996). Survival often depends on the development of strategies to cope with suboptimal conditions and to use optimal ones as fully as possible. Precise timing of key events in the span of a life cycle is a key trait for organisms faced with a seasonally shifting environment. The timing of the reproductive cycle is a good example of this phenomenon; in a substandard environment premature flowering can have severe implications for relative fitness. For plants dependent on pollinators for reproduction,

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flowering also must to be timed with the seasonal availability of other organisms (Hegland et al., 2009). As an irreversible process in most species, the timing of the reproductive transition in plants is especially critical (Kobayashi and Weigel, 2007).

The topic of how plants are able to recognize what constitutes optimal conditions for flowering has been an active area of research for almost a century. Wightman Garner and Henry Allard, two researchers at the USDA, were the first to empirically describe that the duration of light in a 24-hour period is a key cue for the induction of flowering in many plant species. Originally interested in explaining why soybeans planted sequentially over the summer decreased in days to flower as they were planted later in the season, they sought to find the casual variable behind the phenomenon. Over the course of two years from 1918 to 1920, they experimentally manipulated exposure of plants to light and dark cycles by moving plants from a common outdoor plot into darkened sheds. Through the careful control of light and dark duration to simulate different seasonal light conditions, they were able to determine critical durations of light or darkness that are required for induction of flowering in over 12 plant species and many different cultivars. This general principle of an exhibited response triggered by a change in day length, they coined “photoperiodism” (Garner and Allard, 1920). This revolutionary idea changed the thinking about seasonal responses by suggesting that the mechanism for sensing seasonal changes could be tied specifically to the sensing of duration of light in a given day. In addition, they found that plants could be grouped into three different groups groups by their flowering response. Some plants flower as day length increases in late spring (long-day plants), some flower as day length wanes as autumn begins (short-day plants), and some plants flower at certain times regardless of the photoperiods (day-neutral plants) (Garner and Allard, 1920).

The determination of day-length as a critical regulator of flowering time left several questions with regard to the physiology of the flowering response. Where was day-length sensed in the plant and how is the signal for floral induction carried throughout the organism? Elegant grafting experiments performed first by the Russian physiologist Mikhail Chailakhyan determined that a mobile signal from leaf scions exposed to inductive photoperiods could induce flowering in non-induced graft stock (Chailakhyan, 1937; Chailakhyan, 1968). Experimental evidence suggested that the transmissible signal could be universal or nearly universal among flowering plants, for instance grafts in which leaves from induced short-day *Kalanchoë blossfeldiana* (stock) and long-day *Sedum spectabile* plants (scion) were able were able to induce flowering when grafted to plants of the opposite response type (Wellensi, 1967; Zeevaart, 2006). Grafts between different species were also often found to lead to flowering induction (Zeevaart, 1976; 2006). These observations led Denis Carr and Lloyd Evans to propose a model for two-step floral induction (Carr, 1967; Evans, 1971). The first stimulus would be involved in the sensing of photoperiod and the incorporation of other endogenous and environmental factors, which would induce the secondary stimulus that was potentially universal and transmitted from the leaf.

The search for the chemical basis of florigen remained elusive and gradually fell out of favor until contributions from *Arabidopsis* that facilitated the discovery of FT protein as a key candidate. The discovery FT as a mobile signal in *Arabidopsis* along with recognition that its function is conserved in a range of distantly related plant species (Corbesier et al., 2007),

has cemented the role of FT as a universal florigen (Abe et al., 2005; Kobayashi and Weigel, 2007; Kojima et al., 2002; Tamaki et al., 2007; Wigge et al., 2005). Increasingly, as our understanding of the photoperiodic sensing mechanism has expanded, we have found that similar regulatory networks govern flowering plant species other than *Arabidopsis*, and that the mechanism of photoperiodic flowering induction is highly conserved (Song et al., 2010).

In the following article, we will review developments in understanding the molecular mechanism of the photoperiodic flowering response through the model organism *Arabidopsis thaliana*, as well as recent discoveries that highlight the modulation of the photoperiodic sensing mechanism to accommodate both external environmental factors such as light quality through the action of photoreceptor proteins as well as internal physiological status through the sensing of internal photosynthetic accumulation.

II. PHOTOPERIODIC FLOWERING AND THE EXTERNAL COINCIDENCE MODEL

The key question that emerged with the discovery of photoperiodic flowering responses was the mechanism for how photoperiod was sensed. Since the early 18th century with the experiments of the astronomer De Mairan, plants have been known to have oscillatory leaf movements that occur in 24-hour cycles even in the absence of light, as if a light stimulus was present (De Mairan, 1729). These rhythms, which show a period of around 24 hours (hence circadian), show an inherited entrainment to the rotation of the earth that persists even after many generations of exposure to alternative day lengths in the laboratory (Bünning, 1960). This internal “clock” has extreme selective value in the regulation of daily output changes to the internal biochemical processes of the cell and the organism, which we can now appreciate given the advances in molecular biology in the last decades (Baudry and Kay, 2008). The connection between the internal clock and photoperiodic responses, however, was not immediately clear. First proposed by Erwin Bünning in 1936, and refined later by Colin Pittendrigh the “external coincidence” model, as it came to be known, proposed that photoperiodic phenomena could be explained by the interaction of light stimuli and the clock (Bünning, 1936; Pittendrigh and Minis, 1964). The clock would set the pace of the 24-hour rhythm, and define a period of photosensitivity to which exposure to light would be inductive for a photoperiodic response (Pittendrigh, 1972). In non-inductive photoperiods, the presence of darkness during the sensitive period of the response would result in no elicited reaction. In contrast, the encroachment of light into the photosensitive part of the circadian cycle, brought upon by longer inductive photoperiods, would cause a physiological response (see Figure 1).

For more than thirty years, it remained controversial that the endogenous circadian clock regulated the photoperiodic flowering response. Key experiments that unequivocally linked flowering to the clock were performed by Murray Coulter and Karl Hamner on the short-day plant *Glycine max* in 1964, by giving light pulses at different time points after transfer of plants into continuous darkness. One of the prevailing counter-hypotheses of the time posited that night duration was the primary cue for the photoperiodic response, and that this was mediated by the turnover kinetics of the photoreceptor phytochrome. According to this hypothesis, for short-day plants, in which photoperiods below a certain threshold are

inductive, directing light pulses at different times of night should affect the photoperiodic flowering response equally as long as a certain night length was prevented. It was found, instead, that light pulses during the night (referred to as night breaks) affected the flowering response in a rhythmic fashion (Carpenter and Hamner, 1964; Coulter and Hamner, 1964). Additional experiments performed by Halaban in 1968 in the short day plant *Coleus frederici* showed that the phases in which flowering was inhibited by night break pulses of plants always correlated with leaf movement position rather than the duration of night (Halaban, 1968a; b). This was true for plants placed under several different photoperiods. These early findings helped to cement the clock as a crucial component in determining photoperiodic flowering responses.

A. GENETICS OF PHOTOPERIODIC FLOWERING IN *ARABIDOPSIS*

Most *Arabidopsis* accessions that were initially collected for use in the laboratory belong to the summer annual class of wild *Arabidopsis*, mainly due to the ease of flowering without vernalization treatment and compact stature. Interestingly, some of the earliest mutations described in *Arabidopsis* are part of the regulatory framework that determines the photoperiodic flowering response, as mutations in these genes often convert compact summer annual accessions into phenotypes with long vegetative phases of growth. Mutagenic screens performed by Gyorgy Redei in 1962 isolated *gigantia (gi)* and *constans (co)* (as supervital mutants), far earlier than the forward genetic screens that would later more clearly define the regulatory networks that govern the flowering response (Rédei, 1962).

The advent of molecular markers in *Arabidopsis* in the late 1980's by Maarten Koorneef and colleagues enabled the systematic categorization of genes involved in the regulation of flowering time and mapping of their associated loci. Initial genetics of late flowering mutants of Arabidopsis found that *CO*, *GI*, and *FT* were likely components of the same regulatory pathway (Koornneef et al., 1991).

B. CO-FT MODULE IN *ARABIDOPSIS*

The *co* and *gi* mutant phenotype initially interested researchers studying the genetics basis of flowering time because these mutants exhibited a "day neutral" phenotype (Park et al., 1999; Putterill et al., 1995). Under inductive long day conditions, they flowered much later than wild type plants, but flowered about the same as wild type under non-inductive short day conditions. Additional phenotypic analysis led to the conclusion that *CO* is a limiting factor of flowering under short day conditions and that *CO* can promote flowering in a dose dependent manner under inductive photoperiods (Putterill et al., 1995). Transgenic analysis of plants expressing *CO* under a dexamethasone inducible construct found that plants could be induced to flower regardless of the external photoperiod when *CO* is highly expressed (Simon et al., 1996). Generation of mutants involved in the regulation of the circadian clock and light signaling also commonly affected the photoperiodic flowering response. Mutations in *LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), EARLY FLOWERING 3 (ELF3), TIMING OF CAB EXPRESSION 1 (TOC1), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), PSEUDO-RESPONSE REGULATOR 5 (PRR*5), *PRR7*, *PRR9*, and *CRYPTOCHROME 2 (CRY2)* all displayed

aberrant flowering phenotypes, which suggested that the clock on a molecular level was key to the proper induction of a photoperiodic response (El-Din El-Assal et al., 2001; Hicks et al., 1996; Ito et al., 2008; Nelson et al., 2000; Park et al., 1999; Sato et al., 2002; Schaffer et al., 1998; Somers et al., 2000). *CO* mRNA abundance was found to show a pronounced circadian oscillation in long day conditions, and was found to continue to occur after plants entrained to long day conditions were transferred to continuous light (Yanovsky and Kay, 2002). This suggested that the circadian clock regulated *CO* transcription. Additionally, the *CO* transcriptional pattern was significantly affected by mutations in clock components such as *toc1-1*, resulting in early flowering. *toc1-1* mutants have a shortened circadian period to about 21 hours; when circadian periods were shortened to compensate for the short period defect in *toc1-1*, however, proper *CO* expression and function was restored. *CO* transcripts continue to oscillate in short day conditions, but CO protein was initially shown to be highly unstable and actively degraded in the dark (Valverde et al., 2004; Yanovsky and Kay, 2002). This discrepancy between transcript abundance and protein stability explains how the constriction of active CO protein to the afternoon of long days enables a photoperiodic response, and fits nicely with our understanding of the external coincidence model in reference to photoperiodic phenomena (Figure 1). Coupled with experimental evidence that CO was a transcriptional activator of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999; Onouchi et al., 2000; Samach et al., 2000) and that *FT* was directly involved in signaling the activation of floral meristem differentiation, a CO-FT module in which clock and light regulated CO would perceive photoperiodic information and signal for the induction of downstream flowering response through the activation of *FT* transcription began to take shape.

Thus in line with earlier experimental data from the 1960's and 1970's, molecular evidence suggested that the circadian clock could regulate the photoperiodic response, in this case through the transcriptional and post translational regulation of CO, and that this could lead to flowering under inductive conditions. Since this discovery, the regulation of photoperiodic flowering pathway has become increasing complex, and many factors have been shown to regulate CO and FT through a variety of mechanisms (Andres and Coupland, 2012).

III. CURRENT MOLECULAR MECHANISM OF PHOTOPERIODIC FLOWERING IN ARABIDOPSIS

In *Arabidopsis*, long days promote flowering through the function of FT protein (Andres and Coupland, 2012; Wigge, 2011). The protein, a mobile florigen, is synthesized in phloem companion cells of leaves and translocated to the shoot apical meristem where the floral primordia are formed (Corbesier et al., 2007). The timing of flowering is strongly correlated with the relative amount of *FT*; the high levels of *FT* transcript in longer photoperiods influence more rapid flowering compared with the low levels in shorter photoperiods (Kobayashi et al., 1999). The transcriptional activator CONSTANS (CO) protein directly induces the expression of *FT* gene in a day length-dependent manner (Samach et al., 2000). *CO* gene expression is controlled by the circadian clock (Suárez-López et al., 2001), and CO protein abundance is modulated by light signaling, which CO protein is stabilized in the

afternoon of long days (Song et al., 2012b; Valverde et al., 2004). Together, these processes explain how day length is determined and how the floral transition is mediated under inductive photoperiod.

A. REGULATION OF *CO* TRANSCRIPTION

To accurately control the timing of seasonal flowering in *Arabidopsis*, the circadian clock-regulated *CO* expression is a crucial mechanism to precisely measure the difference in day length. *CO* transcription is controlled by many circadian clock proteins, such as CCA1, LHY, and PRRs. These clock proteins directly or indirectly regulate the gene expression of *CYCLING DOF FACTORs* (*CDFs*), transcriptional repressors of *CO* (Song et al., 2010). *CDF1* directly binds to the *CO* promoter and represses its transcription in the morning redundantly with other CDF proteins, CDF2, CDF3, and CDF5 (Fornara et al., 2009; Imaizumi et al., 2005; Sawa et al., 2007). The expression level of *CDF1* gene is positively regulated by CCA1 and LHY proteins (Nakamichi et al., 2007), which are most abundant at dawn (Schaffer et al., 1998; Wang and Tobin, 1998). Consequently, the expression level of CDF1 transcript remains high during the morning (Imaizumi et al., 2005). In the afternoon, the abundance of *CDF* transcripts is reduced through the function of four PRR family members, TOC1, PRR5, PRR7, and PRR9. These PRR proteins physically associate with the *CCA1* and *LHY* loci and repress *CCA1* and *LHY* gene expression (Huang et al., 2012; Nakamichi et al., 2010). TOC1, PRR5, PRR7, and PRR9 proteins also negatively regulate the expression of *CDF1* gene (Ito et al., 2008; Nakamichi et al., 2007). In addition, PRR5 and PRR7 directly binds to the *CDF2* and *CDF5* loci to and repress their transcription (Liu et al., 2013b; Nakamichi et al., 2012) Coincident with their similar roles to *CDF*1 in *CO* regulation *CDF2*, *CDF3*, and *CDF5* transcripts are also high in the morning (Fornara et al., 2009). Clock regulation of *CDF* expression, which keeps *CO* expression low in the morning, lays the groundwork for determining the photosensitive period later in the afternoon of long days, preventing early flowering in shorter photoperiods.

In long days, the repression of *CO* gene expression by CDF proteins is released through the function of FKF1-GI complex in the afternoon (Sawa et al., 2007). FKF1 protein is a blue light photoreceptor (Imaizumi et al., 2003; Sawa et al., 2007) and possesses an E3 ubiquitin ligase activity that mediates proteasome-dependent degradation of target proteins (Imaizumi et al., 2005). Once the expression patterns of FKF1 and GI proteins coincide with light in the afternoon, FKF1 absorbs blue light and is activated. Then, the blue light-activated FKF1 forms a protein complex with GI. The FKF1-GI complex recognizes *CO* repressors, the CDF proteins, and removes those repressors by ubiquitin-dependent degradation on the *CO* promoter (Sawa et al., 2007). FKF1 homologs, ZEITLUPE (ZTL) and LOV KELCH PROTEIN2 (LKP2) proteins, both of which interact with FKF1 and GI proteins, are also involved in the destabilization of CDF2 protein (Fornara et al., 2009). Removal of CDF proteins by the function of FKF1 protein constricts the action of CDF repressors to the morning of long days and facilitates the *CO* gene to be expressed during the late afternoon, while light still remains in the day (Figure 2). Maintaining this window of *CO* expression to the late afternoon allows for the subsequent peak of activation *FT* at dusk of long days, enabling the photoperiodic flowering response.

In contrast to long days, the expression of FKF1 and GI proteins is out of phase in short day conditions. No functional complex between the proteins exits in the daytime under these conditions, which results in the accumulation of *CO* transcripts only during the dark period, which subsequently causes an extremely low level of *FT* expression throughout the day. Transcriptional regulation of *CO* gene expression thus is critical for sensing day length and differentiating between inductive and non-inductive photoperiods to coordinate the flowering response (Sawa et al., 2007).

Once *CO* repression by CDF proteins is relieved, four bHLH transcription factors, FLOWERING BHLH 1 (FBH1), FBH2, FBH3, and FBH4, activate *CO* expression (Ito et al., 2012). These FBH proteins directly bind to E-box elements in the *CO* promoter and redundantly induce *CO* expression in the late afternoon and the dark under both long and short day conditions (Figure 2). It is proposed that FBH mediated *CO* activation is conserved in other plant species because overexpression of *FBH* homologue genes in rice and poplar highly upregulates *CO* transcripts in *Arabidopsis* (Ito et al., 2012). To date, our knowledge of transcriptional repression of *CO* is much more developed than it's activation (Song et al., 2013), and more work needs to be done to determine additional factors involved as well as time dependent impacts of *CO* activators on *CO* transcription.

B. POSTTRANSLATIONAL REGULATION OF CO PROTEIN

Along with the transcriptional regulation of *CO* gene, the posttranslational regulation of CO protein is crucial for the day length-dependent *FT* activation. In both long and short day conditions, the highest accumulation of *CO* mRNA occurs in the dark (Suárez-López et al., 2001). However, the expression of *FT* peaks at dusk in long days (Suárez-López et al., 2001). Various light signaling and proteasome-dependent protein degradation mechanisms have been shown to control CO protein stability and allow the protein to accumulate only in the late afternoon of long days, which accounts for day length-dependent *FT* expression (Jang et al., 2008; Lazaro et al., 2012; Liu et al., 2008b; Song et al., 2012b; Valverde et al., 2004). Red light delays flowering through the destabilization of CO protein, and far-red and blue light promote flowering through the stabilization of the protein (Valverde et al., 2004). PHYTOCHROME A (PHYA) and PHYB mediate far-red and red light responses, respectively, and CRY2 and FKF1 mediate blue light responses. Two RING finger E3 ubiquitin ligases, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1), negatively regulate CO protein stability (Jang et al., 2008; Lazaro et al., 2012; Liu et al., 2008b).

CO protein is stable under far-red light and unstable under red light in wild-type *Arabidopsis* plants. In addition, the amount of the protein is reduced in a *phyA* mutant background throughout the daytime and, by contrast, increased in a *phyB* mutant background especially in the morning (Valverde et al., 2004). In natural conditions, the ratio of red to far-red light is high during the daytime and relatively low at dusk. Reflecting this, the levels of CO protein are reduced in the morning and high in the late afternoon; this seems to indicate that PHYA and PHYB antagonistically modulate the stability of the protein.

Recent evidence has suggested that the PHYB dependent regulation of CO protein stability is quite complex, and may contain several factors that both positively and negatively affect

CO. Mutations in *PHYTOCHROME-DEPENDENT LATE FLOWERING (PHL)*, cause a late flowering phenotype in long days, similar to other photoperiodic flowering pathway components (Endo et al., 2013). Double mutant combinations with *phyB* abolish the late flowering phenotype, suggesting that *PHL* affects the ability of PHYB to repress flowering. *PHL* does not appear to regulate *CO* transcription, but CO protein and PHL interact. PHL protein thus a likely factor involved in sheltering CO from PHYB dependent degradation (Endo et al., 2013). Similarly, it has been found that the VASCULAR PLANT ONE ZINC-FINGER1 (VOZ1) and VOZ2, two NAC domain transcription factors, interact with PHYB, and positively regulate flowering in long days. Like PHYB, *VOZ1* and *VOZ2* are expressed in the cytoplasm and are translocated into the nucleus (Yasui et al., 2012). Their expression is also vascular specific, together with other photoperiodic flowering components (Yasui et al., 2012). The discovery of these factors adds a new layer of complexity with regard to PHYB regulation of photoperiodic flowering, but exact mechanisms for how PHYB destabilizes CO protein remain to be determined. How these PHYB dependent positive regulators of flowering fit into the larger framework of antagonistic PHYB and PHYA signaling with regard to CO will have important implications for light quality dynamics and their impact on the photoperiodic response.

The HOS1 E3 ubiquitin ligase mediates degradation of CO protein in the morning by directly interacting with CO (Lazaro et al., 2012). Another E3 ubiquitin ligase COP1 forms a protein complex with SUPPRESSOR OF PHYA-105 1 (SPA1). The COP1-SPA1 complex binds to CO protein and degrades the protein in the night. Other SPA proteins, SPA2, SPA3, and SPA4, physically interact with CO protein and redundantly regulate the destabilization CO protein(Jang et al., 2008; Laubinger et al., 2006; Saijo et al., 2003). CRY2 is also involved in CO stabilization through protein complex formation with SPA1 (Zuo et al., 2011). The binding of photoactivated-CRY2 to SPA1 enhances the interaction between CRY2 and COP1 in response to blue light, resulting in the suppression of COP1-SPA1 activity and in turn the accumulation of CO in the daytime (Zuo et al., 2011). This function of CRY2 partially explains how blue light accelerates flowering through the stabilization of CO protein and induction of *FT* transcripts.

While we have a good idea of which factors contribute to CO protein stabilization and destabilization, the relationship between these factors throughout the day and how they compete or interact dynamically for CO protein needs to be further clarified. As has been discussed, three photoreceptors, PHYA, PHYB, and CRY2 and two E3 ubiquitin ligases, HOS1 and COP1, regulate CO protein stability. However, functions of the photoreceptors cannot fully account for the question about how CO protein is stabilized only at the end of day in long day conditions because those photoreceptors are constitutively expressed through the day (Mockler et al., 2003). The function of another blue light photoreceptor FKF1 provides a clue to answer the question. FKF1 protein physically interacts with CO protein in a blue light enhanced manner, and the FKF1-CO interaction increases CO stability at a specific time of day, in the afternoon, under long day conditions (Song et al., 2012b). Together with the similar expression profile of those proteins (Imaizumi et al., 2003; Valverde et al., 2004), the blue light enhanced FKF1-CO interaction supports the notion that FKF1 determines the timing of CO stabilization and that the expression of *CO* gene under

light in long day condition is crucial for *FT* induction through CO protein accumulation, in which both gene expression and the protein accumulation are regulated by FKF1 function. As the core clock components CCA1 and LHY regulate the timing of *FKF1* (Imaizumi et al., 2003), the circadian regulation of the FKF1 photoreceptor function is likely the molecular basis of the photosensitivity phase proposed in the external coincidence model in *Arabidopsis*.

C. TRANSCRIPTIONAL REGULATION OF THE *FT* GENE

The photoperiodic flowering pathway serves as a conduit for a large variety of environmental parameters that convert external information and integrate it into *FT* signal. These environmental signals merge to control the *FT* expression through numbers of transcription factors (cite Song TiPS 2013). Several classes of transcriptional repressors exist in the regulation of *FT* gene expression. SCHLAFMÜTZE (SMZ) gene encodes an *APETALA2* (*AP2*)-related transcription factor that binds to the 3′-UTR of *FT* locus and represses *FT* transcription (Mathieu et al., 2009), and the expression of the gene is negatively regulated by GI function mediated through a microRNA pathway (Jung et al., 2007). GI protein positively regulates miRNA172 (miR172) accumulation in long days. The miR172 targets *SMZ* transcripts and decreases amount of the transcripts (Mathieu et al., 2009). TEMPRANILLO 1 (TEM1) protein directly associates with the 5′-UTR of *FT* gene and represses the gene expression throughout the day in long day conditions. TEM2 is involved in the regulation of *FT* expression redundantly with TEM1 (Castillejo and Pelaz, 2008). In addition, GI protein interacts with TEM1 and TEM2 in the nucleus in tobacco cells and probably changes activities of TEM proteins (Sawa and Kay, 2011).

Interestingly, the *CO* transcriptional regulator CDF1 also associates with the *FT* promoter near the transcriptional start site and represses *FT* transcription in the morning(Song et al., 2012b). Other CDF proteins (CDF2, CDF3, and CDF5) also likely regulate *FT* gene expression. The repression of *FT* transcription by CDF1 is released by the function of FKF1-GI complex on the *FT* promoter in the afternoon (Song et al., 2012b), concomitantly with the removal of CDF1 repression on the *CO* promoter. Together with CO protein stabilization, these observations suggest that FKF1 protein controls *FT* induction through a multiple-feed forward motif, which allow strong activation of flowering signals in long day conditions.

In the activation of *FT* transcription, two classes of transcription factors play major roles. A member of B-box transcription factor family, CO, acts as a strong activator of *FT* expression (Putterill et al., 1995; Robson et al., 2001; Tiwari et al., 2010). CO protein contains two functional motifs; two B-box domains at the N-terminus and the CCT (CONSTANS, CONSTANS-like, and TOC1) domain at the C-terminus (Robson et al., 2001). The protein associates with the *FT* promoter and activates *FT* gene expression through two modes of action (Song et al., 2012a; Song et al., 2012b; Tiwari et al., 2010; Wenkel et al., 2006); one is that CO directly binds to the CONSTANS (CO) responsive element (CORE) via the CCT motif (Tiwari et al., 2010), and the other is that CO is recruited by the CCAAT box-binding proteins including selected subunits of Nuclear Factor-Y (NF-Y) and ASYMMETRIC LEAVES 1 (AS1) that both physically interact with CO protein (Song et al., 2012a; Wenkel

et al., 2006). *FT* induction is largely CO-dependent; the relative abundance of *FT* highly accumulates when *CO* expression is constitutive, regardless of day length (Valverde et al., 2004). Another transcription factor family that contains basic helix-loop-helix (bHLH) domain including CRYPTOCHROME-INTERACTING BASIC HELIX–LOOP–HELIX 1 (CIB1), CIB2, CIB4, and CIB5, are involved in *FT* induction (Liu et al., 2008a; Liu et al., 2013c). CIB1 protein forms a complex with CRY2 protein in a blue light-dependent manner and acts as a *FT* activator through directly binding to the *FT* promoter (Liu et al., 2008a). The blue light-dependent CIB1 accumulation is positively regulated by function of ZTL and LKP2, but not by FKF1 (Liu et al., 2013a). All other CIB proteins also interact with CRY2 *in vitro* but only CIB2 and CIB5 form complexes with CRY2 *in vivo* (Liu et al., 2013c). CIB proteins redundantly regulate *FT* transcription. CIB1 protein forms hetero-dimer complexes with other CIBs, and the hetero-dimerization increases the DNA-binding affinity of CIB1 protein to the specific cis-element in the *FT* promoter (Liu et al., 2013c). As described above, blue light signaling play a pivotal role in the regulation of *FT* induction through degradation of *FT* repressors and stabilization of *FT* activators in *Arabidopsis*.

D. MOVEMENT OF FT PROTEIN

Where a florigen is synthesized differs from where it functions; therefore, understanding how the florigen moves is also of great interest in the photoperiodic flowering pathway. FT protein, once synthesized in companion cells in the leaves, is loaded into the phloem and migrates towards its eventual destination at the shoot apex. Initial debate upon the discovery of FT as a primary component of the florigen occurred over whether the mobile signal was *FT* mRNA or FT protein (Corbesier et al., 2007; Huang et al., 2005; Jaeger and Wigge, 2007; Yoo et al., 2013b). Multiple studies have since confirmed that the movement of FT protein explains the florigenic signal. Grafting experiments in *Cucurbita moschata* in particular have proved a useful system for the study of FT movment. RT-PCR and mass spectrometry analysis on phloem sap detected no *FT* transcript but observed FT protein (Lin et al., 2007). Cross species grafting experiments using *Cucurbita moschata* and *Cucurbita maxima*, and subsequent analysis also showed that FT peptides belonging to the induced scion were detected in the phloem sap, but not *FT* mRNA (Yoo et al., 2013a). Additional work in this system has given a picture in which FT movement is regulated in different ways as it moves. Mutations in FT that prevent movement into the shoot apex have been shown to have the capacity to move through the companion cell to sieve tube element barrier. This is supported by evidence that protein size affects the ability of tagged FT to enter the phloem and that specific regions of FT protein are important for movement out of the phloem and into the shoot apex (Yoo et al., 2013a). This suggests a combination of FT movement by diffusion through the companion cell and into the phloem stream and a more active transport mechanism through the plasmodesmata of cells to move FT protein into the cells of the shoot apex (Yoo et al., 2013a). Several candidate proteins for interaction or facilitated movement of FT have been identified, but their roles need to be further clarified and a more nuanced model for FT movement at each step needs to be elucidated (Liu et al., 2012; Yoo et al., 2013a).

Once FT reaches the shoot apex, a complex cascade of interactions occurs that leads to the activation of downstream developmental patterning arrays that give rise to floral meristem

initiation. FT protein interacts with the bZIP transcription factor FD and 14-3-3 to activate transcription of downstream floral targets such as *APETALA1 (AP1)* and *LEAFY (LFY*) (Abe et al., 2005; Kardailsky et al., 1999; Taoka et al., 2011; Wigge, 2011). Modeling of the interactors at the shoot apex has shown that maintenance of steady state levels of FT and other interactors at the shoot apex are necessary to maintain and push the reprogramming of the vegetative meristem forward into the inflorescence meristem (Jaeger et al., 2013). This mechanism is reminiscent of classical feed-forward genetic mechanisms found in *Drosphila* development (Thuringer and Bienz, 1993). This suggests that threshold levels of FT movement may be critical for the reproductive transition, and it will be interesting to see experimentally the quantitative effects of FT protein on the floral transition. Classical grafting experiments have shown that cross species grafts for floral induction can induce some partners but be insufficient for others, suggesting that threshold levels of FT may be different between species (Evans, 1971). Modeling at the shoot apex of these interactions in other species may have interesting implications for the dynamics of the reproductive transition across evolutionary lines.

IV. PHOTOSYNTHATES AS A COMPONENT OF THE PHOTOPERIODIC FLOWERING STIMULUS

Photosynthesis and photosynthetic assimilates have been also recognized in historical experiments to be involved in seasonal flowering, but determining the relationship between inductive photoperiods, the florigenic signal and the photosynthetic status of the plant could not easily be disentangled in the past and is far from concrete in the present (see (Evans, 1971; Zeevaart, 1976) for review of historical work). Recent molecular genetics evidence suggests that photosynthetic components can act in leaves in a photoperiodic manner to contribute in tandem to the known photoperiodic signaling pathway. This new information sheds light on older experimental data demonstrating that photosynthetic status may alter ability to respond to optimum photoperiod in long days in *Arabidopsis*.

A. EARLY EVIDENCE FOR THE INVOLVEMENT OF PHOTOSYNTHESIS IN THE PHOTOPERIODIC FLOWERING RESPONSE

Many experiments have been performed historically to determine the effect of changes in photosynthetic activity on the transition from vegetative to reproductive development. Although photoperiod remains a strict determinant of flowering in many species, the capacity of a plant to respond to an introduced inductive photoperiodic signal can depend on other factors. Experiments that use the application of DCMU, an inhibitor of photosynthesis, showed that flowering could be severely delayed in *Lollium temulentum*, a long-day grass (Evans, 1966). However, DCMU seemed to have no effect on many short-day species, but not universally (Evans, 1971). Prolonged growth in elevated CO_2 coupled with inductive day lengths has been observed to accelerate flowering in several long-day species (Reekie et al., 1994). In contrast to these results, however, experiments utilizing albino *Arabidopsis* mutants grown on 1% glucose could still be induced to flower, suggesting that carbon availability rather that photosynthesis influences the flowering response (Brown and Klein, 1971). Recently, DCMU treatment and removal of CO_2 have also been shown to influence the period of the circadian clock under free running conditions. Thus photosynthetic output

could presumably effect downstream pathways such as photoperiodic components to change gene expression, in a manner similar to the photoperiodic flowering response in several circadian clock mutant backgrounds (Haydon et al., 2013).

Although inhibition and increase in photosynthetic activity seemed to be involved in flowering induction, it was not clear where in the plant photosynthates were acting, nor was it clear whether they were acting through the same mechanism or separately from the floral stimulus. During the 1980s and 1990s when the idea of a universal transmissible signal had fallen out of favor (Zeevaart, 2006), several studies demonstrated a marked increase in sucrose or glucose at the shoot apex of both long-day and short-day species around the time of the flowering induction (Lejeune, 1993; Milyaeva, 1996; Mirolo, 1985; Perilleux, 1997). Yet, in *Sinapsis alba* during a single displaced short day (8 hrs of light at the end of a subjective 16-hr day) and in *Lolium tementulum* in a single long day, an appreciable mobilization of carbohydrates to the shoot apex did not occur until after the floral stimulus left the leaf (Bodson et al., 1977; Perilleux, 1997). This lead Bodson and colleagues to speculate whether photosynthates could lead to floral induction in the leaves rather than at the shoot apex.

Arabidopsis, like *Sinapsis alba*, can be induced to flower in a single long day or in a displaced short day. Mutations in *PHOSPHOGLYCERATE/BIS PHOSPHO-GLYCERATE MUTASE (PGM)* result in the inability to accumulate starch. *pgm* mutants have low rates of floral induction and no increase in sucrose exudates from the leaves in a single displaced short day treatment compared to wild type plants or *pgm* exposed to one long day. This flowering repression of *pgm* in displaced short days, however, could be partially restored by application of sucrose at their apices (Corbesier et al., 1998). Laurent Corbesier and colleagues concluded that sufficient sucrose mobilization from the leaves was needed for flowering induction, and that both a florigenic signal as well as a photosynthetic component was required for the proper photoperiodic flowering response.

B. PHOTOSYNTHATES ACT IN THE LEAVES TO PROMOTE FLOWERING

Recent work regarding trehalose-6-phosphate has provided more detailed insight into the involvement of photosynthates in the leaf. Trehalose-6-phosphate (T6P) increases parallel to sucrose in the leaves and its levels correlate to increasing starch synthesis (Ponnu et al., 2011). It has been implicated as a signal for carbohydrate status in the plant; although new research has shown substantial starch accumulation cannot be induced by T6P alone (Martins et al., 2013). In *Arabidopsis*, T6P increases at dusk similar to the pattern *FT* displays in long days (Imaizumi et al., 2003; Wahl et al., 2013). Loss of *TREHELOSE-6-PHOSPHATE SYNTHASE 1* (*TPS1*) markedly reduces the dusk peak of *FT* and delays flowering in long days. Together this evidence suggests a link between photosynthetic assimilation, long day induction of *FT*, and flowering. Expression of *CO* was only minimally altered in *tps1* mutants, suggesting that increase in *FT* transcripts is *CO* independent (see Figure 4) (Wahl et al., 2013).

Experimental evidence from *Sinapsis alba*, which is closely related to *Arabidopsis*, has also shown that photosynthate production during phases of the day can influence the flowering response. High light intensity provided by fluorescent lamps coupled with removal of CO_2

from the air failed to promote flowering when the treatment occurred during the first eight hours of a long day cycle. However, flowering was strongly induced when the treatment occurred during the last eight hours of the daytime. To our knowledge parallel work has not been done in *Arabidopsis*, however, removal of CO_2 throughout the entire day from *Arabidopsis* plants transferred from short days to long days resulted in a significant down regulation of *FT* transcription compared to normal CO_2 controls (King et al., 2008). If T6P indeed interacts with the photoperiodic pathway to induce flowering in leaves, a time dependent sensitivity to photosynthate accumulation could explain how T6P promotes *FT* transcription only at dusk. It will be interesting to see if T6P levels are strongly reduced by removal of CO_2 early in the day; however, this remains to be tested.

King and colleagues demonstrated that high-intensity (270 μmol m^{-2} s^{-1}) fluorescent light, presumably increasing photosynthetic intake, led to relatively early flowering in short days compared to normal (100 μmol m^{-2} s^{-1}) light intensity in *ft* mutants. This indicates that photosynthesis possibly can override the lack of *FT* signal under short day conditions (King et al., 2008). Clarifying a possible mechanism, Wahl and colleagues found that unlike the *ft* mutant, a *TPS1* deficiency delayed flowering in *Arabidopsis* in short days as well as long days, indicating that T6P could interact with floral signals besides *FT* (Wahl et al., 2013). Further, loss of *TPS1* resulted in reduced expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) *SPL3*, *SPL4* and *SPL5* at the shoot apex. The SPL protein family is the known component of the age-dependent flowering pathway in *Arabidopsis*. Reduced *SPL* expression appeared to be accomplished partially through and partially independently of miR156, which delays the vegetative-reproductive phase transition. Mature miR156 was initially higher in *tps1* mutants compared to wild type, and although it declined to wild type levels over time, *SPL3*, *4*, and *5* accumulated more slowly in *tps1* mutants (Wahl et al., 2013). Finally, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FRUITFUL* (*FUL*) were not altered in the *tps1* mutants, although they have been implicated as inducing *FT* downstream of the SPL proteins in the leaves (Wahl et al., 2013). It seems that T6P acts to regulate *FT* in the leaves mainly independently of the age-dependent pathways, while it acts to induce flowering directly at the shoot apex in response to plant age (Samach et al., 2000; Teper-Bamnolker and Samach, 2005). Because of this, T6P probably occupies a role as a stimulus of flowering in both a photoperiodic and non-photoperiodic context based on tissue specificity.

The FT protein is still the primary component of the transmissible signal in the lengthening days of spring and summer in *Arabidopsis*. Now, it is becoming clear that photosynthetic bi-products can lead to induction of *FT* at the leaf level, additively with the established photoperiod sensing mechanism through *CO*. This synergy appears to be long-day specific. Under short day conditions, it is common to increase the light intensity in photoperiodic studies to normalize the radiative energy received by plants when comparing plants grown in long days and short days to around 120 μmol m^{-2} s^{-1}. In these conditions, *FT* induction does not occur. Therefore, greater accumulation of photosynthates from high-intensity light in short days seems not to over-ride the requirement of late-afternoon light for *FT* induction. Further, it appears that photosynthesis enhances the photoperiodic response, but cannot completely abrogate it, as *FT* did not decline to short-day levels when CO_2 was removed

from the air (King et al., 2008). By what mechanism do photosynthesis and photosynthates interact with the photoperiodic pathway to induce *FT* and flowering? Because *FT* induction through T6P is likely *CO* independent, a heretofore-unknown factor or pathway must be involved in *FT* transcriptional regulation in response to photosynthetic accumulation. Clearly, more work to determine the effects of T6P on photoperiodic pathway components is needed.

Earlier studies into the mechanisms of photoperiodic flowering and photosynthetic involvement in the flowering response highlight interactions between age and photoperiod that we do not fully understand. Many early experiments that were able to induce flowering by a single inductive long day did so by first growing their plants in short days for several weeks (Corbesier et al., 1998; Evans and Wardlaw, 1966; King et al., 2008). It appears, therefore, that age or carbohydrate status increases the amount or reduces the threshold requirement of the floral stimulus, or both. The activity of T6P at the shoot apex, proposed as a fail-safe to ensure flowering will occur even in the absence of inductive conditions (Wahl et al., 2013), suggests one mechanism. The decline of miR156 in the leaves over time resulting in up regulation of *FT* suggests another (Srikanth and Schmid, 2011). Although T6P seems to act independently of FT at the shoot apex, it is possible these two pathways act in parallel to modify plant response. A better understanding of how age and the carbohydrate statuses of the plants interact with photoperiodic induction either at the leaves or the shoot apex is critical to determine the threshold FT necessary to promote flowering under different timescales and spatial contexts.

V. CONCLUSIONS

While our current understanding of the underlying mechanisms that confer a photoperiodic flowering response in *Arabidopsis* are now better understood, the number of factors that are involved in the process makes it a very complex system of interactors. Circadian clock control of a variety of *CO* and *FT* regulators, light perception through photoreceptors, as well as photosynthetic status through T6P can affect the photoperiodic response synergistically to promote flowering in *Arabidopsis* as days get longer in the springtime. Brought into a larger context of all of flowering time regulation, there are a maze of players whose roles and domains may not be easily defined and whose outputs affect feedback within the system. One of the greater challenges in the future will be understanding how the plant is able to assimilate information regarding day length, light quality, temperature, precipitation, photosynthetic status, developmental age and other external and physiological characteristics and to incorporate this information in a way that is meaningful towards timing the floral transition. To this end, systems level approaches will be necessary to untangle the influence of so many factors on one output, and this will be critical if we are to understand how flowering time functions under natural conditions. At a surface level, we assume the large amount of redundancy, overlap, and crosstalk within and among flowering pathway regulators must be necessary and of selective value in coordinating the flowering response; but is this the case? As our mechanistic knowledge of flowering improves, we should continue to look out among natural populations in *Arabidopsis* and other species to see whether what we presume is indeed the case. Can we see that these factors affect fitness as plants expand and contract across geographic ranges, climates, and latitudes?

As detailed, work in *Arabidopsis* has established that the CO-FT module is critical for day length sensing, and recent developments have confirmed the highly conserved nature of this mechanism for flowering and it's co-option for other photoperiodic outputs across the angiosperm lineage (Böhlenius et al., 2006; Kloosterman et al., 2013; Song et al., 2010). While the limited information we have on other species points to this similarity, much work is needed to better characterize mechanisms of photoperiodic sensing in other plants. With the improved genomic and functional systems at our disposal, hopefully these will shed light on the commonalities and divergence of seasonal adaptation and how plants utilize that information to survive, and hopefully how we can use that knowledge to better adapt the plants which we depend on to flourish in changing habitats.

Acknowledgments

This work was supported by a Pre-Doctoral Developmental Biology Training Grant (5T32HD007183) from the National Institutes of Health to G.S.G., the National Science Foundation Graduate Research Fellowship Program to H.K.S., funding from the Next-Generation BioGreen 21 Program (SSAC, PJ009495) to Y.H.S., and the National Institutes of Health (GM079712) to T.I.

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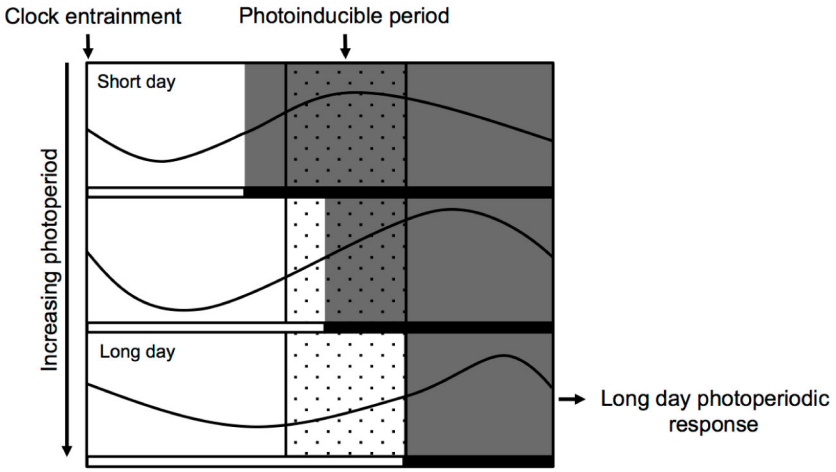

Fig. 1.

The external coincidence model for photoperiodic phenomena:

The following example represents a photoperiodic response that occurs in the afternoon of long days, as in photoperiodic flowering in *Arabidopsis*. The circadian clock generates a rhythm that determines a specific period of the day in which a light signal can induce the response. This period is similar regardless of day length. In short day conditions the photoinducible period does not coincide with a light signal, so no response occurs. As days lengthen with the coming of spring and summer, light begins to encroach on the photoinducible period, eliciting the photoperiodic response. Light serves a dual purpose; to reset the clock at dawn and dusk and to be present or absent during the photoinducible phase, to promote or halt the response.

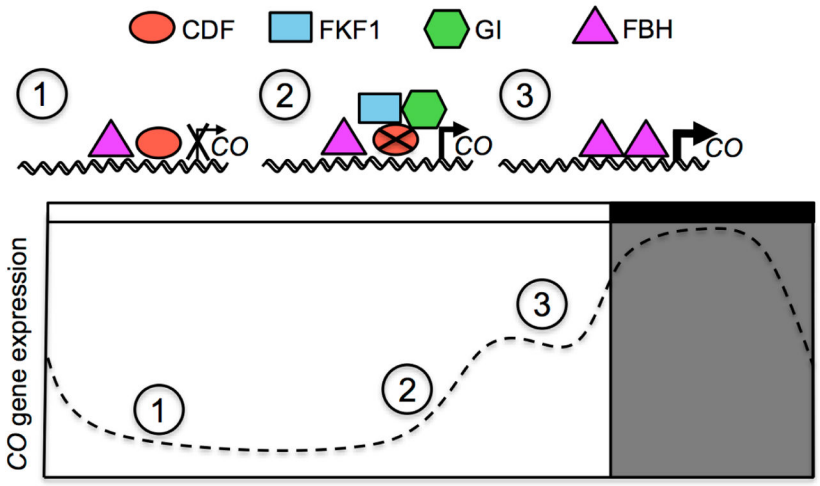

Fig. 2.

CONSTANS (CO) oscillatory transcription is dependent on multiple factors throughout the day: *CO* gene expression changes throughout the day. In inductive long-day conditions for flowering in *Arabidopsis*, the peak of *CO* expression is constrained to the afternoon before dusk. In the morning, CDF family transcription factors bind to the *CO* promoter to repress transcription. Beginning in the afternoon, FKF1 and GI form a protein complex that ubiquitinates CDFs through an F-box protein function on FKF1 and targets them for proteasomal degradation, freeing the *CO* promoter from repression. FBH transcriptional activators are then recruited to the *CO* genomic locus, resulting in increased transcription of *CO* before dusk. Constraining *CO* mRNA expression to the late afternoon, and stabilization of resultant CO protein results in *FT* expression at dusk and promotes flowering in long days.

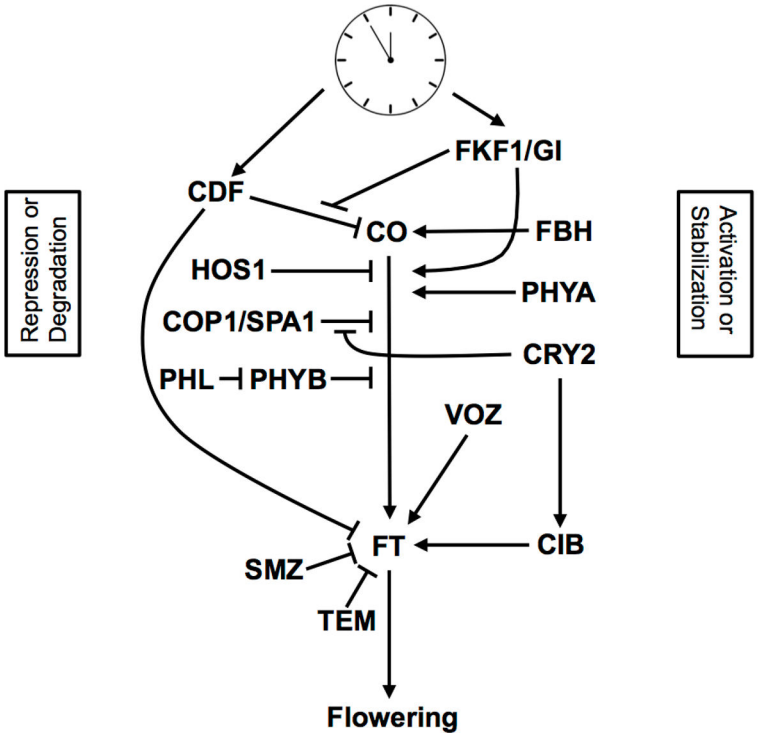

Fig. 3.

Regulators of the photoperiodic flowering pathway: Flowering under inductive long days requires a peak of *FT* expression in the late afternoon. *CO* transcription, CO protein stability, and *FT* transcription are critical to the photoperiodic flowering response. Blue light promotes flowering through FKF1 dependent degradation of CDFs and stabilization of CO protein, direct activation of *FT* through *CIB* transcription factors, and stabilization of the COP1/SPA1 complex by CRY2, which normally destabilizes CO protein in the dark. Red light inhibits flowering through destabilization of CO protein by PHYB. Far-red light promotes flowering through increased stability of CO protein by PHYA. Low temperature destabilizes CO protein through HOS1. The promotion or inhibition of each respective component can affect the flowering output, and thus serves to integrate multiple environmental signals such as day length, light quality, and temperature.

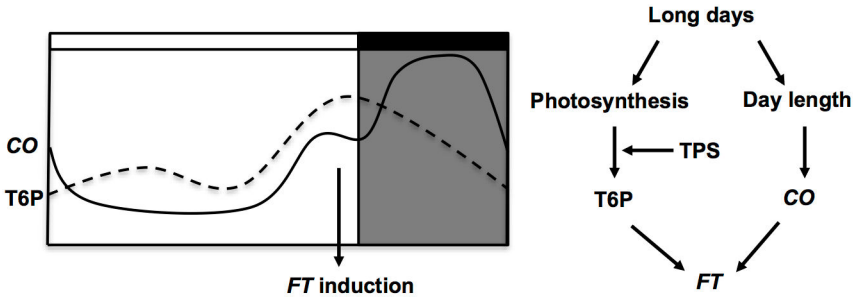

Fig. 4.

Trehalose-6-phospate regulates *FT* expression in long days: T6P levels peak in the afternoon of days in *Arabidopsis*, which coincides with the afternoon peak of *FT* transcription. Loss of function of *tps1*, the enzyme which produces T6P, results in a significant reduction in the dusk peak of *FT* expression. *CO* expression levels are unaltered by the *tps1* mutation, suggesting that T6P regulation of *FT* in leaves occurs in a *CO* independent manner. Inductive long days may thus produce a photoperiodic flowering response through FT via multiple regulatory pathways.