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# Review. Photoperiodic control of flowering time

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## Abstract

The rotation of the earth results in periodic changes in environmental factors such as daylength and temperature; the circadian clock is the endogenous mechanism responsible for day-length measurement, and allows plants to anticipate these fluctuations and modulate their developmental programs to maximize adaptation to those environmental cues. Flowering represents the transition from a vegetative to reproductive phase and is controlled by complex and highly regulated genetic pathways. In many plants, the time of flowering is strongly influenced by photoperiod, which synchronizes the floral transition with the favourable season of the year. Over the last decade, genetic approaches have aided the discovery of many signalling components involved in the photoperiod pathway and here, we highlight the significant progress made in identifying the molecular mechanisms that measure daylength and control flowering initiation in Arabidopsis, a long day (LD) plant, and in rice, a short day (SD) plant. Some components of the Arabidopsis regulatory network are conserved in other species, but the difference in the function of particular genes may contribute to the opposite photoperiodic flowering response observed between LD and SD plants. The specific regulatory mechanisms involved in controlling CONSTANS (CO) expression and stability by the circadian clock and the different photoreceptors will be described. In addition, the role of *FLOWERING LOCUS T* (*FT*), as part of the florigen, and several other light signalling and circadian-dependent components in photoperiodic flowering will be also discussed.

**Additional key words**: circadian clock, *CONSTANS*, florigen, *FT,* photoperiodism, photoreceptors.

## Resumen

**Revisión. Control fotoperiódico del tiempo de floración**

La rotación diaria de la tierra provoca cambios periódicos en la duración del día o en la temperatura, y el reloj circadiano es un mecanismo endógeno responsable de la medida de la duración del día; este oscilador molecular permite a los organismos anticiparse a dichos cambios y adaptar su desarrollo de manera adecuada. La floración representa la transición desde una fase vegetativa del crecimiento a una reproductiva, y está controlada por diferentes rutas, muy complejas y altamente reguladas. En muchas plantas, esta transición está controlada principalmente por la duración del día o fotoperiodo, el cual sincroniza la floración con la estación más favorable del año. En la última década, diferentes estudios genéticos han facilitado la caracterización de muchos componentes de señalización involucrados en la ruta del fotoperiodo y en esta revisión se discute el progreso reciente que se ha llevado a cabo en la identificación de los mecanismos moleculares que permiten medir la duración del día, y que controlan el tiempo de floración en Arabidopsis, una especie de día largo, y en arroz, un especie de día corto. Aunque los componentes de las rutas reguladoras de Arabidopsis parecen conservarse en otras especies, la diferencia de función de genes concretos parece contribuir a la respuesta opuesta a la duración del día observada entre especies de día largo y de día corto. Se describen los mecanismos reguladores específicos que participan en la expresión y estabilidad de CONSTANS (CO) por el reloj circadiano y por diferentes fotorreceptores. Además, también discutiremos el papel del locus *FLOWERING LOCUS T* (*FT*), como parte del florígeno*,* y de otros componentes dependientes del mecanismo del reloj circadiano o de la señalización por luz en el control por fotoperiodo del tiempo de floración.

**Palabras clave adicionales**: *CONSTANS*, florígeno, fotoperiodismo, fotorreceptores, *FT,* reloj circadiano.

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## Introduction[1]

The question of how plants monitor environmental cues and trigger the initiation of flowering at the right season has attracted the interest of plant biologists for decades. The duration of daylight changes with a predictable pattern along the year, providing a reliable environmental signal for the varying seasons. The ability to sense and respond to changes in daylength is known as photoperiodism, and is a widespread phenomenon found in both plants and animals that allows these organisms to adapt to seasonal changes in their environment. The importance of daylength in controlling seasonal responses was already proposed by Tournois and Klebs back in the early 1900's; these researchers working independently suggested that the duration rather than the quantity of light is a major determinant in plant development (Tournois, 1912, 1914; Klebs, 1913). However, it was Garner and Allard (1920) that, using controlled photoperiodic conditions, showed for the first time that daylength can determine the time of flowering, being the first photoperiodism phenomenon documented. Their studies on soybean and tobacco led to the proposal that flowering would only occur if the duration of the daily light period was sufficiently short. These authors classified plants into three photoperiodic groups according to their flowering response to daylength. In long day (LD) plants flowering is promoted by daily periods of light longer than a critical daylength, whereas plants that accelerate flowering in response to daylength below a critical threshold are called short day (SD) plants. Day-neutral (DN) plants flower at the same time irrespectively of the photoperiodic conditions.

Following these and other observations that established the central role of daylength perception in controlling plant development, several models have attempted to explain the basis of the photoperiodic responses. A first simple model proposed that the gradual accumulation of a substance is required to trigger a physiological response; the amount of this chemical can increase up to a threshold level only in photoperiodic inductive conditions. For instance, in the case of photoperiodic flowering, increasing the length of darkness should either promote (in SD plants) or inhibit (in LD plants) the accumulation of a product that results in flowering induction. According to this hourglass model, once a threshold duration is reached, further increments should have no consequences on flowering; however, the analysis of the floral responses of many plant species to cycles of 8 h of light and increasing hours of darkness provides strong evidence for the involvement of the circadian system in the measurement of photoperiodic time. In contrast to the prediction of the hourglass model, the floral response fluctuates rhythmically under these conditions, and a maximum response is achieved every time the total length is 24 h or a multiple of it, whereas at intermediate cycle lengths the response is much lower (Thomas and Vince-Prue, 1997). Therefore, it would be the presence or absence of light at specific times of the day rather than the duration of the periods of light or darkness that is important for photoperiodic responses. The effect on flowering of night-breaks is consistent with this view; pulses of light during the night period can effectively prevent flowering of SD plants and this floral response follows circadian rhythms. These observations strongly support the clock hypothesis postulated by Bünning (1936), who proposed more than seventy years ago that the mechanism controlling daily movements in leaves or petals, subsequently named the circadian clock, was also the basis of photoperiodic time measurement. This circadian clock generates a rhythm with a period close to 24 h and is responsive to light only at a particular phase of the cycle. When a plant displaying a photoperiodic flowering response is grown under a daylength regime that causes it to be exposed to light at this particular phase, flowering is

[1] Abbreviations used: *AP* (*APETALA)*, bHLH (basic helix-loop-helix), *CCA* (*CIRCADIAN CLOCK ASSOCIATED*), CDF (CYCLING DOF FACTOR), CK (casein kinase), *CO* (*CONSTANS*), COP (CONSTITUTIVELY PHOTOMORPHOGENIC), CRY (CRYPTOCHROMES), DET (DE-ETIOLATED), DN (day-neutral), EE (evening element), Ehd (early heading date), *ELF (EARLY FLOWERING),* EST (expressed sequence tag), *FHY* (*FAR-RED ELONGATED HYPOCOTYL)*, FKF (FLAVIN BINDING KELCH REPEAT F-BOX), *FLC* (*FLOWERING LOCUS C*), FMN (FLAVIN MONONUCLEOTIDE), *FT* (*FLOWERING LOCUS T*), *Hd* (*Heading-date),* GI (GIGANTEA), LD (long day), *LHY* (*LONG ELONGATED HYPOCOTYL),* LKP (LOV KELCH PROTEIN), *LUX (LUX ARRHYTHMO),* miRNA *(*microRNA), PAS (PERIOD-CIRCADIAN-PROTEIN/AH-RECEPTOR-NUCLEAR-TRANSLOCATOR-PROTEIN/SINGLE-MINDED-PROTEIN), PFT (PHYTOCHROME AND FLOWERING TIME), PHOT (PHOTOTROPIN), PHY (PHYTOCHROME), PIF (PHYTOCHROME INTERACTING FACTOR), PIL (PHYTOCHROME INTERACTING FACTOR-LIKE), PRR (pseudo-response regulator), QTL (quantitative trait loci), RFI (RED AND FAR-RED INSENSITIVE), RVE (REVEILLE), SAM (shoot apical meristem), SD (short day), *SFT* (*SINGLE FLOWER TRUSS*), SOC (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS), *SPA* (*SUPPRESSOR OF PHYA)*, *SRR* (*SENSITIVITY TO RED LIGHT REDUCED*), *SVP* (*SHORT VEGETATIVE PHASE*), *TIC* (*TIME FOR COFFEE*), TOC (TIMING OF CAB EXPRESSION), *TSF* (*TWIN SISTER OF FT*), ZTL (ZEITLUPE).

induced if the plant shows a LD response, or repressed in the case of a SD plant. A number of years later, Pittendrigh, working on insects, introduced a new turn in Bünning's postulates and proposed the external coincidence model that includes the role of light in entraining the clock to the solar cycle to explain photoperiodic responses (Bünning, 1960; Pittendrigh and Minis, 1964). Recent studies addressing the photoperiodic control of flowering have provided strong support for the external coincidence hypothesis and molecular components of the mechanism responsible for daylength discrimination have been identified (reviewed in Yanovsky and Kay, 2003; Searle and Coupland, 2004; Corbesier and Coupland, 2005; Baurle and Dean, 2006; Imaizumi and Kay, 2006; Jarillo and Piñeiro, 2006).

Besides these observations concerning the timekeeping mechanism, a crucial finding in the understanding of the photoperiodic regulation of flowering was the identification of leaves as the site of daylength perception. Since photoperiod is measured in the leaves but the flowering response is evoked in distal meristems, the existence of a mobile signal termed the «florigen» was postulated (Chailakhyan, 1936a,b, 1937). This florigen was defined as a graft-transmissible substance(s) that is generated in the leaves in response to photoperiodic inductive conditions and moves through the phloem to stimulate the initiation of flowering in the shoot apical meristem (SAM). The florigen was proposed to be a universal signal that could induce flowering in grafts of different species, even if they display diverse photoperiodic responses. The nature of this substance has remained elusive for decades, despite the effort dedicated to its characterization.

In this review we discuss recent progress in identifying components involved in regulating the photoperiodic control of flowering; much of these advances come from analyses performed in the model species Arabidopsis (*Arabidopsis thaliana*), a LD plant, and rice (*Oryza sativa*), a SD plant (Yanovsky and Kay, 2003; Hayama and Coupland, 2004; Searle and Coupland, 2004; Corbesier and Coupland, 2005; Imaizumi and Kay, 2006). The molecular mechanisms underlying the photoperiodic responses in other plant species have been poorly characterized; only how the flowering response to daylength is achieved in plants such as tomato (*Solanum lycopersicum*) and *Pharbitis nil* has begun to be analyzed and is briefly summarised (Hayama *et al.*, 2007; Mizoguchi *et al.*, 2007). We discuss recent data related to the specific regulatory mechanisms controlling *CONSTANS* (*CO*) expression and stability by the circadian clock and several photoreceptors, and that provide the molecular basis for our current understanding of the external coincidence model in the control of photoperiodic flowering (Yanovsky and Kay, 2002; Valverde *et al.*, 2004; Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). We also describe the involvement of the floral integrator *FT (FLOWERING LOCUS T)* in this process, as well as its proposed role as an essential component of the florigen (Lifschitz *et al.,* 2006; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007). Finally, we depict other light signalling and circadian-dependent proteins that participate in the regulation of photoperiodic flowering.

## The circadian system in photoperiodic flowering

Daylength measurement depends on the ability of plants to detect light and the existence of a timekeeping mechanism referred to as the circadian clock. As in other organisms, the plant circadian system consists of input pathways that provide temporal information to the clock, the central oscillator mechanism itself, responsible for driving rhythms with a period close to 24 h, and a number of output pathways that regulate metabolic and developmental processes using the temporal information provided by the clock; the participation of the circadian clock in the control of biological activities allows plant species to anticipate and adapt to periodic environmental changes, maximizing their opportunities to survive successfully (Mas, 2005; McClung, 2006; Hotta *et al.*, 2007) (Fig. 1). The control of flowering by daylength is a key determinant of seasonal patterns of flowering, and is a process regulated by one or more of these output branches of the clock. To measure daylength and achieve this photoperiodic regulation, the core oscillator determines the daily rhythms in output genes, and these can set the light sensitive phase for triggering the floral transition when plants are exposed to appropriate photoperiodic conditions.

### Light perception and entrainment of the circadian clock

Light is perceived by photoreceptors and represents the main input pathway to the clock; the pace of the clock is reset by light every day allowing the progressive

adjustment of the clock to the time of dawn, so that the mechanism of the oscillator remains synchronized with external cycles of light and dark (Franklin *et al.*, 2005; Jiao *et al.,* 2007). Plants have evolved an array of photoreceptors to detect light over a large range of fluence rates and wavelengths, including the PHYTOCHROMES (PHY), which absorb in the red and far-red region of the spectrum, and the CRYPTOCHROMES (CRY), PHOTOTROPINS (PHOT), and the ZEITLUPE (ZTL)/ LOV KELCH PROTEIN 2 (LKP2)/FLAVIN BINDING KELCH REPEAT F-BOX 1 (FKF1) family, all of which absorb blue and UV-A light (Yanovsky and Kay, 2003; Jiao *et al.*, 2007).

*Phytochromes*

In higher plants PHYs constitute families with both distinct and overlapping functions in light perception (Quail, 2002). Arabidopsis contains five PHYs (A-E) with PHYA playing the most prominent role in LD perception. In fact *phyA* mutants show a flowering delay when grown in SD conditions that are extended for several hours with incandescent light, enriched in far-red light that promotes flowering (Johnson *et al.*, 1994). By contrast, red light-responsive phytochromes in Arabidopsis only have secondary roles in the photoperiodic regulation of flowering time, and in fact, Arabidopsis wild type plants do not discriminate SD from LD under red light (Mockler *et al.*, 2003). However, PHYB contributes to daylength perception in Arabidopsis through its interaction with PHYA and CRY2 (Mockler *et al.*, 2003). In contrast to Arabidopsis, rice *phyA* mutants do not display significant alterations in flowering time (Takano *et al.*, 2001), whereas mutations in either rice *phyB* or *phy*C cause moderate early flowering under LD conditions (Ishikawa *et al.,* 2005; Takano *et al.*, 2005). However, *phyA* mutations, in combination with *phyB* or *phyC,* caused dramatic early flowering of double mutant plants (Takano *et al.*, 2005), suggesting that these phytochromes are the main photoperiodic photoreceptors in rice. Consistent with this, rice *se5* mutant, affected in the biosynthesis of the phytochrome chromophore, is insensitive to photoperiod and flower early in all photoperiodic conditions (Izawa *et al.*, 2000). Intriguingly, in tomato no flowering phenotypes have been associated with mutations affecting *PHY* genes, even though flowering in this species is affected by light intensity and at certain point by photoperiod (Samach and Lotan, 2007).

The activity of phytochromes is crucial for the light-mediated entrainment of the clock (Fig. 1). Arabidopsis *phy* mutants have a normal clock function under continuous dark, excluding a direct effect of these photoreceptors on the oscillator (Devlin and Kay, 2000). Single and multiple combinations of Arabidopsis *phy* mutants analyses in red, far-red or blue lights showed that the loss of one or more photoreceptors caused a lengthening in the period of the clock and suggest partially overlapping roles for different PHYs in clock entrainment (Somers *et al.*, 1998a; Devlin and Kay, 2000).

The phytochrome-dependent input pathway to the clock may be mediated through interaction with basic helix-loop-helix (bHLH) proteins such as PHYTOCHROME INTERACTING FACTOR 3 (PIF3). Irradiation of Arabidopsis plants with red light induces the binding of phytochrome to PIF3, whereas a pulse of far-red light releases phytochrome from the complex (Ni *et al.*, 1999). PIF3 binds to a G-box sequence motif present in the promoters of two central components of the clock, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LONG ELONGATED HYPOCOTYL* (*LHY*), inducing their transcription. Indeed, the induction of *CCA1* and *LHY* was reduced in transgenic plants expressing *PIF3* antisense RNA (Martínez-García *et al.*, 2000). The lack of circadian defects in *pif3* mutants could be accounted for by other members of the PIF family such as PIF4 (Huq and Quail, 2002) and PIF5/PHYTOCHROME INTERACTING FACTOR-LIKE 6 (PIL6) (Fujimori *et al.*, 2004; Nozue *et al.*, 2007), that might have redundant functions in the light signal transduction to the clock and compensate for the loss of *PIF3* function. However, the absence of circadian alterations in *PIF3* antisense and *PIF3* overexpression lines (Kim *et al.*, 2003a; Monte *et al.*, 2004; Oda *et al.*, 2004; Viczian *et al.*, 2005) poses a question on a direct role for PIF3 in the oscillator, although it might still modulate the light input to the clock. In addition, some PIF/PIL proteins can also interact with TIMING OF CAB EXPRESSION 1 (TOC1/PRR1), an Arabidopsis pseudo-response regulator (PRR) whose central role on clock function will be discussed in the following section (Yamashino *et al.*, 2003; Fujimori *et al.*, 2004; Ito *et al.*, 2007). TOC1 and PIF/PIL interactions may occur at the *CCA1* and *LHY* promoters, enhancing their expression in a light-dependent fashion.

Mutations in *SENSITIVITY TO RED LIGHT REDUCED 1* (*SRR1*), another PHYB signalling pathway component, cause a number of circadian defects such as shortening of the period of leaf movement and *TOC1*

and *CCA1* expression (Staiger *et al.,* 2003), as well as early flowering and long hypocotyl in red light (Hall *et al.,* 2002; Staiger *et al.,* 2003). Because SRR1 protein does not interact directly with PHYB, other components must lie between them.

*FAR-RED ELONGATED HYPOCOTYL1* (*FHY1*) and *FHY3,* related to PHYA signalling, are required for phase shifting of leaf movement in response to far-red light (Yanovsky *et al.*, 2001). FHY3 was also associated with the gating of PHY signalling into the circadian clock (Allen *et al.*, 2006). Furthermore, mutations in *SUPPRESSOR OF PHYA1* (*SPA1*) led to a reduction in the free-running period of *TOC1* and *CCA1* expression (Ishikawa *et al.*, 2006).

Light may also signal to the oscillator through DE-ETIOLATED1 (DET1) (Song and Carre, 2005), and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Ma *et al.*, 2003), negative regulators of PHY and CRY signalling. Mutations in *DET1* and *COP1* genes cause reduced circadian period, possibly through the inhibition of LHY degradation (Song and Carre, 2005).

### *Blue light receptors*

Blue light promotes flowering in Arabidopsis (Goto *et al.*, 1991), and CRY2 regulates flowering time redundantly with CRY1 and PHYA under these conditions (Lin and Shalitin, 2003; Mockler *et al.*, 2003). Associations between PHYs and CRYs are supported by functional interactions (Ahmad *et al.*, 1998; Mas *et al.*, 2000), and in fact, CRY2 binds physically to PHYB antagonizing its inhibitory effect on flowering initiation (Mockler *et al.*, 1999; Mas *et al.*, 2000). Both CRY1 and CRY2 have partially overlapping functions in clock entrainment (Somers, 2005), and CRY1 may also act downstream of PHYA in red light signalling (Devlin and Kay, 2001). Double mutants *cry1 cry2* still show robust rhythmicity (Devlin and Kay, 2000), indicating that cryptochromes do not form a part of the central circadian oscillator in plants as they do in mammals (Cashmore, 2003). Because a quadruple photoreceptor mutant of *cry1 cry2 phyA phyB* still keeps track of time and retains circadian rhythmicity (Yanovsky *et al.*, 2000), it is possible that photoreceptors like PHYC, PHYD and PHYE or ZTL and ZTL-like proteins may provide light input to the clock (Imaizumi *et al.*, 2005; Kim *et al.*, 2007a; Sawa *et al.*, 2007).

In rice, three cryptochromes, OsCRY1a, OsCRY1b and OsCRY2, have been identified and only the latter is involved in the promotion of flowering time (Hirose *et al.*, 2006). In tomato, overexpression or silencing of *CRY2* had no effect on the developmental timing of the transition to flowering, although the rate of leaf production was altered causing a delayed appearance of flowers (Giliberto *et al.*, 2005).

ZTL/LKP2/FKF1 proteins, another family of blue light receptors, are also involved in the control of flowering time and circadian rhythms in Arabidopsis (Nelson *et al.*, 2000; Somers *et al.*, 2000; Jarillo *et al.*, 2001; Schultz *et al.*, 2001; Imaizumi *et al.*, 2003). All three proteins bear a PAS/LOV domain that binds FMN (Imaizumi *et al.*, 2003), an F-box domain devoted to recruit proteins for ubiquitination and target them for subsequent degradation (Han *et al.*, 2004), and six kelch repeats mediating the establishment of interactions between proteins. Phototropins contain a chromophore binding domain very similar to the PAS signal-sensor motif present in ZTL/LKP2/FKF1, but in contrast to these, they do not appear to have a role in the control of flowering time (Christie, 2007).

*ztl* mutants show an array of defects on circadian rhythmicity and clock-controlled gene expression, flowering late only under LD (Nelson *et al.*, 2000; Somers *et al.*, 2000; Jarillo *et al.*, 2001; Imaizumi *et al.*, 2003; Kevei *et al.*, 2006). Although *ZTL* mRNA is constitutively expressed, ZTL protein levels show a daily pattern of oscillation (Kim *et al.*, 2003c), and this rhythmic expression of ZTL is necessary to sustain a normal circadian period by controlling the proteasome-dependent degradation of TOC1 (Somers *et al.,* 2000; Mas *et al.*, 2003b; Han *et al.*, 2004). TOC1 also interacts with PRR3, modulating TOC1 stability by hindering ZTL-dependent TOC1 degradation (Para *et al.*, 2007). However the degradation of TOC1 is not sufficient to explain the *ztl* phenotypes, and in fact, ZTL also targets the TOC1 homologue PRR5 for degradation (Kiba *et al.*, 2007). These observations, together with the ability of ZTL to bind PHY and CRY (Jarillo *et al.*, 2001), argue for a role of ZTL in the light input to the clock. GIGANTEA (GI), another clock-component (Fowler *et al.*, 1999), is essential to establish and sustain oscillations of ZTL by a direct protein-protein interaction (Kim *et al.*, 2007a). GI stabilizes ZTL *in vivo* and the ZTL-GI interaction through the LOV domain of ZTL is strongly enhanced by blue light. Notably, a mutation in the LOV domain eliminates blue-light-enhanced binding of GI to ZTL. These data are consistent with a function of ZTL as a blue-light photoreceptor, which facilitates its own stability through a blue-light-enhanced GI inter-

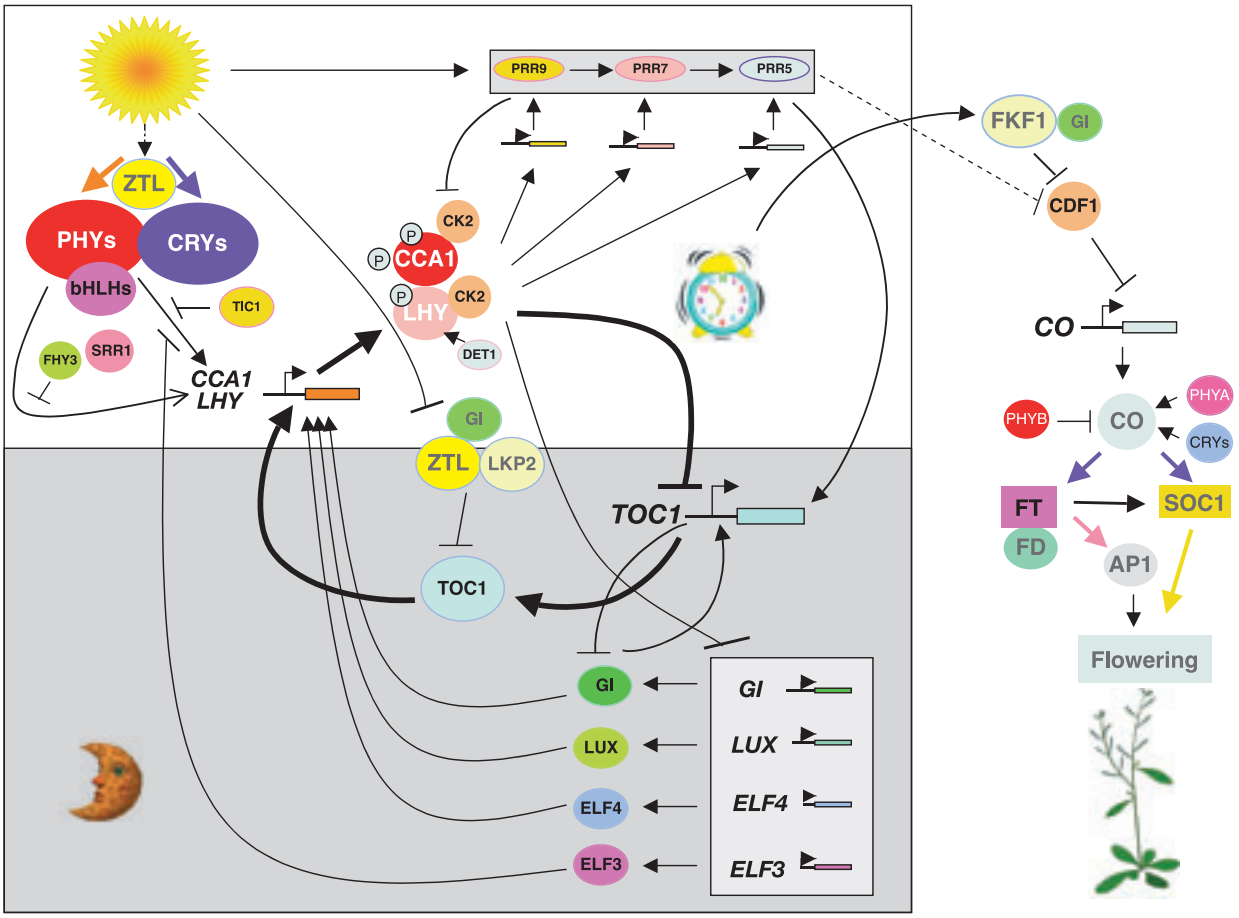


**Figure 1.** Clock-controlled photoperiodic induction of flowering in Arabidopsis. CO promotes flowering in response to LD mediating the control of flowering by the circadian clock. Phytochromes and cryptochromes entrain and condition the negative-feedback loop that comprises the central oscillator. ZTL family of proteins may also act as blue light photoreceptors, using the LOV domain as a flavin binding site, and mediating the effects of light/dark cycles on the phase and period of the circadian clock. Light input to the clock via phytochrome may occur through complexes with bHLHs, such as PIF factors, which bind to a G-box motif in *CCA1* and *LHY* promoters. SRR1, a PHYB signalling pathway component, has also effects on the clock. Different proteins such as ELF3, TIC1 and FHY3 act as clock-gated negative regulators of light input to the clock.
A reciprocal regulation between TOC1 and CCA1/LHY defined the first basic loop for the clock mechanism in Arabidopsis. When CCA1/LHY levels fall late in the day, TOC1 may activate the transcription of *CCA1/LHY,* thus forming the outline of a transcriptional feedback loop highlighted with thick lines. CCA1 and LHY are phosphorylated by CK2, which may make them substrates for the SCF complex and target them for degradation by the proteasome. Light may also signal to the oscillator through DET1 regulating LHY degradation. LHY/CCA1 also act as negative regulators of *ELF3, ELF4, LUX* and *GI,* which positively regulate the transcription of *LHY/CCA1.* ZTL, and possibly LKP2, target TOC1 and PRR5 for degradation via the ubiquitin system, playing some role in protein turnover of clock-associated components. GI is essential to establish and sustain oscillations of ZTL by a direct protein-protein interaction. At least two other loops are thought to interlock with the CCA1/LHY/TOC1 loop. In the first one, *TOC1* may be activated by a hypothetical evening-expressed protein that itself is repressed by TOC1 and suggested that may be GI. PPR5, PPR7 and PPR9 proteins, also regulated positively by *CCA1* and *LHY,* may close another regulatory feedback loop. Shaded area indicates activities peaking in the subjective night, and white area indicates activities peaking during the subjective day.
Clock-controlled expression of genes involved in the light signalling pathways and flowering-time regulation provide the organism with the ability to anticipate and adapt to periodic changes in the environment. The initiation of flowering begins when *CO* expression coincides with light. FKF1 is required to increase *CO* transcription at dusk, controlling the stability of CDF1, which is a repressor of *CO* transcription. FKF1 and GI form a complex that is recruited to the *CO* promoter in the late afternoon to regulate *CO* expression, providing a mechanistic view of how the coincidence of light with circadian timing regulates photoperiodic flowering. PRR9, PRR7 and PRR5 activate *CO* expression during the daytime predominantly by repressing *CDF1* repressor. Blue and far-red lights stabilize CO through PHYA and CRY2, whereas red light acting through PHYB destabilizes the protein. Active CO protein can directly increase expression of the *FT* and *SOC1* genes, promoting the transition to flowering. FT protein, as part of the florigen, is transported to the SAM where interacts with FD to induce the expression of *AP1,* that play a pivotal role in specifying floral meristems during the floral transition.

action. The cycling of GI protein may confer a post-translational rhythm on ZTL protein. This mechanism of establishing and sustaining robust oscillations of ZTL results in the high-amplitude TOC1 rhythms necessary for proper clock function (Kim *et al.*, 2007a).

The over-expression of *LKP2* gene also provokes a number of circadian alterations, suggesting that this protein may function within or close to the circadian clock (Schultz *et al.*, 2001). In fact, this protein may have a similar function to ZTL in mediating TOC1 degradation since LKP2 interacts with both Skp1-like proteins and TOC1 (Yasuhara *et al.*, 2004). The potential redundancy of ZTL and LKP2 is underscored by the fact that neither *ztl* nor *lkp2* mutants are arrhythmic, whereas plants overexpressing either ZTL or LKP2 are, presumably due to increased degradation of TOC1 (Somers *et al.*, 2004). The *ztl lkp2* double mutant has a phenotype similar to that of *ztl*, suggesting that the effects of *lkp2* on circadian function are subtle (Somers, 2005).

The third member of this family, FKF1, is structurally quite similar to ZTL and LKP2 but, in contrast to *ztl*, the *fkf1* mutant has a weaker effect on circadian clock regulation (Nelson *et al.*, 2000). FKF1 appears to function in the regulation of flowering time as we will discuss in the next section. *FKF1* mRNA is itself clearly circadian clock-regulated, peaking towards the end of the day (Imaizumi *et al.*, 2003). FKF1 has been implicated in a mechanism that activates *CO* expression by light, controlling the stability of CYCLING DOF FACTOR 1 (CDF1), which is a repressor of *CO* transcription (see section «Photoperiodic induction of flowering in Arabidopsis» below; Imaizumi *et al.*, 2005).

Therefore, both light-labile CRY2 and PHYA together with FKF1 seem to be the most important photoreceptors discriminating day and night in Arabidopsis (Fig. 1); their combined ability to detect the wide range of light qualities that exist in nature might ensure the correct onset of plant developmental programs that rely on light perception. Photoreceptors mediate light input to the clock, but they are themselves regulated by the clock, creating regulatory feedback loops that play a central role in the circadian gating of photic signals to the clock (Fig. 1). In addition, light can also regulate the expression of clock genes such as *CCA1*, *LHY*, *GI*, *ELF4*, *PRR9*, etc (Wang *et al.,* 1997; Matsushika *et al.*, 2000, 2002; Tepperman *et al.*, 2001; Kim *et al.*, 2003b; Farre *et al.*, 2005; Kikis *et al.*, 2005; Locke *et al.*, 2005b), or the accumulation of clock proteins by modulating their translation as in the case of LHY and PRR7 that display light-dependent daily rhythms (Kim *et al.,* 2003b; Farré and Kay, 2007); the stability of clock components that can be targeted for degradation, as it occurs with TOC1 and ZTL proteins, may also be influenced by light (Mas *et al.*, 2003b; Kim *et al.*, 2007a). TOC1 protein levels are constantly elevated in *ztl*, consistent with a role for ZTL in the degradation of TOC1, and the interaction between these proteins (Mas *et al.*, 2003b) has been proposed to be regulated by light, making photo-activated ZTL unable to get in contact with TOC1. A blue-light-enhanced GI interaction with ZTL may be essential to establish and sustain oscillations of this protein (Kim *et al.*, 2007a).

### *Central oscillators*

The rhythmic behaviour of the circadian clock resides in an endogenous oscillator with a period length close to 24 h that can be entrained to daily oscillations in light and temperature (Mas, 2005; McClung, 2006; Hotta *et al.*, 2007). In plants, the circadian system is likely to consist of more than one clock. There is compelling evidence for independent oscillators in each cell. It is also possible that there are cell-specific oscillators and multiple oscillators in individual cells (see Gardner *et al.*, 2006, for a review). Genetic studies support a role for the circadian clock in a regulatory pathway involved in the control of flowering time in response to daylength en Arabidopsis. Most of the mutants isolated on the basis of their altered circadian phenotypes such as *toc1* (Somers *et al.*, 1998b), *ztl* (Somers *et al.*, 2000) and *lux arrhythmo* (*lux)* (Hazen *et al.*, 2005; Onai and Ishiura, 2005) also exhibit defects in the regulation of flowering time. Conversely, many mutants initially selected for their defects in photoperiodic flowering also display alterations in other output pathways of the clock as well as aberrant circadian rhythms; that is the case of *early flowering 3* (*elf3*) (Hicks *et al.*, 1996), *lhy* (Schaffer *et al.*, 1998), *gi* (Fowler *et al.*, 1999; Park *et al.*, 1999), and *elf4* (Doyle *et al.*, 2002; McWatters *et al.*, 2007).

Transcriptional feedback loops are a feature of circadian clocks both in animals and plants (Mas, 2005). Based on their circadian behaviour, two Arabidopsis transcription factors, CCA1 and LHY, and the pseudo-response regulator TOC1 were proposed as core components of the clock (Fig. 1) (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Strayer *et al.*, 2000; Alabadi *et al.*, 2001). *CCA1* and *LHY* fulfil required criteria for being clock components, showing circadian oscillations

of transcript and protein levels in plants kept in continuous light (Schaffer *et al.*, 1998; Wang and Tobin, 1998). In addition, plants over-expressing *CCA1* and *LHY* genes exhibited arrhythmicity as well as late flowering phenotype. Moreover, *LHY* and *CCA1* were shown to be partially redundant genes, absolutely required to sustain circadian rhythms (Green and Tobin, 1999; Alabadi *et al.,* 2002; Mizoguchi *et al.*, 2002). CCA1/LHY-like proteins, named REVEILLE (RVE), have been identified (Zhang *et al.,* 2007); some of them are clock-controlled genes and oscillate at both mRNA and protein levels.

On the other hand, TOC1 integrates the environmental information to coordinate circadian responses (Mas *et al.*, 2003a). *toc1* mutations shorten the period of multiple rhythms and cause early-flowering (Millar *et al.*, 1995; Somers *et al.*, 1998b). This essential component of the oscillator is a nuclear protein containing an atypical response-regulator-receiver domain and two other motifs, the CCT and an acidic domain, suggesting a role in transcriptional regulation (Makino *et al.*, 2000; Strayer *et al.*, 2000). *TOC1* is itself circadian regulated and participates in a feedback loop to control its own expression (Strayer *et al.*, 2000) (Fig. 1). In transgenic TOC1-over-expressing plants, the circadian rhythm of *CAB2* expression was damped and the circadian profiles of potential clock-associated genes *CCA1*, *LHY*, *GI* and *CCR2* were all markedly altered, implicating TOC1 as a player within, or close to, the central oscillator (Makino *et al.*, 2002). *TOC1/PRR1* and related *PRR3, 5, 7* and *9* genes of Arabidopsis are transcribed with a circadian rhythm and accumulate sequentially after dawn in the order PRR9-7-5-3-TOC1, suggesting that the PRR family of proteins is closely associated with circadian clock function (Matsushika *et al.*, 2000; Mizuno, 2004). The functional characterization of the *PRR* genes has been performed by analysing loss of function mutants and transgenic over-expressing lines (Mizuno, 2004; Matshusika *et al.*, 2007). Specific circadian phenotypes, such as altered rhythms under continuous light, changes in flowering time and altered sensitivity to red light during photomorphogenesis have been described for each of the *prr* mutants (Yamamoto *et al.*, 2003; Mizuno, 2004; Mizuno and Nakamichi, 2005; Matsushika *et al.*, 2007).

TOC1 appears to positively regulate *LHY* and *CCA1* expression, whereas LHY and CCA1 negatively regulate *TOC1*, and this reciprocal interaction establishes the outline of a transcriptional feedback loop initially proposed as the molecular basis for clock rhythm (Alabadi *et al.*, 2001) (Fig. 1). LHY/CCA1 act redundantly in the late night and early day by binding to a *TOC1* promoter region that contains a sequence over-represented in a cluster of evening-phased genes (the evening element, EE, AAATATCT), and repressing its expression (Harmer *et al.*, 2000; Alabadi *et al.*, 2001, 2002; Mizoguchi *et al.*, 2002; Harmer and Kay, 2005). In addition, recent observations indicate that *TOC1* circadian induction is accompanied by clock-controlled cycles of histone acetylation that favor transcriptionally permissive chromatin structures at the *TOC1* locus (Perales and Mas, 2007). At dawn, *TOC1* repression relies on the *in vivo* circadian binding of CCA1, while histone deacetylase activities facilitate the switch to repressive chromatin structures and contribute to the declining phase of *TOC1* waveform around dusk. The chromatin remodeling activities relevant at the *TOC1* locus are distinctively modulated by photoperiod, suggesting a mechanism by which the clock sets the phase of physiological and developmental outputs.

Besides the transcriptional level of regulation, daily phase specification may involve differential binding properties or phosphorylation status of clock components at distinct circadian phases or different interacting partners recruited to the promoters that modulate CCA1/LHY/RVE function. Interestingly, CCA1 and LHY are phosphorylated in vitro by casein kinase 2 (CK2) (Sugano *et al.*, 1998; Sugano *et al.*, 1999; Daniel *et al.*, 2004), and this modification is necessary for their circadian oscillator function in Arabidopsis. Overexpression of CK2 regulatory subunits alters the function of the Arabidopsis clock, resulting in period shortening of genes peaking at different phase angles and reduced daylength sensitivity (Portoles and Mas, 2007).

Nevertheless, several lines of evidence suggest that the CCA1/LHY/TOC1 model might not fully account for the complex regulation of clock function. First, *cca1 lhy* mutants are not completely arrhythmic (Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002). Moreover, rhythms in *EARLY FLOWERING 3* (*ELF3)* expression persist in mutants constitutively overexpressing *LHY* (Hicks *et al.*, 2001). On the same way, the model cannot explain why mutations and overexpression of *TOC1* both lead to decrease in *CCA1* and *LHY* (Hayama and Coupland, 2003). Indeed, it is unclear whether TOC1 is directly responsible for regulating *CCA1* and *LHY* expression and how this might be achieved (Hayama and Coupland, 2003; Millar, 2004). Consistent with this, modelling studies show that available data cannot be explained by a single feedback loop (Locke *et al.*, 2005a); this

may imply that other components are required for the proper functioning of the core oscillator and currently somewhat complicated interlocking multi-loop models are favourably envisaged (Gardner *et al.*, 2006; McClung, 2006). At least two other loops are thought to interlock with the CCA1/LHY/TOC1 loop (Fig. 1). Locke *et al.* (2005b) proposed a second loop in which *TOC1* is activated by a hypothetical evening-expressed protein that itself is repressed by TOC1 and suggested that may be GI. Very recently, it has been proposed that PRR5, PRR7 and PRR9, also regulated positively by *CCA1* and *LHY*, close a third regulatory feedback loop (Farré *et al.*, 2005; Harmer and Kay, 2005; Nakamichi *et al.*, 2005a,b; Farre and Kay, 2007). PRR5/7/9 are negative regulators of *CCA1*/*LHY* because *CCA1* and *LHY* transcripts accumulate in *prr7* and *prr7 prr9* mutants (Farré *et al.*, 2005), and *CCA1* is permanently transcribed in the *prr5 prr7 prr9* triple mutant (Nakamichi *et al.*, 2005b). PRR5/7/9 and TOC1 are thought to be mutually repressive (Mizuno and Nakamichi, 2005). Moreover, *PRR7* and *9* are partially redundant genes essential for temperature responsiveness of the Arabidopsis circadian clock (Salomé and McClung, 2005). Emphasizing the close association of the PRRs proteins to clock function, the *prr5 prr7 prr9* triple mutant is essentially arrhythmic under all conditions tested (Nakamichi *et al.*, 2005b). However, overexpression of PRR3, PRR5 and PRR9 has only small period effects (Matsushika *et al.*, 2002; Sato *et al.*, 2002; Murakami *et al.*, 2004), suggesting that additional factors are required for full PRR function. On this way, overexpression of PRR7 leads to severely compromised circadian rhythms (Farré and Kay, 2007). These transgenic lines display significantly reduced levels of *CCA1* and *LHY* RNA, providing further evidence for a transcriptional feedback loop between PRR7 and these transcription factors. Altogether, these observations suggest that the Arabidopsis circadian oscillator is composed of several interlocking positive and negative feedback loops, a feature broadly conserved between fungi, plants and animals. A consistent multi-loop clock model has recently been built through mathematical simulation (Locke *et al.*, 2006; Zeilinger *et al.*, 2006).

TOC1 is nucleus localized and has been proposed to stimulate *CCA1* and *LHY* transcription; however, evidence supporting that TOC1 can bind DNA is still missing. The expression of *CCA1-LHY* is dependent on at least four other genes expressed with *TOC1* in the evening: *ELF3*, *ELF4*, *GI* and *LUX* (Fowler *et al.*, 1999; Park *et al.*, 1999; Liu *et al.,* 2001a; Doyle *et al.*, 2002; Hazen *et al.*, 2005; Onai and Ishiura, 2005) (Fig. 1). Loss of function of *ELF3*, *ELF4* and *LUX* cause early flowering while plants with mutations in *GI* exhibit a late flowering phenotype.

ELF3 binds to PHYB and modulates light signalling to the oscillator, acting as a clock-gated negative regulator of light input to the clock (Covington *et al.*, 2001; Liu *et al.*, 2001a). Another gene, *TIME FOR COFFEE* (*TIC*), encoding a nucleus-acting clock regulator working close to the central oscillator, may have a similar effect to *ELF3* on gating light input to the clock, although during a distinct phase (Hall *et al.*, 2003; Ding *et al.*, 2007). The clock in the *tic* mutants may be arrested in the subjective morning, whereas in *elf3* mutants the clock arrests in the subjective night (McWatters *et al.*, 2000; Hall *et al.*, 2003); indeed, a double mutant *elf3 tic* is completely arrhythmic in light and darkness, indicating that these clock components act at different circadian times.

*ELF4 is* closely linked to the circadian oscillator; *elf4* mutants show similar phenotypes to those displayed by *elf3* mutants, raising the possibility that ELF3 and ELF4 work in close proximity in a pathway controlling clock function (Doyle *et al.*, 2002). A strong reduction of *LHY* and *CCA1* transcripts is observed in *elf4* mutants (Doyle *et al.*, 2002), whereas *CCA1* and *LHY* also negatively regulates *ELF4* expression (Kikis *et al.*, 2005). Furthermore, *ELF4* could also act together with *TOC1* to induce *LHY*/*CCA1*, emphasizing the complex interactions that underlie clock function (McWatters *et al.*, 2007).

*LUX* encodes a small putative Myb transcription factor necessary for activation of *CCA1* and *LHY* expression (Hazen *et al.*, 2005; Onai and Ishiura, 2005). *CCA1* and *LHY* are repressed in the *lux* mutants, whereas *TOC1* is activated. Moreover, CCA1 and LHY bind to the *LUX* promoter and repress its expression (Hazen *et al.*, 2005), as they do with *TOC1*.

The clock-controlled gene *GI* encodes a nuclear protein involved in the photoperiodic control of flowering in Arabidopsis (Fowler *et al.*, 1999; Park *et al.*, 1999; Huq *et al.*, 2000). *gi* mutations affect the expression of central components of the clock such as *CCA1*/*LHY* (Fowler *et al.*, 1999; Park *et al.*, 1999; Mizoguchi *et al.*, 2002)*;* in the same way, *GI* expression is regulated by *LHY*/*CCA1*, indicating that is under control of the clock and is, therefore, a clock output (Park *et al.*, 1999; Mizoguchi *et al.*, 2002). *GI* mRNA rhythms are also perturbed in *elf3* mutants, suggesting that *GI* acts downstream from *ELF3* (Fowler *et al.*, 1999). On the other hand, GI may have a role in PHYB signalling, participating in a light input pathway to the clock (Park

*et al.,* 1999; Huq *et al.*, 2000), and interacts with ZTL-like proteins (Kim *et al.*, 2007a; Sawa *et al.*, 2007). All these observations have favoured the debate about the place of GI in relation to the clock and recently it has been reconsidered as a clock-associated gene (Locke *et al.*, 2005b; Mizoguchi *et al.*, 2005). Besides the complex interaction with components of the oscillator, *GI* mediates between the circadian clock and the floral integrators in the photoperiodic control of flowering, an output pathway that will be discussed in the next section. In fact, *GI* regulates *CO* expression, which is down-regulated in *gi* mutants, whereas over-expression of *CO* in *gi* mutant corrects its late flowering phenotype (Suárez-López *et al.*, 2001).

## Photoperiodic induction of flowering in Arabidopsis

The regulation of flowering time in response to daylength, one of the output pathways of the clock, has become a model of how photoperiodic mechanisms might work in Arabidopsis (Fig. 1). CO is probably a transcriptional regulator that plays a crucial role in the photoperiodic induction of flowering in this species (Puterill *et al.*, 1995; Robson *et al.*, 2001; Suárez-López *et al.*, 2001). *co* mutants flower late only under LD, whereas CO over-expression causes early flowering both under LD and SD (Puterill *et al.*, 1995; Onouchi *et al.,* 2000). CO by itself does not bind DNA but it is likely to participate in a CCAAT-box-binding complex involving HAP proteins (Ben-Naim *et al.*, 2006; Wenkel *et al.*, 2006; Cai *et al.*, 2007).

In response to LD exposure, CO is responsible for the activation of the so-called floral integrator genes, such as *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) that act as strong activators of flowering initiation (Putterill *et al.,* 2004; Baurle and Dean, 2006). *CO* expression is controlled by the circadian clock and suffers different daily oscillations depending on daylength conditions: in Arabidopsis plants grown under non-inductive SD conditions, the period of *CO* expression is largely confined to darkness; however, under LD photoperiods, that promote flowering, *CO* mRNA peaks during the evening before the lights are off and stay high until dawn, overlapping with the illuminated part of the day (Fig. 2). Therefore, *CO* has been postulated to function as a mediator between the circadian clock and the floral integrators *FT* and *SOC1* (Suárez-López *et al.*, 2001).

Growing evidence supports the view that the precise time of *CO* expression is crucial for daylength discrimination. Altering the peak of *CO* expression relative to subjective dusk by shortening or lengthening the duration of the day cycle from 24 to 21 or 30 h showed that the expression of *CO* during the light period correlated with *FT* up-regulation and early flowering (Roden *et al.*, 2002; Yanovsky and Kay, 2002). Similarly, in mutants like *toc1* where the clock run faster, the peak of *CO* expression occurs earlier under SD and overlaps the light period; this change in the pattern of *CO* expression correlates with increased *FT* expression and early flowering. In contrast, in *toc1* mutants grown under SD conditions but reducing the duration of the day to 21 h, the peak of *CO* expression is restricted again to the dark period and these plants flower as late as wild-type plants (Yanovsky and Kay, 2002). Altogether, these observations indicate that light-dependent activation of CO protein is central in daylength measurement and photoperiodic induction of flowering.

The quality of light influences flowering in a specific way; while blue and far-red lights promote flowering in Arabidopsis through the action of PHYA, CRY1 and CRY2 photoreceptors, red light-activated PHYB delays flowering (Johnson *et al.*, 1994; Guo *et al.*, 1998; Yanovsky and Kay, 2002; Valverde *et al.*, 2004). Consistent with this, blue or far-red lights stabilize CO whereas in red light or darkness, CO is degraded with the involvement of the ubiquitin-proteasome pathway (Valverde *et al.*, 2004). These data provide support for the model of external coincidence to explain photoperiodic control of flowering (Bünning, 1936). Many factors are likely to participate in the regulation of CO protein stability, and some aspects of this regulation are still unclear. It remains unknown why CO protein abundance peaks in the late afternoon or evening on LDs, but not in the early morning when *CO* mRNA level is also high. A possibility is that the abundance or activity of proteins that participate in CO degradation may be controlled by the circadian clock such that they fluctuate throughout the light phase of a LD (Valverde *et al.*, 2004). Proteins such as COP1, SPA proteins and ZTL family proteins, involved in light responses or photoperiodic flowering, may regulate CO stability. COP1 is responsible for the proteasome-mediated degradation of HY5, LAF1, PIF3, HFR1 or PHYA (Seijo *et al.*, 2003; Seo *et al.*, 2003, 2004; Bauer *et al.*, 2004; Duek and Fankhauser, 2005). The four-member SPA protein family of Arabidopsis, which acts in concert with COP1 to suppress photomorphogenesis in dark-

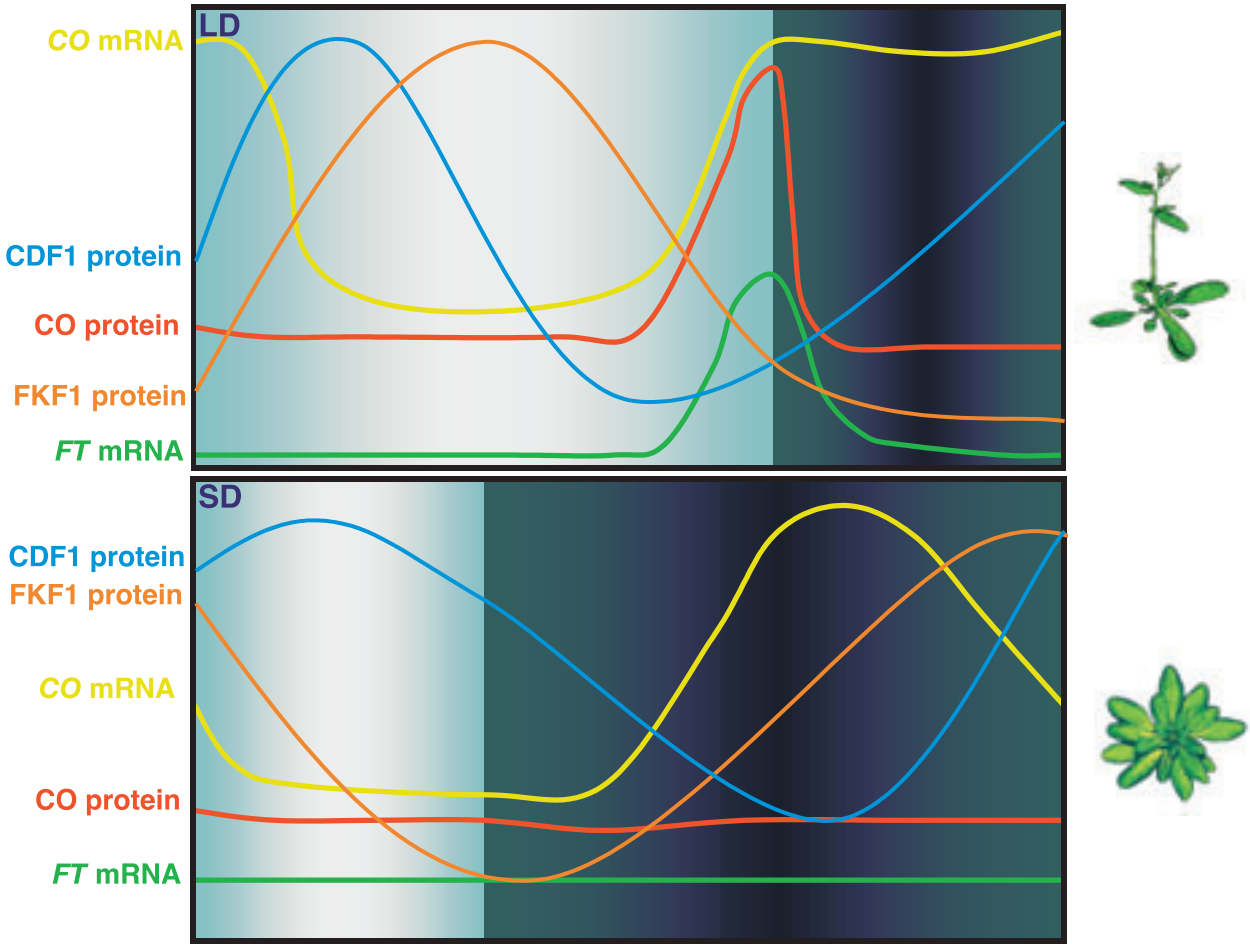


**Figure 2.** The expression of CONSTANS provides the molecular basis for day length perception in Arabidopsis. The expression patterns of *CO* and *FT* mRNAs and CO, CDF1 and FKF proteins under LD (long day) and SD (short day) are shown in the cartoon. Under LD, high levels of the CDF1 protein at the beginning of the day prevent *CO* expression; FKF1 destabilizes CDF1 protein in the presence of light (left side of the panels), allowing an increase in the levels of *CO* mRNA during the evening. In this way, under long photoperiods *CO* mRNA coincides with light during a significant part of the daily cycle; since CO protein is stable in light, CO can activate *FT* expression under LD and promote flowering (upper panel). In contrast, under SD conditions *CO* mRNA is only abundant during the period of dark (right side of the panel), the CO protein does not accumulate and therefore *FT* is not expressed, resulting in delayed flowering (lower panels).

grown seedlings, is essential for photoperiodic flowering (Laubinger *et al.*, 2006). Interestingly, SPA proteins interact physically with CO *in vitro* and *in vivo*, suggesting that SPA proteins regulate CO protein function. *spa1 spa3 spa4* triple mutants are insensitive to photoperiod and exhibit strongly increased CO protein levels, which are not caused by a change in *CO* gene expression. Taken together, these results suggest that SPA proteins regulate photoperiodic flowering by controlling the stability of the floral inducer CO (Laubinger *et al.*, 2006). On the other hand, ZTL family of proteins are also capable of interacting with CO/COL family proteins (Fukamatsu *et al.*, 2005), suggesting their possible participation in the proteasome degradation of the CO protein.

Besides the stability of CO protein, light also modulates *CO* expression at the transcriptional level; mutants affected in the blue light photoreceptor FKF1 flower late under inductive LD and lack the peak of *CO* expression that occurs late in the afternoon in LD grown plants, suggesting that FKF1 is required for this peak that facilitates the coincidence between *CO* expression and light (Fig. 2) (Imaizumi *et al.*, 2003). *FKF1* and *GI* genes are expressed similarly and regulate *CO* transcription (Fowler *et al.*, 1999; Suárez-López *et al.*, 2001; Imaizumi *et al.*, 2003); besides, it has been recently

proposed that FKF1 and GI proteins form a complex in a blue-light dependent manner that is required for day-length measurement (Sawa *et al.*, 2007). Although FKF1 does not regulate the stability of the GI protein, the timing of this interaction appears to be crucial to regulate daytime *CO* expression. In fact, FKF1 function is dependent on GI and mediates the degradation of CDF1, a *CO* repressor (Imaizumi *et al.*, 2005), facilitating the peak of *CO* expression before dusk. Moreover, GI, FKF1, and CDF1 proteins associate with *CO* chromatin and the FKF1-GI complex is recruited to the *CO* promoter in the late afternoon to regulate *CO* expression, providing a mechanistic view of how the coincidence of light with circadian timing regulates photoperiodic flowering (Niwa *et al.*, 2007; Sawa *et al.*, 2007). RED AND FAR-RED INSENSITIVE 2 (RFI2), a RING-domain zinc finger protein, is another relevant factor for shaping the *CO* mRNA profiles, although its precise role has not been defined (Chen and Ni, 2006). On the same way, PRR9, PRR7 and PRR5 have also been implicated in the activation of *CO* expression during the daytime predominantly by inactivating *CDF1* repressor (Nakamichi *et al.*, 2007). Therefore, circadian clock and light-signaling pathways are integrated at the level of CO, ensuring that only under LD does the activation of CO allow the accumulation of *FT* to levels that are sufficient to promote flowering. Together with the post-transcriptional regulation of CO protein (Valverde *et al.*, 2004) that we discussed earlier, this regulation represents an essential adaptive mechanism that allows plants to select the most favourable season for successful flowering.

Although *FT* is an important target, it might not be the only gene directly regulated by CO in the photoperiodic flowering pathway. CO also activates the transcription of a close homolog of *FT*, *TWIN SISTER OF FT* (*TSF*), in a similar way to *FT* regulation (Michaels *et al.*, 2005; Yamaguchi *et al.*, 2005). *TSF* daily expression pattern is quite similar to *FT,* although the tissue-specific patterns of expression are different; *FT* is expressed in the leaf phloem whereas *TSF* is expressed in the stem phloem, suggesting that both proteins play a similar role in the promotion of flowering.

## Spatial control of photoperiodic flowering

Several decades ago, a number of grafting experiments based on the exposure of different organs of the plant to specific photoperiodic conditions demonstrated that daylength is perceived in the leaves (Knott, 1934). As a result, it was postulated the existence of a floral stimulus of unknown nature, that had to be produced in the leaves of photoperiodic species upon exposure to the right daylength regime; this substance(s) had to be transported through the phloem to the SAM, where the floral developmental program was triggered (Zeevart, 2006; Corbesier and Coupland, 2007). Elucidating the site where CO is required to promote floral initiation is a key aspect to understanding the photoperiodic regulation of flowering. Recent data indicate that CO acts in the vascular tissue and not in the SAM to activate *FT* and promote flowering (An *et al.*, 2004; Ayre and Turgeon, 2004); the expression of a *GUS* reporter gene in *CO::GUS* plants is strongly detected in the phloem of leaves and stems (Takada and Goto, 2003; An *et al.*, 2004). Consistent with this, *FT* is expressed in the vascular tissue (Takada and Goto, 2003). Moreover, the expression of *CO* controlled by promoters of genes expressed in the companion cells of the phloem complements the flowering time phenotype of the *co* mutant (Ayre and Turgeon, 2004), something that does not take place when *CO* is expressed from meristem specific promoters (An *et al.*, 2004), suggesting that CO is required in the vascular tissue to promote the floral initiation in response to LD.

*FT* is expressed in the leaves in response to photoperiod, but the FT protein acts in the SAM to promote gene expression, suggesting that a product of *FT* may be transported to the meristem as part of the florigen. Several reports have provided growing evidence that the mobile signal is the FT protein itself rather than mRNA in different species such as Arabidopsis, tomato, rice and cucurbits (Huang *et al.*, 2005; Lifschitz *et al.,* 2006; Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Lin *et al.*, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007). The floral stimulus, but not detectable mRNA of genes similar to *FT*, crossed the junction between grafted tomato plants (Lifschitz *et al.,* 2006). In the case of Arabidopsis, *FT* mRNA is required only transiently in the leaf (Corbesier *et al.*, 2007). In addition, FT fusion proteins expressed specifically in phloem cells move to the apex and move long distances between grafted plants, concluding that FT protein acts as a long-distance signal that induces Arabidopsis flowering (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007). *FT* is required for the activation in the meristem of *SOC1* (Searle *et al.*, 2006). In the apex, FT interacts with a transcription factor, FD, to induce

the expression of floral meristem identity gene *APETALA1* (*AP1*), that play a pivotal role in specifying floral meristems during floral transitions (Abe *et al.*, 2005, Wigge *et al.*, 2005; Liu *et al.*, 2007) (Fig. 1). It is likely that other molecules such as microRNAs (miRNAs) have supporting roles to the florigen, as some of them have been detected in phloem sap as well (Yoo *et al.*, 2004).

Consistent with the role of *FT* as an integrator of flowering signals, this locus is a direct target of *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*), repressors that mediate flowering responses to winter temperatures or to moderate changes in ambient temperature, respectively (Searle *et al.,* 2006; Lee *et al.,* 2007). In addition, chromatin modifications in the genomic regions of *FT* prevent inappropriate expression of this gene that acts as a floral switch (Piñeiro *et al.,* 2003; Takada and Goto, 2003; Germann *et al.,* 2006; Imaizumi and Kay, 2006; Turck *et al.,* 2007).

## Conservation of the photoperiodic flowering response in rice

Photoperiodic control of flowering initiation is widespread among plant species, although it remains unknown whether the components involved in the photoperiodic induction of flowering in Arabidopsis are conserved, especially in those plant species displaying different photoperiodic responses, such as SD and DN plants. Genomic and genetic comparison of components involved in the photoperiodic control of flowering in Arabidopsis and rice, a SD plant, argues for the conservation of regulatory networks in both species (Izawa *et al.*, 2003; Izawa, 2007a). Indeed, the cloning of the rice quantitative trait loci (QTL) *Heading-date1* (*Hd1*), *Hd3a* and *Hd6,* responsible for natural variation in flowering time or heading date, supports this view. *Hd1* encodes a protein with high similarity to CO (Yano *et al.*, 2000); *Hd3a* is highly similar to Arabidopsis *FT* (Kojima *et al.*, 2002); and *Hd6* encodes the α-subunit of the CK2 protein (Takahashi *et al.*, 2001), which has a crucial role in the regulation of clock function in different organisms.

*CCA1*/*LHY*- and *TOC1*-like genes, other members of the family of pseudo-response regulators of Arabidopsis, as well as *GI*, *ZTL*, *LKP2*, *FKF1*, *CDF1*, *ELF3*, *ELF4*, *LUX*, etc., are also found in the rice genome (Doyle *et al.*, 2002; Izawa *et al.*, 2003; Murakami *et al.*, 2003, 2007a,b; Staiger *et al.*, 2003; Hayama and Coupland, 2004; Izawa, 2007a; Nakamura, 2007). A gene related to *CCA1*/*LHY* exhibits circadian rhythms with a phase similar to that of Arabidopsis *CCA1* (Izawa *et al.*, 2002, 2003). A quintet of PRR-like proteins reminiscent to that described in Arabidopsis is also found in rice (OsPRR73, OsPRR37, OsPRR95, OsPRR59 and OsPRR1) (Murakami *et al.*, 2005, 2007b). *OsPRR37* maps close to the *Hd2*-QTL (Yano *et al.*, 2001), identified as the major component enhancing the photoperiod sensitivity of flowering in Nipponbare variety; indeed, the Kasalath *OsPRR37* allele bears a severe mutation in the coding region (Murakami *et al.*, 2005). This locus is highly related to the barley pseudo response regulator *Ppd-H,* a locus involved in adaptation to photoperiod in this species (Turner *et al.*, 2005).

PHYs are likely to play an essential role in the regulation of photoperiodic flowering responses in rice, as suggested by the severe early flowering and insensitivity to daylength displayed by the *photoperiod sensitivity 5* (*se5*) mutant; Se5 is similar to the heme oxygenase involved in the biosynthesis of the PHY chromophore in Arabidopsis (Fig. 3A) (Izawa *et al.*, 2000). Circadian rhythms are largely unaffected in *se5* mutants and the expression of clock output genes is not altered, indicating that rice PHYs must participate in a pathway regulating photoperiodic flowering at least in part independently of the clock (Fig. 3A); this pathway is required to repress the expression of rice *FT* homologue genes (Izawa *et al.*, 2002), and may act similarly to the Arabidopsis PHYB-mediated pathway required to repress flowering independently of the clock, where phyB signals to PHYTOCHROME AND FLOWERING TIME 1 (PFT1) to regulate *FT* expression in response to suboptimal light conditions (Cerdan and Chory, 2003). Arabidopsis PFT1, with a putative orthologue in rice (gi 115478458), has been recently proposed as the Med25 subunit of the plant Mediator complex that mediates communication between transcription regulatory proteins and core promoters, establishing interactions with the C-terminal domain of the largest Pol II subunit (Bäckström *et al.*, 2007).

The transcriptional analysis of *se5* mutants led to the confirmation that a *GI* ortholog is present in rice. *OsGI* is expressed at lower levels in *se5*, is circadian-clock regulated and it follows a temporal pattern of expression similar to that of *AtGI* (Hayama *et al.*, 2002). *OsGI* overexpression causes a delay in flowering of rice plants, and increased expression of *Hd1;* in contrast, *Hd3a* expression was significantly reduced, suggesting that *OsGI* acts to promote *Hd1* expression

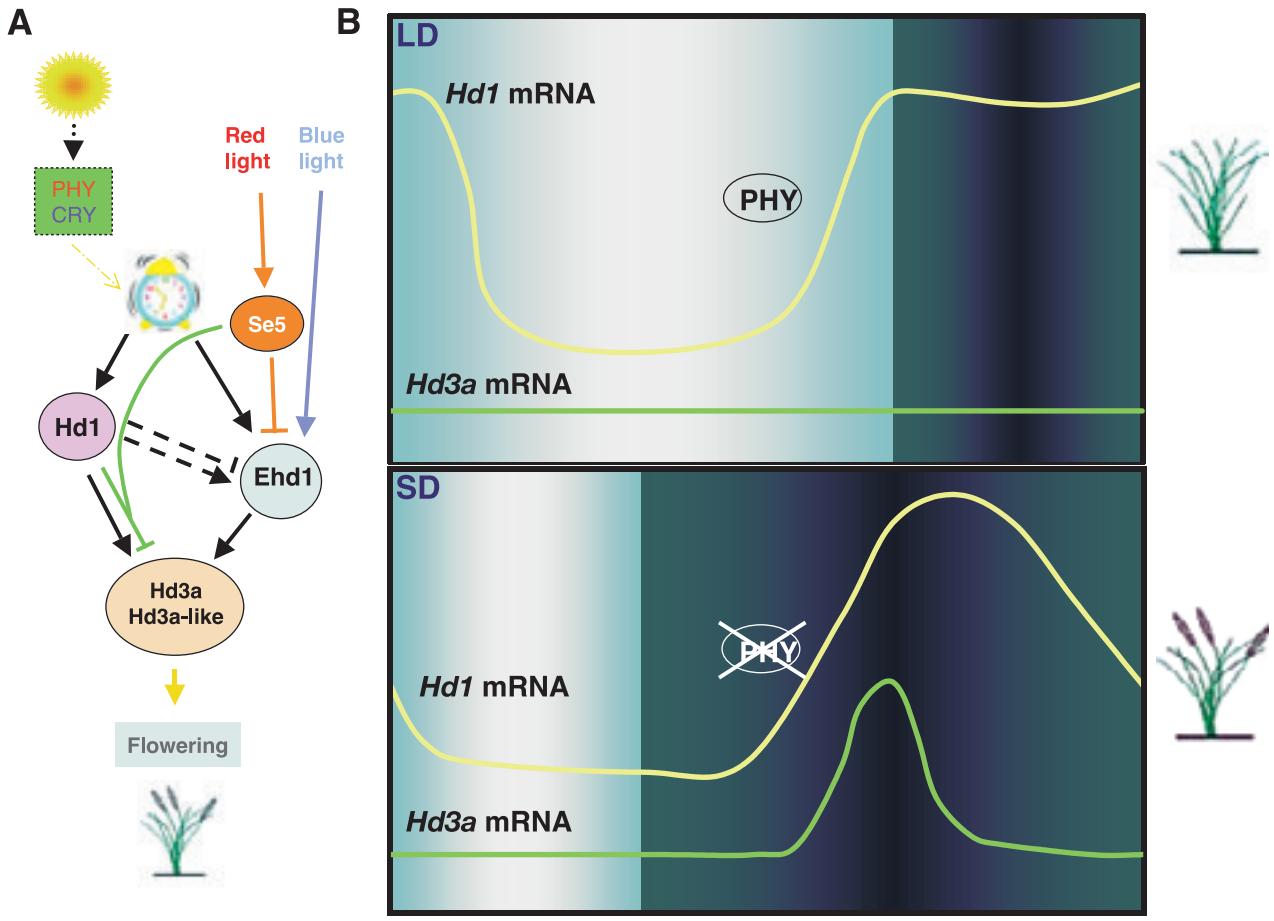


**Figure 3.** Molecular basis for photoperiodic flowering in rice. A) Genetic components involved in the regulation of photoperiodic flowering in rice. The circadian clock regulates *Hd1* (*CO* orthologue) expression, while both light and the clock control the expression of *Ehd1*. *Hd1* and *Ehd1* are likely to interact (dashed line) to finely tune the time of heading in rice, although the mechanisms involved remain poorly understood. Under SD, both Hd1 and Ehd1 promote flowering by activating *Hd3* (*FT* orthologue) expression; in contrast, in LD conditions Hd1 delays flowering (green line), and this repression is PHY-dependent. B) Schematic representation of *Hd1* and *Hd3a* mRNAs during a 24 hour cycle under LDs (upper panel) and SDs (lower panel). Under LDs, Hd1 protein is exposed to day light and, in a process mediated by PHYs, acts to repress *Hd3a* expression, delaying heading of rice plants. In contrast, in SDs, in the absence of active PHYs, Hd1 protein acts to activate *Hd3a* expression and promote heading.

in LD, perhaps through a mechanism similar to that operating in Arabidopsis, but *Hd1* activity results in repression of *Hd3a* expression under LD conditions (Hayama *et al.*, 2003). These observations are consistent with the phenotype of *hd1* mutants that flower earlier than wild-type and have higher levels of *Hd3a* mRNA under LD (Izawa *et al.*, 2002; see below).

Recent data with transgenic plants have shed some light on how the same genes lead to opposite flowering responses in Arabidopsis and rice (Fig. 3A,B). As in Arabidopsis, overexpression of *Hd3a*, the likely *FT* orthologue in rice, accelerates flowering (Kojima *et al.*, 2002). However, in contrast to Arabidopsis, *Hd3a* expression in wild type plants is induced under SD (Kojima *et al.*, 2002), and this daylength-dependent regulation of *Hd3a* is mediated by *Hd1*, which exhibits diurnal patterns of expression similar to those of Arabidopsis *CO* under LDs and SDs (Izawa *et al.*, 2002; Kojima *et al.*, 2002; Hayama *et al.*, 2003; Shin *et al.*, 2004). *Hd1* promotes heading under SD but represses it under LD, because loss of *Hd1* function causes early flowering and increased transcription of *Hd3a* under LDs, but late flowering and decreased *Hd3a* mRNA levels under SD (Yano *et al.*, 2000; Izawa *et al.*, 2002; Kojima *et al.*, 2002). In rice, coincidence of *Hd1* expression and exposure to light may generate

LD signals that inhibit *Hd3a* transcription and prevent flowering; the floral repression activity of Hd1 under LDs depends on Se5, and *se5 hd1* plants never flower earlier than each single mutant under LDs, suggesting that both genes inhibit flowering within the same genetic pathway. In fact, *se5 hd1* double mutants flower later than *se5* mutant, indicating that, in the absence of *Se5, Hd1* can promote flowering under LDs (Izawa *et al.*, 2002). Therefore, Hd1 represses flowering under LDs, conditions in which this protein is expressed in the evening, overlapping with the period of light. The coincidence of Hd1 with the illuminated part of the day leads to *Hd3a* repression, and this process probably requires the participation of active phytochrome. In contrast, under SDs, Hd1 is only expressed during the period of darkness, when active phytochrome is absent. In these conditions, Hd1 protein is capable to induce Hd3a expression and consequently promote heading (Fig. 3B). Other factors must be involved in this regulatory mechanism so that activation of *Hd3a* under LDs can be achieved in *hd1* mutant background. Intriguingly, the repressive function of Hd1 requires the involvement of PHYB; loss of PHYB activity leads to early flowering in rice (Ishikawa *et al.,* 2005; Takano *et al.*, 2005), similar to the effects of *phyB* mutations in Arabidopsis (Reed *et al.,* 1993), albeit for a different reason: while PHYB antagonizes the activation of *FT* by CO in Arabidopsis (Cerdán and Chory, 2003; Halliday *et al.,* 2003; Valverde *et al.,* 2004; Endo *et al.,* 2005), it stimulates the repression of Hd3a by Hd1 in rice (Ishikawa *et al.,* 2005). Conservation of the floral pathways CO/FT and Hd1/Hd3a strongly suggests that Hd3a is also involved in transmissible signals in rice and molecular confirmation of this role has been obtained recently (Tamaki *et al.*, 2007).

The molecular basis for the dual role of Hd1 protein in both activation (SD) and repression (LD) of flowering remains obscure. The *CO* orthologue from the SD plant *Pharbitis nil* can complement the flowering time defects of the Arabidopsis *co* mutant (Liu *et al.*, 2001b); in the same way, the *Hd1* orthologue from wheat, a LD plant, can complement defects in the rice *Hd1* gene (Nemoto *et al.*, 2003), suggesting that protein structural differences among CO orthologues are unlikely to explain distinct photoperiodic flowering responses across species. In fact, recent results support the notion that different regulatory mechanisms might underlie the photoperiodic flowering responses found in plant species. For instance, SD response in *Pharbitis* is controlled by a dedicated light sensitive clock, set by dusk, that activates two putative orthologs of *FT* (Pn*FT1* and Pn*FT2*) transcription in darkness, a different mechanism for measuring daylength than those described for Arabidopsis and rice (Hayama *et al.*, 2007).

In addition, despite the conservation of key components of the photoperiod-promoting pathways in rice, Arabidopsis and other species, specific factors might still exist in some of these species but not in others. A two-component signaling cascade is integrated into the conserved pathway in the photoperiodic control of flowering in rice (Doi *et al.*, 2004). Early heading date 1 (Ehd1) is a transcription factor that confers SD promotion of flowering regulating *FT-like* and MADS box gene expression in the absence of a functional allele of *Hd1*. *Ehd1*, without orthologues in Arabidopsis, is expressed only in the presence of blue light signals with an *Hd1*-deficient background (Izawa, 2007a). Ehd1 could participate in developmental or environmental signals mediated not only by light but also by phytohormones, which may affect flowering time in some plant species (Samach *et al.,* 2000). At least two factors that regulate *Ehd1* expression have been isolated; one of them, *Oryza sativa* LEC2 and FUSCA LIKE 1 (OsLFL1) is a B3 transcription factor that causes late flowering when overexpressed. In these overexpression lines OsLFL1 appears to repress *Ehd1* by binding its promoter (Peng *et al.*, 2007, 2008). In contrast, OsMADS51 acts to promote flowering initiation under SD; *OsMADS51* functions downstream of *OsGI* and upstream of *Ehd1*, activating its expression (Kim *et al.*, 2007b). However, few genes have been found to act downstream of *Ehd1*, suggesting that this gene acts as a floral inducer in the final steps of the photoperiodic pathway in rice. Interestingly during domestication, the adaptation of rice to northern regions by artificial selection may have become possible through the interactions of the *Hd1*- and *Ehd1*-dependent pathways (Izawa, 2007b).

## Day-neutral response of photoperiodic flowering in tomato

Current tomato cultivars with DN responses have been bred using genetic selection. The modern tomato has a mild SD response that is sometimes unnoticed due to other environmental conditions (high light or mild temperatures). The molecular mechanisms underlying the photoperiodic responses of DN plants are poorly characterized and we do not know yet whether the

ancestral tomato had a strong photoperiodic response and became DN or these plants already had a DN response type. Gene expression patterns reveal that tomato has a circadian clock mechanism (Ben-Naim *et al.*, 2006; Facella *et al.*, 2006), and several processes have been reported to be controlled by the circadian clock (Samach and Lotan, 2007). Since photoperiod has little effect on flowering of tomato, mutations in the clock apparatus may not influence tomato flowering; however, recent evidence suggests that tomatoes have a sense of time measurements and that the connection between the circadian clock and one of the output pathways, the flowering response, may be impaired at a certain point (Mizoguchi *et al.*, 2007). Tomatoes possess *GI*, *LHY, CO* and *FT* homologs (Ben-Naim *et al.*, 2006; Lifschitz *et al.*, 2006; Niinuma *et al.*, 2007). The *GI* and *LHY* homologs show diurnal and circadian expression patterns, and may have similar functions to those of Arabidopsis. This suggests that these genes may also be involved in the regulation of the circadian time in tomato (Niinuma *et al.*, 2007). Unexpectedly, overexpressing the Arabidopsis *CO* gene or tomato *CO*-like genes in tomato did not seem to affect flowering time, even when the overexpression of a tomato *CO* gene does have a strong effect in Arabidopsis flowering (Ben-Naim *et al.*, 2006). This might suggest that *CO*-like genes are not linked to flowering in tomato, although this possibility should be tested first by analyzing loss-of-function alleles of tomato *CO* homologs.

Mutations in an *FT* homolog in tomato (*SINGLE FLOWER TRUSS*; *SFT*) delays flowering, which indicates that *SFT* in tomato actually functions as a floral activator in a similar way to those Arabidopsis and rice homologs (Lifschitz and Eshed, 2006; Lifschitz *et al.*, 2006). SFT-dependent graft-transmissible signals complement all developmental defects in *sft* plants. Since no *SFT* mRNA could be detected in the recipient, either SFT protein is the mobile signal or SFT may elicit a secondary mobile signal (Lifschitz *et al.*, 2006). Whether the *GI*, *LHY* and *CO* tomato homologs are affecting the expression of *FT* or how is the expression of the *FT* gene regulated are still open questions.

The analysis of several thousands expressed sequence tags (ESTs) revealed that 30% of the genes identified appear to be unique to tomato (Yamamoto *et al.*, 2005). Then, novel players may have pivotal roles in the control of flowering time in tomato. Moreover, the isolation of tomato mutants with delayed or accelerated flowering phenotypes and the identification of genes responsible for these phenotypes will be crucial steps for the comparison of mechanisms underlying the three classes of the photoperiodic flowering responses in plants.

## Future perspective

In recent years, we have gained a better understanding of the molecular mechanisms by which plants sense photoperiodic changes within the leaf and integrate the information to set their developmental fate in the shoot apex. While central aspects of how photoperiod is perceived and how the signal is released from the leaves to the SAM are known, we do not completely understand the photoperiodic flowering process. Despite the significant progress achieved, a number of key questions remain unanswered. New components involved in the regulation of *CO* and *FT* expression and activity need to be identified and the functional interactions between known factors, as well as newly identified, must be explored. Given that the spatio-temporal regulation of the *CO* and *FT* expression patterns is crucial to this pathway, it will be essential to determine the expression pattern of the genes involved to contemplate the molecular mechanisms underlying their regulation. The manipulation of the spatial distribution of gene activity by tissue-specific overexpression or through more specific approaches such as the ablation of gene function in specific tissues or cells, already employed with *FT* and *TSF*, would be useful to study if clock components working upstream of CO, and CO itself, are required in phloem companion cells. The use of Arabidopsis, rice and tomato as model species will allow the identification of new factors involved in the regulation of flowering and other photoperiod-mediated processes as well as increase our knowledge of how these factors interact to enable the perception and response to daylength. Finally, one question remains and will be the focus in the coming years; this is, understanding the nature, regulation and the mode of action of the signals generated in the sites of daylength perception as well as establishing how developmental programs are triggered in the sites where the evoked responses occur. In fact, the conserved CO/FT module is being used in other photoperiod-dependent processes, such as tuberization in potatoes and bud dormancy in trees (Martínez-García *et al.*, 2002; Böhlenius *et al.*, 2006). The identification of downstream elements of such module that provide specificity in these developmental processes should in turn also inform our understanding of floral induction.

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