Journal of Plant Biology, June 2007, 50(3) : 257-265 **REVIEW**

Plants Measure the Time

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All eukaryotes, including plants, and most prokaryotes have developed elaborate mechanisms to anticipate external environmental changes associated with the Earth's rotation. These mechanisms are mediated by a circadian clock, which regulates several physiological and biochemical processes. Microarray experiments using Affymetrix chips that included about 8000 of the 27000 *Arabidopsis* genes have demonstrated that as much as 6% of that genome may be under the control of this clock. While our understanding of such mechanisms is lagging, molecular genetics studies of *Arabidopsis* have allowed us to make great progress toward identifying and characterizing components of the plant circadian clock since its first component was isolated in 1995. The generation of 24-h rhythms by this clock appears to rely on mechanisms similar to those found in other organisms. However, an entirely different set of molecular components are recruited to perform these functions in *Arabidopsis*. In this review, we introduce useful and powerful approaches for identifying clock-associated genes and determining how they can act together in the interlocking feedback loops that comprise this particular clock.

Keywords: *Arabidopsis*, circadian clock, circadian rhythms, entrainment

CIRCADIAN RHYTHMS

Many aspects of physiology and metabolism show rhythmic variations within a 24-h period. In plants, gene transcription, stomatal opening, leaf movement, and hypocotyl elongation all exhibit circadian rhythms (Dowson-Day and Millar, 1999; Harmer et al., 2000; Somers et al., 1998b). These patterns persist under constant environmental conditions, unlike strictly diurnal rhythms that are driven entirely by environmental signals. Circadian rhythms have several distinctive properties (Somers, 1999). First, they all exhibit a free-running period of approximately 24 h. Their second property is that of 'entrainability', the process by which rhythms are synchronized to environmental signals such as light/dark or rhythmic temperature cycles. The third property is temperature compensation over a physiological temperature range. Whereas a 10°C rise in temperature (Q_{10}) increases the rate of biochemical reactions two- to three-fold, the Q_{10} values for a particular circadian period are between 1.0 and 1.1. For example, in *Arabidopsis*, this period varies by no more than 2.5 h over a 20°C alteration in temperature (Somers et al., 1998b). Compensation is an important property for maintaining a constant pace under changing conditions. Finally, the fourth and perhaps hallmark property of circadian rhythms is their ability to persist in the absence of environmental signals. This trait implies regulation by an endogenous and self-sustaining oscillator, i.e., the circadian clock.

This clock is often described as having three domains – an oscillator plus input and output pathways. The oscillator consists of a set of components that negatively regulate their own transcription, thereby generating rhythmicity. Through input pathways, external signals are transduced to the oscillator, which then can adjust to changes in day/night cycles through daily re-settings. A network of output pathways then modulates a variety of cellular and physiological activities (Kay and Millar, 1995). A simple model for such a circadian clock is shown in Figure 1. However, as new information emerges, the boundaries between these three domains have become blurred. For example, in *Arabidopsis*, the expression of clock input components such as a photoreceptor, PHYTOCHROME B (PHYB), is regulated by the circadian clock, which therefore also constitutes an output rhythm (Bognar et al., 1999; Harmer et al., 2000).

Nevertheless, this model has been useful as a conceptual framework for probing the functioning of new clock components within the circadian regulatory network.

The molecular mechanisms of a circadian oscillator are best understood in other organisms, such as *Drosophila*, *Neurospora*, *Cyanobacteria*, and mammalian species (see reviews by Dunlap, 1999; Young and Kay, 2001). Elements of a similar oscillator have also been identified in *Arabidopsis* (Alabadi et al., 2001). In all of these examples, the circadian oscillator (Fig. 1) has an autoregulatory negative feedback loop. Its positive elements promote the transcription of other clock genes whose products play a role as negative elements in that loop. These negative elements then interfere with the activity of the positive components to turn off their own transcription.

APPROACHES FOR *ARABIDOPSIS* CLOCK RESEARCH

Morphological rhythms

Overt circadian rhythms might be viewed as the hands of the clock, such that the development of easily assayed circadian markers is essential for clock research. Plants exhibit rhythmic morphological changes and gene expression rhythms. For example, *Arabidopsis* has circadian rhythms in its leaf movements, stomatal conductance, and hypocotyl expansion. About 270 years ago, the French scientist Jean Jacques d'Ortous de Mairan reported endogenous

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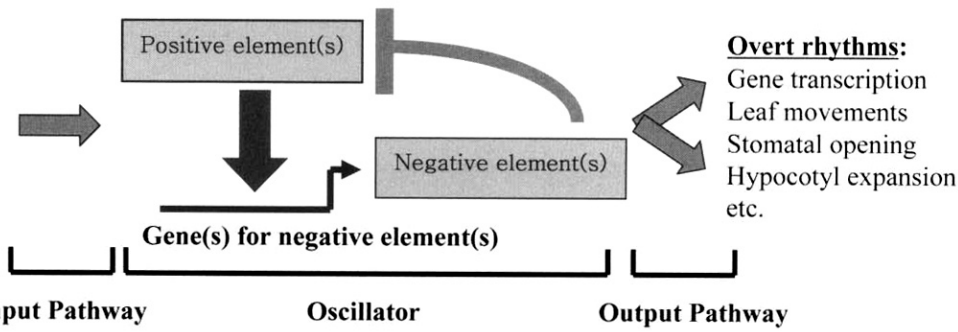

Figure 1. Simple model for organization of circadian system. Oscillator consists of negative-feedback loop that generates signal each 24-h period. Its positive element(s) activate transcription of its negative element(s), which interfere with activity of positive elements to negatively regulate their own transcription. This oscillator can be entrained in response to daily environmental cues, e.g., light/dark or temperature cycles through input pathways. Timing signal is relayed by output pathways, resulting in such overt rhythms as for gene expression, leaf movement, stomatal opening, and hypocotyl elongation.

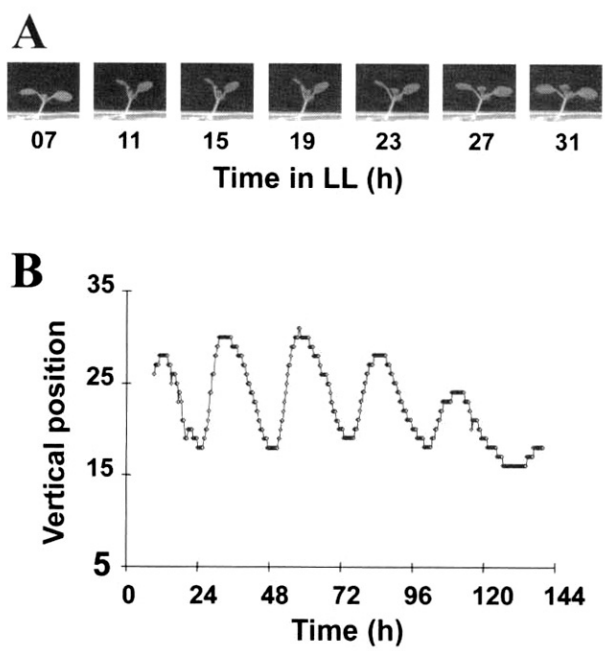

Figure 2. Leaf movement rhythm in constant light. (**A**) Cotyledon movement in *Arabidopsis*. Plants were entrained under 12L12D (12-h light/12-h dark cycle) for 8 d, then released into LL. Numbers indicate time elapsed after release. (**B**) Circadian rhythm for movement of leaves, as imaged by time-lapse video camera for 6~7 d. Vertical positions were determined for leaves.

biological rhythms by observing leaf movements in *Mimosa* (Somers, 1999). These plants opened their leaves during the day and closed them at night (Fig. 2). When placed under constant illumination, their leaf movements persisted with a 24-h period, as though the plants could anticipate day and night (Fig. 2). This was the first experimental evidence for the maintenance of rhythmicity in the absence of external signals. Since then, leaf-movement assays have been used in *Arabidopsis* to test for circadian clock dysfunctions in various mutants. *Arabidopsis* seedlings also show rhythmic patterns in their hypocotyl elongation. Although defective inhibition is a classic phenotype of photoreception mutants, aberrant elongation is correlated with circadian clock dysfunctions. For example, mutations in a clock input component, *EARLY FLOWERING 3* (*ELF3*), result in the abolishment of daily periods of growth arrest and show abnormal hypocotyl elongation (Dowson-Day and Millar, 1999). Circadian rhythms in stomatal aperture are also well-characterized (Lumsden and Millar, 1998). In *Arabidopsis*, stomata are open during the day and closed at night (Somers et al., 1998b).

Gene-expression rhythms

Although RNA blot assays can provide an estimate of average periodicity over an entire population, it is not possible to measure the period length for individual *Arabidopsis* seedlings by this technique. Furthermore, RNA analyses require plant destruction, thereby precluding the isolation of mutants with defective patterns of rhythmicity. Therefore, use of the firefly luciferase (*LUC*) reporter gene as a circadian marker has accelerated the identification of circadian clock mutants in *Arabidopsis*. Bacterial enzymes, such as β-glucuronidase (GUS) or β-galactosidase (lacZ), have also been used as reporter genes in plants and *Drosophila*. However, their activities are too stable to accommodate the measurement of circadian rhythmicity of gene expression. In contrast, because LUC activity is unstable *in vivo*, changes in its activity reflect alterations in *LUC* mRNA levels (Millar et al., 1992, 1994). This property allows for the accurate observation of rhythmic gene expression *in vivo* and also in real time.

A *LUC* gene can be coupled to the promoter of a circadian-regulated gene, such as the chlorophyll a/b-binding protein gene (*CAB*). In fact, the *CAB::LUC* fusion was the first rhythmic reporter to be used in clock research. *CAB* encodes a major component of the thylakoid membrane in chloroplasts, where it participates in the process of capturing light for photosynthesis (Green et al., 1991). Circadian changes in the expression of *CAB* mRNA were first observed by Kloppstech (1985). In peas grown under light/dark cycles, levels of *CAB* mRNA are minimal at midnight, begin to increase 2 h before the lights come on, and then peak in the morning. This rhythmic expression of *CAB* transcript also persists in constant light (LL) (Otto et al., 1988). The anticipation of dawn under light/dark cycles (LD) and the free-running rhythm under continuous illumination demonstrate the regulation of *CAB* transcription by an endogenous circadian clock. A similar circadian rhythmicity of *CAB*

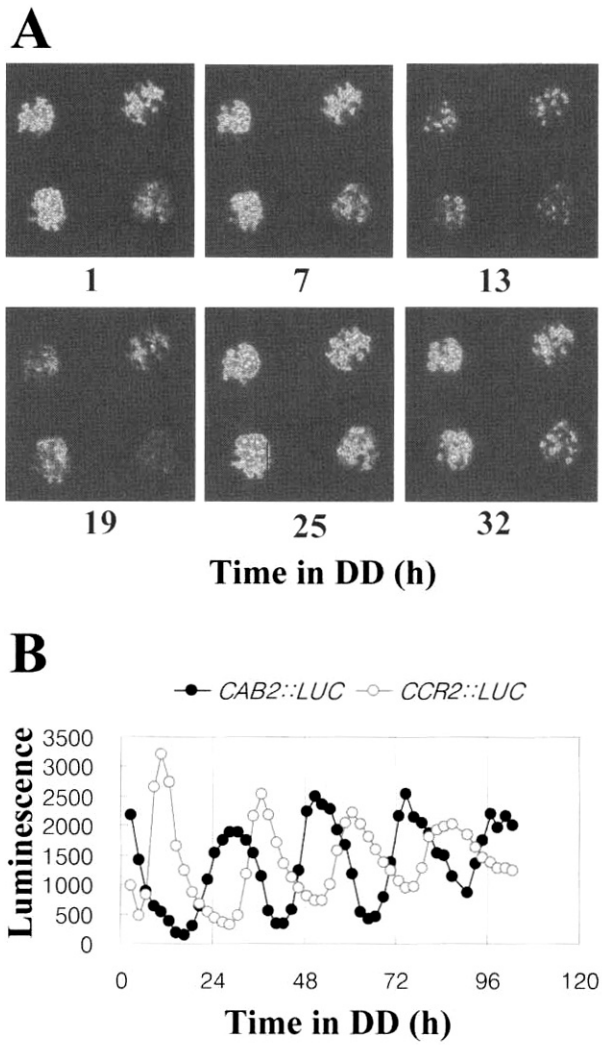

Figure 3. Circadian-regulated luminescent reporter genes. (**A**) Luminescence under constant darkness. Seeds carrying *CAB2::LUC* marker were sown in groups of 25 to 30. Plants were entrained under 12L12D for 8 d, then released into DD. Transgenic plants expressing luciferase emitted luminescence after being sprayed with luciferin substrate. Luminescence was imaged with photon-counting camera every 2 h, for 10 or 25 min each, over 5~6 d. Numbers indicate time elapsed after release into DD. (**B**) Oscillation of circadian-regulated genes. Luminescence activity driven by *CAB* and *CCR2* promoters was quantified using software after photons were counted by camera at each circadian time point.

expression has been observed in many other higher plants (Lumsden and Millar, 1998). Those that carry the *CAB::LUC* reporter construct emit luminescence when sprayed with the substrate luciferin. Images can be taken by a photon-counting camera at different circadian times, and the light emitted by individual plants can then be quantified (Fig. 3).

However, because expression of the *CAB* gene is regulated by light (Karlin-Neumann et al., 1988), analysis of circadian changes in *CAB::LUC* expression is complicated. For example, under light/dark cycles, the expression of *CAB::LUC* in wild-type *Arabidopsis* shows a peak at 2 to 4 h after lights-on and a trough at midnight. Advances or delays in the peak of *CAB* expression in a mutant background may be due to altered light regulation or to a defect in the clock itself. Furthermore, it is difficult to assay *CAB* expression under continuous dark conditions (DD). Thus, the development of a new circadian marker with robust rhythmicity in dark-adapted seedlings is important to the investigation of light-independent circadian functioning in plants. An attractive candidate is the gene that encodes the COLD AND CIRCADIAN REGULATED 2 (CCR2) protein. CCR2 is a glycine-rich protein with RNA-recognition motifs (Carpenter et al., 1994). *CCR2* mRNA levels exhibit diurnal oscillations. The phase of rhythm for *CCR2* expression is opposite that of *CAB*, with a peak in the evening rather than morning (Fig. 3; Carpenter et al., 1994; Heintzen et al., 1997; Staiger and Apel, 1999). Its rhythmic expression continues in both LL and DD (Carpenter et al., 1994), thus implying that the expression of *CCR2* transcript is under the control of the clock and is independent of light effects.

THE *ARABIDOPSIS* CLOCK: THE CORE OSCILLATOR

No homologous sequences for the clock genes found in other organisms have been found anywhere in the *Arabidopsis* genome sequence, suggesting that the clock for this genus might be composed of unique components. However, the basic mechanism by which the *Arabidopsis* oscillator works is likely to be conserved, possibly consisting of elements arranged in a transcriptional feedback loop (Alabadi et al., 2001).

Negative elements within the oscillator

LATE ELONGATED HYPOCOTYL (*LHY*), a gene encoding a MYB-like transcription factor, has been isolated on the basis of the late-flowering and long-hypocotyl phenotypes of its dominant gain-of-function mutant allele. *LHY* transcript is rhythmically expressed, peaking at dawn (Schaffer et al., 1998). Strikingly, constitutive overexpression of *LHY* driven by a strong constitutive viral promoter causes arrhythmic expression of clock-controlled genes and abnormal leaf movements under both LL and DD. In addition, *LHY* represses its own transcription (Schaffer et al., 1998). These results suggest that this gene functions as a component of a negative feedback loop, which may constitute the oscillatory mechanism of the circadian clock.

If *LHY* does encode such an essential component, we would expect that plants lacking *LHY* functioning would be arrhythmic. However, an *lhy* null mutant, *lhy-12*, still displays circadian rhythmicity (Mizoguchi et al., 2002). Thus, *LHY* itself is not indispensable for clock functioning, probably because its role is redundant with that of a closely related homologue, CIRCADIAN CLOCK-ASSOCIATED1 (CCA1). The LHY and CCA1 proteins share about 90% sequence identity in the MYB DNA-binding domain and 46% identity in the entire protein sequence. Both *LHY* and *CCA1* are expressed rhythmically, with a similar phase, and negatively regulate their own and each other's expression (Schaffer et al., 1998; Wang and Tobin, 1998). Overexpression of either gene causes arrhythmic phenotypes, and mutants lacking activity by either will exhibit similar short-period rhythms

(Green and Tobin, 1999; Mizoguchi et al., 2002). These observations suggest that *LHY* and *CCA1* might have overlapping functions within the circadian clock. Consistent with this, the combined effects of mutations at both *LHY* and *CCA1* cause complete disruption of free-running rhythmicity after two or three cycles (Mizoguchi et al., 2002).

Closing the feedback loop

When comparing its feedback-loop model with that from other organisms, an additional *Arabidopsis* element, for which expression is expected to peak in the evening, is thought to be required for closing the loop. Mutant screening via forward genetics has successfully isolated this missing link. Mutations at *TIMING OF CAB EXPRESSION1* (*TOC1*) result in a short period for all rhythms tested (Millar et al., 1995; Somers et al., 1998b; Mas et al., 2003). *TOC1* encodes a protein with homology to bacterial two-component response regulators. Expression of *TOC1* mRNA is clock-controlled, peaking in the evening (Strayer et al., 2000). Studies of the relationship between *TOC1* and *LHY/CCA1* have led to analyses of the *TOC1* promoter that contains the motif AAAATATCT, known as the Evening Element (EE) (Harmer et al., 2000). Mutations in EE are manifested as an arrhythmic expression of the *LUC* reporter gene and a failure in binding of the LHY and CCA1 proteins to this element *in vitro* (Alabadi et al., 2001). Therefore, the EE is believed to be important for direct clock-regulation by LHY/CCA1.

Data from the research described above suggest that a feedback loop generated by LHY/CCA1 and TOC1 might form the basis of the clock mechanism. LHY and CCA1 may act as negative elements of a feedback loop (Schaffer et al., 1998; Wang and Tobin, 1998) while TOC1 would be a positive element (Alabadi et al., 2001). Elevated expression of LHY and CCA1 in the morning may repress *TOC1* expression, leading to a decline in *LHY* and *CCA1* transcripts over the course of a day. Decreased levels of LHY and CCA1 may de-repress the *TOC1* gene such that its transcription resumes early in the night. Likewise, elevated levels of

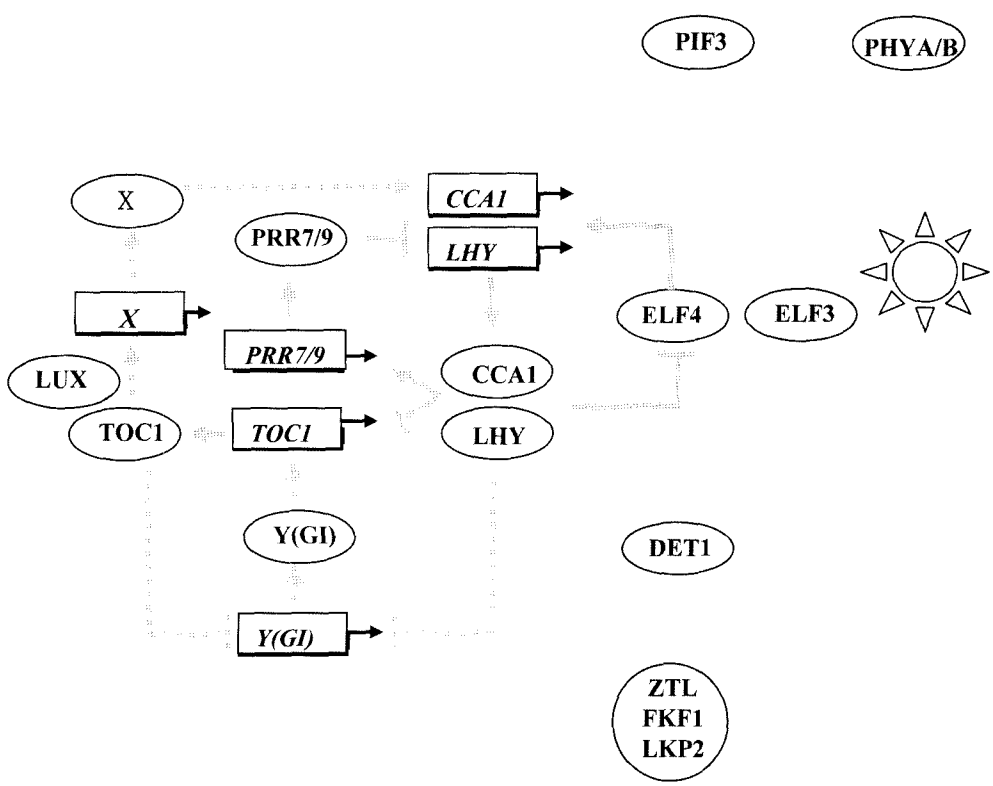

Figure 4. Interaction among clock-associated genes in *Arabidopsis*. Genes and proteins are indicated by squares and ovals, respectively. Elements and interactions in light-input pathways are in orange, while components and interactions associated with oscillatory mechanisms are in green. Hypothetical pathways are indicated as dotted lines. Locke et al. (2006) has proposed two hypothetical genes, *X* and *Y*, to account for oscillator that matches properties of *Arabidopsis* clock. *X* is required for activation of *LHY* expression, while Y is a gene activated by light and promotes *TOC1* expression. Y is repressed by both LHY and TOC1. LHY and CCA1 proteins repress *TOC1* transcription. GI is one candidate for Y. Hence, TOC1 and Y form loop. Farre et al. (2005) have proposed another loop in which PRR7/9 act as negative regulators for LHY/CCA1, and LHY/CCA1 act as positive regulators for PRR7/9 through direct binding to their promoters. These two loops might be coupled by currently unknown X. LUX, a MYB-like transcription factor, is core component of oscillator. LHY/CCA1 represses *LUX* by binding to EE in *LUX* promoter, while LUX is required for high level of *LHY/CCA1* transcripts. As shown, ELF4 might form loop independent of TOC1-dependent loops. In etiolated plants, transcription of *LHY/CCA1* is activated by PIF3 transcription factor. Under light, however, further transcription of *LHY* is inhibited, while translation of pre-existing *LHY* transcript is promoted (Kim et al., 2003). LHY inhibits expression of *ELF3* mRNA, but ELF3 promotes expression of *LHY* transcript, possibly by repressing inhibitory effect of illumination. Light may also target clock components for degradation through actions of ZTL, FKF1, and LKP2. Targeted degradation of TOC1 by ZTL is preferentially achieved in dark; light inhibits action of ZTL on TOC1 degradation. Role of DET1 in LHY degradation is controversial; DET1 can be involved in targeting LHY for degradation. However, mutations in *det1* accelerate degradation of LHY in light-independent manner, suggesting that DET1 can also inhibit turnover of LHY protein.

TOC1 protein at night may promote the transcription of *LHY* and *CCA1*, thereby forming a negative feedback-loop circuit.

An oscillator or oscillators?

A simplified model for the *Arabidopsis* clock is depicted in Figure 1. However, increasing data suggest that this model is incomplete. First, several circadian oscillations are still detected in LL and DD in *lhy-11 cca1-1* double mutants, suggesting that at least one additional protein might be capable of substituting for their function (Mizoguchi et al., 2002). Second, oscillation of *ELF3* is not disrupted by *LHY* overexpression, implying that such oscillation might be independent of the LHY-dependent oscillator(s) (Hicks et al., 2001). Third, this simplified model also cannot account for why overexpression of *TOC1* leads to reductions in *LHY* and *CCA1* expressions (Makino et al., 2002). Finally, TOC1 has not been shown to bind to either the *LHY* or the *CCA1* promoter (Strayer et al., 2000). Thus, it is not clear whether TOC1 is directly responsible for regulating their expressions. If that is the case, another question would be how this might be achieved. These observations suggest that other components may also be required for proper functioning of the oscillator.

Locke et al. (2006) have proposed two additional elements, conveniently called X and Y, that may be incorporated into those loops. In their model, *TOC1* is activated by an unknown evening-expressed protein (Y) that is induced by light. TOC1 acts to induce *LHY* expression by an unknown pathway through element X. Although the identity of Y has not been confirmed, the mathematical data suggest that its functioning might be fulfilled by *GIGANTEA* (*GI*; Locke et al., 2006). *GI* expression follows a circadian pattern, showing a broad peak in the late afternoon. It is also induced by light; its promoter contains several evening elements necessary for LHY-mediated repression. Overexpression or loss-of-function of *GI* affects circadian rhythms in both LL and DD, implying that GI acts within the central clock mechanism (Mizoguchi et al., 2005). Other studies have proposed a third loop, in which CCA1 and LHY act as positive regulators of two *TOC1* relatives, *PSEUDO RESPONSE REGULATOR 7* and *9* (*PRR7* and *9*), by directly binding to a CCA1-binding site (AAAAATCT) in their promoters (Farre et al., 2005). PRR 5/7/9 act as negative regulators of *CCA1/LHY* because the transcripts of *CCA1* and *LHY* accumulate in the *prr7 prr9* double mutants, and *CCA1* is constitutively transcribed in the *prr5 prr7 prr9* triple mutants (Nakamichi et al., 2005). The oscillators, composed of the LHY/CCA1-PRR7/9 loop and the TOC1-Y (GI) loop, are shown in Figure 4.

LUX ARRHYTHMO (LUX; Hazen et al., 2005) and EARLY FLOWERING 4 (ELF4; Doyle et al., 2002) might also be components of the core oscillator. *LUX* encodes a MYB transcription factor like *LHY* and *CCA1*, and has been isolated from mutants that show long hypocotyls and arrhythmic *CAB2* expression. It is expressed late at night and contains an EE in its promoter that can be bound by LHY and CCA1 (Hazen et al., 2005). Mutations in *LUX* cause arrhythmic *CAB2::LUC* and *CCR2::LUC* expression in LL and DD, implying that LUX might also be a core element in the oscillator. Although LUX has an MYB domain, its binding to the *LHY* and *CCA1* promoters has not yet been demonstrated. *ELF4* has been isolated based on the early-flowering phenotype from mutants with lesions in the *ELF4* locus. This gene is required for the maintenance of rhythmic *CAB2::LUC* and *CCR2::LUC* expression, as well as for the light-induced expression of *LHY* and *CCA1*. However, it is not required for the light-dependent expression of TOC1. Hence, it is possible that another oscillator composed of LHY/CCA1 and ELF4, but not TOC1, might be present (Doyle et al., 2002; Kikis et al., 2005).

Moreover, several clock-regulated genes, e.g., *CAB*, *CHALCONE SYNTHASE* (*CHS*), and *PHYB*, show distinctive free-running periods of 23.7, 25.4, and 24.6 h, respectively (Hall et al., 2002; Thain et al., 2002). Therefore, the observations described above raise the possibility of multiple oscillators in *Arabidopsis*. However, mechanisms for the cooperative regulation of rhythms generated by these multiple oscillators remain to be uncovered.

LIGHT INPUT TO THE CLOCK

In *Arabidopsis*, processes necessary for photosynthesis occur early in the day, whereas other aspects of metabolism, such as the activity of catalase or starch-mobilizing enzymes, take place at night (Harmer et al., 2000). Hence, for the circadian oscillators to be meaningful, they should be connected to the environment and be capable of adjustment to changing daily time cues. Such synchronization to exogenous light signals, termed 'entrainment', is mediated by input from multiple photoreceptors, including phytochromes and cryptochromes (Somers et al., 1998a). Early signalling partners of phytochromes have now been identified. FAR-RED IMPAIRED RESPONSE1 (FAR1), SUPPRESSOR OF PHYA 1 (SPA1), and SHORT UNDER BLUE LIGHT1 (SUB1) have been implicated specifically in PHYA-signalling, while PIF3 (PHYTOCHROME INTERACTING FACTOR3), PKS (PHYTOCHROME KINASE SUBSTRATE1), and ELF3 interact with PHYB-signalling (Neff et al., 2000).

Five structurally related phytochrome genes (*PHYA*, *-B*, *-C*, *-D*, and *-E*) have been isolated in *Arabidopsis* (Sharrock and Quail, 1989). Interestingly, the phytochromes contain a PAS (PAR-ARNT-SIM) domain found in other clock-associated proteins, suggesting that they play a role in interactions with clock components (Millar, 1997). While the period of free-running rhythms for *CAB* expression is 24.5 h in LL, it is lengthened in DD to 30 to 36 h, implying that photoreceptors may shorten the period length (Millar et al., 1995). According to Aschoff's (1960) rule, as the light fluence rate increases, the period of free-running rhythms is diminished; in contrast, as the light fluence rate decreases, the period is lengthened. The effects of red-light fluence rate on the free-running period of *CAB::LUC* expression have been examined in *phyA*, *phyB*, *cry1*, and *cry2* mutants (Somers et al., 1998a). There, the *phyA* mutants show a deficient response to low fluence rates for both red and blue light, suggesting that PHYA is specifically involved in the regulation of period length under low rates. By contrast, the *phyB* mutants are defective in their perception of high fluence rates of red light. The function of *PHYD* and *PHYE* in light input to the

clock also has been addressed (Devlin and Kay, 2000). Although both single mutants exhibit a wild-type response to red light, when the *phyA-B-D* and *phyA-B-E* triple mutants are compared with *phyA-B* double mutants, the triple mutants display a slightly longer period of *CAB::LUC* expression than the double mutants at high fluence rates of red light. Therefore, *phyD* and *phyE* play a role in controlling period length under a high fluence of red light. Thus, the function of different phytochromes is not fully redundant; i.e., PHYA mediates responses to low fluence rates of red and blue light, while PHYB, -D, and -E mediate the perception of high fluence rates of red light.

Cryptochromes (CRYs), the classic blue-light photoreceptors, are also present in *Arabidopsis*. For example, *cry1 cry2* double mutants show robust free-running rhythms in LL, indicating that they are not essential for circadian rhythmicity (Devlin and Kay, 2000). However, the length of the free-running period of *CAB* expression rhythm is affected by *cry1* and *cry2* mutations, in a fluence-dependent manner. The *cry1* mutants have a longer period than wild-type plants at both low and high fluence rates of blue light, whereas the *cry2* mutants display a wild-type period over all fluence rates tested. However, the double mutants exhibit a long period of *CAB* expression over the entire range of blue-light fluence rates, indicating that both CRY1 and CRY2 play a role in controlling the period length of the circadian clock.

The phototropin family of photoreceptors in *Arabidopsis* comprises two members: NONPHOTOTROPIC HYPOCOTYL 1 (NPH1; Liscum and Briggs, 1995) and NPH-LIKE 1 (NPL1; Kagawa et al., 2001). *NPH1* encodes a 120-kDa protein with three recognizable domains -- a serine-threonine kinase domain at the C-terminus and two Light/Oxygen/Voltage (LOV) domains at the N-terminus (Christie et al., 1998, 1999). Like NHP1, NPL1 has two LOV domains and a kinase domain (Kagawa et al., 2001). The LOV domain is a degenerated PAS domain that is involved in the binding of flavin chromophore, a cofactor for blue-light photoreceptors in *Arabidopsis*. Therefore, these proteins may function as such photoreceptors (Christie et al., 1998). However, the effects of *nph1* or *npl1* mutations on clock activity have not yet been examined. The influence of multiple photoreceptors in controlling period length seems to be complex but compensatory. For example, PHYA and CRY1 interact with each other *in vitro*, and mediate responses to low fluence rates of both red and blue light (Ahmad et al., 1998; Devlin and Kay, 2000). By recruiting a combination of photoreceptors that can cover overlapping fluence rates and spectral qualities, plants might be able to ensure a constant pace by the circadian clock.

Another interesting component is PIF3, a bHLH-transcription factor. The red-illuminated, active form of PHYB can bind specifically to PIF3, suggesting that this PHYB-PIF3 interaction is important for signal transfer (Ni et al., 1999). PIF3 localizes constitutively to the nucleus and binds to G-box sequences present in the promoters of the *LHY* and *CCA1* genes (Ni et al., 1998; Martinez-Garcia et al., 2000). PIF3 might promote the transcription of *LHY* and *CCA1* because the induction of their transcriptions by red light is reduced in PIF3-deficient seedlings. Thus, PIF3 may function as a molecular switch between the light input pathway and the clock. It might also be involved in re-setting that clock by inducing the expression of those oscillator components, LHY and CCA1. However, that is yet to be proven.

In one way, CRYs and PHYs participate in light input, but they are also rhythmic outputs of the clock (Somers et al., 1998a). Circadian control of light-signalling components enables that clock to modulate the sensitivity of the light input pathway, and it provides a mechanism for the circadian gating of light responses (Millar and Kay, 1996). This gating phenomenon was first described with regard to the light induction of *CAB::LUC* expression (Millar and Kay, 1996). Maximum induction of *CAB* expression takes place during the day, while little or none occurs at night. ELF3 has been suggested as a regulator of the impact of light on the clock in a circadian manner (Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001). In fact, *elf3* mutants show hypersensitivity to light induction of *CAB* during the night, suggesting that the gating mechanism is impaired (McWatters et al., 2000). Strong effects of light on the clock may cause arrhythmicity, and ELF3 is thought to prevent such disruptive effects of light in the middle of the subjective night.

To ensure proper running of the clock, oscillator elements may also be regulated at post-translational levels. Several components of the circadian light-input pathway are believed to be target proteins for ubiquitination in response to light. These include DE-ETIOLATED 1 (DET1), ZEITLUPE (ZTL), FLAVIN KELCH REPEAT F-BOX1 (FKF1), and LOV KELCH PROTEIN2 (LKP2) (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001; Song and Carre, 2005). *det1* was first identified as a mutant that develops light-grown phenotypes in the dark (Chory et al., 1989). Consistent with that phenotype, *det1* shows constitutive expression of light-regulated genes, suggesting that the wild-type *DET1* gene functions as a negative regulator of these genes (Pepper et al., 1994). Surprisingly, the *det1* mutation accelerates the degradation of LHY protein through proteosomal degradation pathways, thereby providing a molecular explanation for the short-period effect in this mutant (Song and Carre, 2005). On the other hand, DET1 is known to enhance the activity of ubiquitin-conjugating enzyme(s) *in vitro* (Yanagawa et al., 2004). Therefore, the two effects of DET1 on protein degradation are contradictory to each other, raising the possibility that its positive or negative influence on ubiquitin-conjugating enzyme(s) might depend on the presence or absence of additional partner(s).

In contrast to LHY and CCA1, the mechanism underlying the daily degradation of TOC1 by ZTL1 and its related genes is well known. The effects of *ztl*, a long-period mutant (Somers et al., 2000), are fluence rate-dependent, suggesting that *ZTL* plays a role in light input to the clock. Two *ZTL*-related genes, *FKF1* and *LKP2*, are also involved in the regulation of circadian rhythms (Nelson et al., 2000; Schultz et al., 2001). Each of these proteins has three distinctive domains: a LOV domain, an F-box motif, and a kelch repeat. The LOV domain is a degenerate PAS domain involved in the binding of a flavin chromophore. Flavins serve as cofactors for blue-light photoreceptors in *Arabidopsis*. Therefore, ZTL, FKF1, and LKP2 may also function as blue-light sensors (Christie et al., 1998). The F-box functions as an E3 ligase in ubiquitin-dependent protein degradation. Therefore, the

properties of light perception and adaptor for ubiquitination may lead us to conclude that ZTL, FKF1, and LKP2 represent novel light-regulated proteolytic systems involved in the degradation of clock-associated molecules. Actually, ZTL has been reported to be a component of the SCF (Skp1/Cullin/F-box) complex, which recruits TOC1 for proteosomal degradation (Mas et al., 2003). TOC1 protein levels are elevated in the *ztl* mutants, demonstrating that ZTL is crucial for the degradation for TOC1. Notably, the TOC1 protein is preferentially degraded in the dark, and light blocks the degradation activity of ZTL on TOC1. Hence, ZTL would be the first putative photoreceptor whose function is repressed by light.

THE CLOCK OUTPUT RHYTHM: PHOTOPERIODISM

One output pathway from the *Arabidopsis* clock is the response to photoperiod that controls the transition from vegetative growth to reproduction. *Arabidopsis* is a facultative long-day plant, which means that long days accelerate flowering. Genetic analysis has identified more than 80 genes important in that process. A subset of these genes specifically affects the promotion of flowering under long days. Mutants lacking in LHY, CCA1, and TOC1 show an altered flowering time, providing evidence for a central role by the circadian clock in photoperiodism. The first link connecting these two parties is *CONSTANS* (*CO*) (Valverde et al., 2004). *CO* encodes a zinc-finger protein that is activated by light and oscillates with a peak at 16 h after lights-on under long days (16-h light/8-h dark cycle), as well as at 20 h after lights-on during short days (8-h light/16-h dark cycle) (Valverde et al., 2004). Under short days, little CO protein is detected, but under long days, the level of CO protein is high. In *Arabidopsis*, the blue-light photoreceptors (CRY1 and CRY2) and the far-red light photoreceptor (PHYA) are believed to stabilize CO protein and allow its accumulation during inductive long days. An elevated level of CO induces the expression of downstream floral activators, such that flowering is accelerated during long days (Searle and Coupland, 2004). This description of a photoperiod-sensing mechanism is consistent with the concept of an external coincidence model that was originally developed by Bunning (1936) and later re-named by Pittendrigh and Minis (1964).

CONCLUDING REMARKS

After a decade of research on the plant circadian clock, a simple oscillator model (Fig. 1) has been embellished to arrive at a more complex oscillator model (Fig. 4). However, it is still premature to claim that the oscillator mechanism is now fully understood. The small oscillators generated by the LHY/CCA1-PRR5/7/9 loop and by the TOC1-Y (GI) loop might actually be coupled to form a large LHY/CCA1-TOC-Y(GI) loop (Fig. 4). If so, this larger loop may provide the flexibility for an oscillator to anticipate dawn and dusk as well as the ability of such a clock to "measure" daylength. However, to gain broad support, this large oscillator model still requires the identification of a missing component (X) that connects the two small oscillators. Study results have suggested that the clock is also involved in the regulation of photoperiodic flowering. Therefore, when the mechanism for this plant circadian oscillator is solved, we may envisage challenging, tailored modifications to that clock in order to develop crop plants that can be regulated independent of specific environmental restrictions.

ACKNOWLEDGEMENTS

Our work was funded by the Global Research Laboratory Program (2006-03870) from KICOS/MOST, Korea, and by the Plant Diversity Research Center (PF06304-02). H.-R.S. was supported by the BK21 Program from Korea Research Foundation.

Received March 6, 2007; accepted March 14, 2007.

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