Shedding light on the circadian clock and the photoperiodic control of flowering

Ryosuke Hayama and George Coupland*

Recently, notable progress has been made towards understanding the genetic interactions that underlie the function of the circadian clock in plants, and how these functions are related to the seasonal control of flowering time. The *LHY/CCA1* and *TOC1* genes have been proposed to participate in a negative feedback loop that is part of the central oscillator of the circadian clock. Furthermore, analysis of a flowering-time pathway has suggested how transcriptional regulation by the circadian clock, combined with post-transcriptional regulation by light, could activate proteins that control flowering time in response to appropriate daylengths.

Addresses
Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, D-50829 Köln, Germany
*e-mail: coupland@mpiz-koeln.mpg.de

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Abbreviations

CAB	*CHLOROPHYLL A/B BINDING PROTEIN*
CCA1	*CIRCADIAN CLOCK ASSOCIATED1*
CO	*CONSTANS*
CRY1	Cryptochrome1
elf3	*early flowering3*
FKF1	*FLAVIN-BINDING, KELCH-REPEAT, F BOX1*
FT	*FLOWERING LOCUS T*
GI	*GIGANTEA*
Hd1	*Heading-date1*
LD	long day
LHY	*LATE ELONGATED HYPOCOTYL*
PhyA	Phytochrome A
PIF3	PHYTOCHROME-INTERACTING FACTOR3
TOC1	*TIMING OF CAB EXPRESSION1*
ZTL	*ZEITLUPE*

Introduction

Fluctuations in daylength are recognized by higher plants and allow them to coordinate the initiation of flowering with changing seasons. The alteration of flowering time in response to daylength (i.e. photoperiod) is mediated by complex interactions between environmental signals and the time-keeping mechanism that is associated with the circadian clock. The circadian clock is an autonomous mechanism that generates endogenous rhythms with a period of approximately 24 hours. For convenience, the circadian system is often divided into three general parts [1]. The central oscillator is the core of the system and generates the 24-hour rhythm. The oscillator is synchronized or entrained to daily cycles of night and day through light and temperature signaling pathways, which are often referred to as input pathways. Output pathways are controlled by the oscillator, and represent a range of biochemical and developmental pathways. Control of flowering by daylength may be triggered by such an output pathway, and the activation of this pathway by daylength may be caused by a requirement that the time at which the activity of the pathway peaks coincides with a time at which the plant is exposed to light.

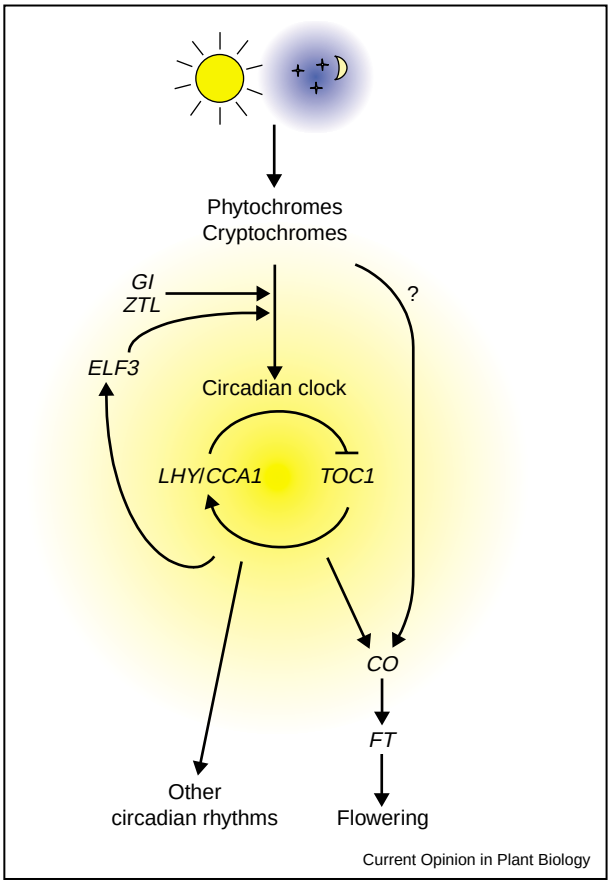
In *Arabidopsis*, mutations that alter the function of the circadian clock have been isolated either by using luciferase marker genes to follow rhythms in output pathways or by identifying mutants that exhibit altered flowering time, a trait that is regulated by the circadian clock. These approaches have identified mutants in which the duration, or period length, of output rhythms is altered. Recent work has focused on the isolation of the genes that are affected by these mutations and on determining whether they act within input pathways, the oscillator or output pathways. These experiments have demonstrated that the distinctions between these three parts of the system are not often clear cut. For example, feed-back loops in which output pathways regulate input to the oscillator mean that some genes simultaneously act in both parts of the system (Figure 1).

Arabidopsis is sensitive to daylength and flowers earlier under long days than under short days. This response is regulated by the circadian clock, and recent work has established molecular-genetic interactions between the control of circadian-clock function and flowering time [2–5]. Studies of this interaction have also suggested that daylength measurement is mediated by transcriptional regulation of gene expression by the circadian clock, combined with post-transcriptional regulation by light. This review focuses on recent advances in understanding the molecular mechanisms involved in the function of the circadian clock and the control of flowering time in *Arabidopsis*.

Regulation of circadian-clock function in *Arabidopsis*

Molecular analyses of the circadian clock in animals and cyanobacteria provided the model of the oscillator as an autoregulatory transcriptional and translational negative-feedback loop [1]. In *Arabidopsis*, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION1*

Figure 1



Model of the circadian system of *Arabidopsis* and its relationship to the flowering-time gene *CO*. Phytochromes and cryptochromes act additively or redundantly as photoreceptors for the light resetting of the circadian clock. *LHY/CCA1* and *TOC1* form a negative-feedback loop in the circadian oscillator; LHY/CCA1 proteins suppress the expression of *TOC1*, which acts as a positive regulator for *CCA1* and *LHY*. The circadian oscillator generates multiple circadian rhythms, including that of the expression of *CO*, which acts as an output for the regulation of flowering time. *CO* activity may also be regulated directly by light signals in a post-transcriptional manner, allowing *CO* to activate under long days and to induce the expression of *FT*, a gene that functions to promote flowering. *ELF3* expression is also regulated by the circadian clock, and acts on light input into the clock to suppress or 'gate' the light signals. This allows the circadian clock to be reset by the dawn signal, and to cycle even under constant light. *ZTL* and *GI* also act on light input into the clock, and ZTL function in the input is accompanied by its interaction with PhyB. The transcript levels of *ZTL* are not regulated by the circadian clock.

(*TOC1*) are candidate genes that have been associated with the central oscillator. CCA1 and LHY are closely related proteins, each with a single MYB-related DNA-binding motif [6,7]. The mRNA transcript levels of these genes oscillate in a similar pattern, peaking in the morning soon after dawn [6,7]. Overexpression of either gene disrupts many circadian rhythms, and loss-of-function alleles in each of them shorten the circadian period by 2–3 hours [6–8,9••]. In addition, the overexpression of either *CCA1* or *LHY* suppresses the expression of CCA1 or LHY [6], suggesting that these genes may form a negative-feedback loop in which they repress their own expression. CCA1 might not feed back directly to repress its own transcription, however, because CCA1 protein appears to be expressed at a time in the cycle, or phase, that is very similar to that of *CCA1* mRNA [6].

The similarity in sequence and function of LHY and CCA1 suggest that they might show partial redundancy, and therefore that the phenotypes of single mutants affected in either of these genes might underestimate the requirement for these genes in the circadian system. Recently, plants in which the activity of both genes was impaired were described, and the effect of *LHY* and *CCA1* on circadian clock function analyzed [9••,10••]. Plants carrying likely null alleles of both genes, that is *lhy-12 cca1-1* double mutants, showed a more severe short-period phenotype in the expression of several clock-controlled genes than either single mutant [9••]. The double mutants showed a severe early-flowering phenotype under short day conditions [9••]. Furthermore, these rhythms were maintained for only two days under continuous light, and thereafter damped out completely [9••,10••]. Similar observations were made under continuous darkness, reducing the likelihood that *LHY* and *CCA1* act only within a light-input pathway to the oscillator [10••]. These results strengthen the idea that these two genes encode components of the oscillator.

TOC1 was originally identified in a mutant that exhibited shortened period length in the circadian rhythm of *CHLOROPHYLL A/B BINDING PROTEIN* (*CAB*) gene expression [11]. This effect was independent of light quality and was observed when plants were entrained by temperature cycles, consistent with *TOC1* acting within the circadian oscillator [12]. The *toc1* mutant also has an early-flowering phenotype. *TOC1* belongs to a novel family of pseudo response regulators and has a characteristic plant-specific motif, called CCT (CO, COL and TOC1). This motif was originally identified in the flowering-time gene *CONSTANS* (*CO*) and is thought to mediate protein–protein interactions and nuclear localization [13–15]. *TOC1* transcript levels oscillate with a peak in the evening. Its overexpression under continuous light increases the period length and severely reduces the amplitude of the oscillations in expression of some circadian clock controlled genes, such as *LHY* and *CCA1*. In contrast, TOC1 overexpression under continuous light increases the period length and severely reduces the amplitude of the oscillations in the expression of some circadian clock controlled genes, such as *LHY* and *CCA1*, whilst completely abolishing the circadian rhythms of the expression of other genes, such as *GIGANTEA* (*GI*), *COLD CIRCADIAN RHYTHM-RNA BINDING2* (*CCR2*) and *CAB* [13].

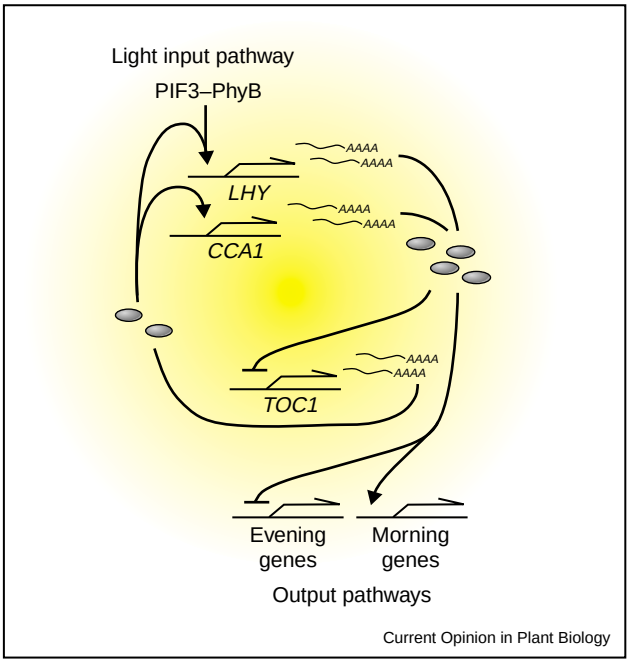
Recently, a model for a feedback loop involving *LHY*, *CCA1* and *TOC1* was proposed [16••]. This model was

initially based on the observation that *LHY/CCA1* and *TOC1* are expressed in opposite phases, with *LHY/CCA1* expression peaking in the morning and *TOC1* expression peaking in the evening. The model proposes that the expression of *TOC1* rises in the evening and participates in the activation of *LHY* and *CCA1* expression the following morning. *LHY* and *CCA1* are also directly activated by light. In turn, LHY and CCA1 are proposed to repress the expression of *TOC1*. Therefore, as *LHY* and *CCA1* expression rises, *TOC1* expression falls, eventually resulting in a reduction in *LHY* and *CCA1* expression as the TOC1 activator is lost. A second cycle then begins with a rise in TOC1 levels. The reduction in *TOC1* expression when *LHY* and *CCA1* are overexpressed supports the roles of LHY and CCA1 as repressors of *TOC1*. Furthermore, *TOC1* expression is elevated in *lhy-12 cca1-1* double mutants [9••,16••].

The repression of *TOC1* by *CCA1/LHY* has been proposed to occur through a promoter region called an evening element, to which CCA1 and LHY protein will bind *in vitro* [16••]. The evening element has been identified in the promoters of several genes whose transcripts oscillate with a peak in the evening, and is also required for the robust cycling of *TOC1* expression [16••,17]. Strikingly, the evening element is closely related in sequence to the *CCA1*-binding site that is present in the *CAB* gene, through which *CCA1* is proposed to activate *CAB* expression in the morning [17,18]. The loss of *TOC1* function reduces the level of *LHY* and *CCA1* mRNAs, supporting the role of TOC1 as an activator of *LHY/CCA1* [16••]. A potential mechanism by which TOC1 may activate *LHY/CCA1* expression was suggested by the interaction of TOC1 and PHYTOCHROME-INTERACTING FACTOR3 (PIF3) in yeast and *in vitro* [13]. PIF3 is a basic helix-loop-helix (bHLH) transcription factor that binds to the *LHY* and *CCA1* promoters, and interacts with phytochrome to mediate light responses [19,20]. Taken together, these results support the idea that *LHY/CCA1* and *TOC1* form a negative-feedback loop within the central oscillator of *Arabidopsis* (Figure 2).

This model is only a framework, however, and there are still questions to be answered. First, it is not clear that TOC1 acts as an activator of *LHY/CCA1*. For example, it is not yet known whether the oscillation of *TOC1* is responsible for generating that of *LHY* and *CCA1*. Furthermore, although a mutation in *TOC1* reduces *LHY/CCA1* levels, so does overexpression of *TOC1* [13]. The relationship between the abundances of *TOC1* and *LHY/CCA1* mRNA is therefore not straightforward. Second, although we know that LHY/CCA1 can repress evening genes by binding to evening elements, we cannot yet explain how other genes that contain evening elements and are expressed in the same phase as *TOC1* respond differently to LHY and CCA1. For example, *GI* and *TOC1* are expressed in a similar phase and the promoters of both

Figure 2



A model of the function of the circadian clock oscillator in *Arabidopsis*. The expression of *LHY* and *CCA1* is activated by light through the function of PIF3, which interacts with the Pfr form of PhyB. These proteins directly suppress the transcription of *TOC1*, an activator of *LHY* and *CCA1*. LHY and CCA1 may also directly activate the transcription of morning genes, whose transcript levels cycle and peak at dawn, and suppress the evening genes, whose transcript levels peak at dusk. As the levels of the LHY and CCA1 proteins fall, the levels of *TOC1* transcript and TOC1 protein are proposed to rise. TOC1 interacts with PIF3, and once more activates the transcription of *LHY* and *CCA1*.

genes contain evening elements that are predicted to bind LHY and CCA1, but *GI* is expressed at a low level in *lhy-12 cca1-1* double mutants whereas *TOC1* is expressed at a high level [9••]. Similarly, we do not know how evening elements, which mediate the repression of gene expression, interact with specific classical CCA1-binding sites, which mediate the activation of gene expression.

Light-input pathways

Genes that are involved in light input into the clock have also been isolated from *Arabidopsis*. Phytochromes (PhyA, PhyB, PhyD, and PhyE) and cryptochromes (Cry1 and Cry2) are photoreceptors that are responsible for red- and blue-light input to the clock, respectively [21,22]. Although the genetic pathway that mediates between these photoreceptors and the circadian clock in light entrainment is not yet clear, some candidate genes have been identified recently.

ZEITLUPE (*ZTL*; also called *ADAGIO1* [*ADO1*]) may be involved in light input into the circadian clock. The *ztl* mutant was originally identified because it exhibits a lengthened period of *CAB* gene expression [23]. This

effect is dependent on light intensity, suggesting that *ZTL* acts through light input to the clock [23]. Recently, the ZTL protein was reported to interact with PhyB and CRY1 [24•]. This suggests that *ZTL* affects the light signaling pathways from photoreceptors to the circadian clock that are involved in resetting the clock. *ZTL* encodes a protein that has a PAS domain, an F-box, and six repeated kelch motifs, suggesting that it might recruit proteins for degradation by the proteosome in a way that is influenced by light [23,24•]. *ZTL* belongs to a family of xthree genes that also includes *FLAVIN-BINDING, KELCH-REPEAT, F BOX1*(*FKF1*) and *LOV KELCH PROTEIN2* (*LKP2*) [25,26]. The *fkf1* mutation appears not to alter the circadian period of clock-controlled genes but alters their phase of expression [25], suggesting that *ZTL* and *FKF1* have different functions in the control of circadian rhythms in *Arabidopsis*.

Many organisms that show robust circadian cycling in continuous darkness become arrhythmic under continuous light [27]. Conversely, *Arabidopsis* and some other plant species show robust circadian rhythmicity under continuous light. One possible explanation for this is that, in these plants, the activity of the light input to the oscillator is restricted or 'gated' by the circadian clock itself. The oscillator is therefore protected from the light signal at particular times of the day and thus continues to cycle. The circadian phenotypes of *Arabidopsis early flowering3* (*elf3*) mutants are consistent with this idea. The *elf3* mutation disrupts the circadian oscillation of *CAB* expression under continuous light but not under continuous darkness [28], consistent with its affecting light input to the clock. The arrythmicity of *elf3* may be due to stopping the circadian clock at a certain phase under continuous light; the expression of *CAB* begins to oscillate once more when plants are shifted from constant light to darkness [29]. Furthermore, gating of *CAB* expression by the circadian clock, a process whereby acute induction of *CAB* by light is repressed if light is given during the night, is abolished in this mutant, suggesting the involvement of *ELF3* in the gating mechanism. These observations suggest that *ELF3* acts to gate light input to the oscillator using a mechanism that is similar to that which gates *CAB* induction: *ELF3* activity enables the oscillator to pass through subjective dusk and to cycle continuously under continuous light. In *Arabidopsis*, the 'light-on' signal is effective in resetting the circadian clock [30]. *ELF3* function may be responsible for this regulation; *ELF3* might gate the light input at dusk so that the circadian clock is reset by the light-on signal. *ELF3* encodes a novel protein that may function as a transcriptional regulator [31•,32•]. Consistent with its putative role in gating light input to the clock, *ELF3* transcript and ELF3 protein levels show a circadian rhythm, and the ELF3 protein interacts with PhyB *in vitro* [32•]. Interestingly, however, constitutive expression of *LHY* may not abolish the oscillation of *ELF3* [31•].

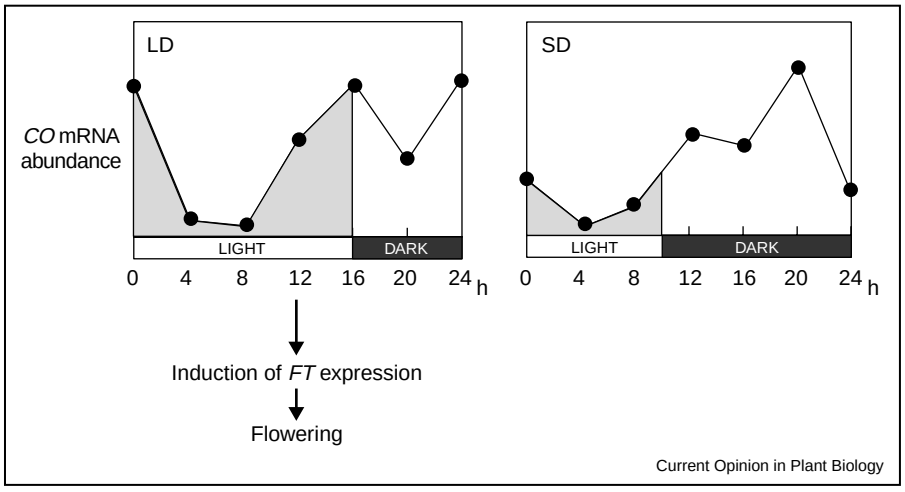
There is a complex relationship between genes that are involved in the entrainment of the circadian clock and those that regulate flowering time. Several mutations that are proposed to affect circadian clock entrainment by light also have dramatic effects on flowering time. Nevertheless, these mutations do not always have consistent effects on circadian rhythms and flowering time. For example, *ztl* and some photoreceptor mutants exhibit increased period length and affected flowering time, but *phyB*, *phyD* and *phyE* mutants are early flowering whereas *ztl*, *phyA* and *cry2* are late flowering [23,33–39]. In addition, *gi* mutations, which also impair a light-input pathway, shorten period length and cause severe late flowering [40–42]. Thus, the effect of these mutations on flowering cannot be fully explained by their effects on circadian rhythms, and the light signaling pathways that are impaired by these mutations probably affect flowering time independently of their effects on circadian rhythms.

Genetic control of flowering time by the circadian clock: a molecular mechanism for daylength measurement in *Arabidopsis*

Two general models have been proposed to explain how the circadian clock controls photoperiodic responses such as flowering. One of these models proposes that the circadian clock is responsible for setting the light-sensitive phase; flowering is promoted or inhibited if the plant is exposed to light during that phase. This model is referred to as the external coincidence model, and has been supported by a number of physiological studies on the control of flowering time. The second model, the internal coincidence model, proposes that flowering is promoted or inhibited under conditions in which two differently entrained rhythms are brought into the same phase. Flowering is not affected under conditions in which these rhythms are out of phase. Detailed analyses have not yet been carried out to verify this model in plants [43].

Although it is not yet clear how the circadian clock regulates the photoperiodic response of flowering at the molecular level, important information has been provided from several studies of the flowering-time gene *CO*. This gene was originally isolated from a mutant that has delayed flowering under long days (LDs) [44]. It encodes a protein that has a CCT domain and two B-box-type zinc-fingers, which were originally identified in animal proteins and are believed to mediate protein–protein interactions [44,45]. The expression of *CO* exhibits a circadian rhythm under continuous light [46••]. Overexpression of *CO* does not alter the period length of *CAB* expression [47], indicating that *CO* is part of an output pathway from the circadian oscillator. A role for *CO* downstream of the oscillator is also suggested by the effects of mutations in several genes that are associated with the function of the circadian clock and that affect flowering time. These mutations alter *CO* expression in ways that are consistent with their effects on flowering-

Figure 3



Expression of *CO* under long and short days in *Arabidopsis*. The diurnal expression of *CO* determines the light-sensitive phase. During long days, plants are exposed to light during the light-sensitive phase (in this case, at dawn and dusk when *CO* is highly expressed). During short days (SD), plants are not exposed to light during periods when *CO* transcripts are most abundant. This results in the induction of the *FT* flowering signal under long days, which promotes flowering in *Arabidopsis*. This mechanism is mediated by the post-transcriptional activation of *CO* by light, which allows CO to promote flowering under long days.

time [46••]. For example, *elf3* causes early flowering and increases *CO* expression, whereas the overexpression of *LHY* reduces *CO* expression and causes late flowering. In addition, the early-flowering phenotype caused by overexpression of *CO* is epistatic to the late-flowering phenotypes that are caused by such mutations [46••]. These observations suggest that *CO* plays a major role in mediating the effect of the circadian clock on the flowering time of *Arabidopsis*.

CO directly activates the expression of another flowering-time gene, *FLOWERING LOCUS T* (*FT*), which promotes flowering and whose expression is activated only under LDs [48–50]. The question of how *CO* is activated under LDs to bring about the expression of *FT* therefore becomes key to understanding the regulation of flowering time by the circadian clock in *Arabidopsis*. *CO* has a diurnal expression pattern with a peak in the night, and this pattern is regulated by photoperiod. Under LDs, the level of *CO* mRNA is high in the early morning, decreases in the middle of the day and increases again during the night; whereas under short days, *CO* transcript levels are relatively low in the early morning and increase during the night [46••]. Thus, if *CO* is activated by light in a post-transcriptional manner, the long-day signal could be generated and flowering would be promoted only under long days (Figure 3). Such a model may also explain the early-flowering phenotypes caused by mutations in genes that encode proposed oscillator components such as LHY, CCA1 and TOC1. These mutations cause short-period phenotypes that might result in the expression of *CO* during the light phase under short-day conditions. The photoreceptors PhyA and Cry2 may be involved in the post-transcriptional activation of *CO*; mutations in the genes that encode these photoreceptors also cause late flowering under LDs and have been implicated in photoperiod response [33,34]. This model for *CO* regulation is a version of the external coincidence model. It proposes that *CO* is responsible for determining the light-sensitive phase through its diurnal expression pattern: if plants are exposed to light at a time when *CO* expression is high, flowering is promoted through the activation of *CO* (Figure 3).

Conclusions and perspectives

Recent molecular and genetic studies using *Arabidopsis* have progressed our understanding of the mechanism associated with the control of circadian-clock function and flowering time. For example, studies of *LHY/CCA1* and *TOC1* have provided the first evidence of their genetic interaction, leading to the generation of a negative-feedback model for the oscillator in *Arabidopsis*. On the other hand, recent analyses of circadian-clock function have also shown that the circadian system is composed of multiple molecular loops that also involve light-input pathways [29,30,31•,32•,40,47], and that the regulatory mechanism that controls the function of the circadian clock is complex. In addition, although several other genes that are associated with circadian-clock function have been identified in *Arabidopsis*, their genetic interactions are largely unknown. Biochemical approaches will provide a deeper understanding of the control of clock function in *Arabidopsis*. Although the clock-associated mechanism that is responsible for daylength measurement is still unclear, the finding that *CO* mediates between the circadian clock and flowering time may provide a route to understanding this mechanism. The identification and

analysis of genes that are involved in the post-transcriptional regulation of *CO* by light may provide information on the genetic interactions that are responsible for the control of daylength measurement in *Arabidopsis*.

We have known that different plant species show distinct responses to daylength since the discovery of photoperiodism in the 1920s. Whether the molecular mechanism responsible for the photoperiodic response of flowering in *Arabidopsis* is conserved in other plant species, especially in those whose flowering is promoted by exposure to short days, is an important question. Recently, quantitative trait loci (QTL) that are associated with the photoperiod sensitivity of rice, a short-day plant, have been isolated [51]. Among them, *Heading-date1* (*Hd1*), *Hd3a*, and *Hd6* have been found to encode proteins that are similar to CO, FT, and CK2, respectively [51–53]. In addition, a rice homologue of the *Arabidopsis* flowering-time gene *GI* has been identified and reported to be expressed in a pattern similar to that of *Arabidopsis GI* [54]. Similarly, an essential role for phytochrome in the control of the photoperiodic response in rice was suggested by the analysis of the *photoperiodic sensitivity 5* (*se5*) mutant, which has a defect in the biosynthesis of phytochrome chromophore [55]. Analysis of these genes, as well as other QTLs [56], will allow us to compare the molecular mechanisms that are responsible for the photoperiodic response of flowering in rice and *Arabidopsis*.

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This paper provides an elegant model of how *CCA1*, *LHY*, and *TOC1* function within the circadian oscillator in *Arabidopsis*. Overexpression of *LHY* and *CCA1* suppresses the expression of *TOC1*. This transcriptional regulation is directly mediated through a promoter region called evening element, which is often found in genes that are expressed predominantly in the evening. Loss of *TOC1* function decreases the transcript levels of both *LHY* and *CCA1*. This is the earliest paper to propose the existence of a negative transcription and translational-feedback loop in a plant species.

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