

## Clockwork green—the circadian oscillator in *Arabidopsis*

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### Abstract

Although rhythmic leaf movement in a higher plant was the first physiological process recognised to be under circadian control, our understanding of the molecular drives underlying circadian rhythms in plants is still limited. Genetic screens for mutants impaired with regard to circadian rhythmicity have identified components critical for clock function in the model plant *Arabidopsis thaliana*, providing a snapshot of interconnected transcription-based feedback circuits at the core of the clockwork. Subsequently, the *Arabidopsis* genome project provided the basis for reverse genetic approaches to uncover additional gene products operating close to the core clockwork. We will review recent progress in the dissection of the molecular mechanisms within the basic oscillator and in the incorporation of additional components into the basic clock model.

**Keywords:** *Arabidopsis thaliana*, *slave oscillator*, *negative feedback loop*

### Introduction

Instrumental to the maintenance of circadian rhythms in physiology, biochemistry and behaviour is the cyclic manifestation of cellular components. Rhythmically expressed proteins form a basic oscillator which in turn passes timing information to downstream components and in this way elicits rhythmicity in their expression or activity (Dunlap 1999).

Like the well-described oscillators of mammals, flies or fungi, the circadian oscillator in *Arabidopsis* is based on interconnected feedback loops through which clock proteins activate and repress transcription of their own genes and thus generate their daily rhythm. Additional regulation of the clock proteins at the post-translational level ensures a period of 24 h. For a comprehensive description of the *Arabidopsis* circadian system we also refer to several recent reviews (Barak et al. 2000; Young & Kay 2001; Eriksson & Millar 2003; Hayama & Coupland 2004; Salome & McClung 2004; Staiger et al. 2006).

Although the circadian clock can operate without cyclic input from the environment, it does not operate entirely on its own: every day the periodic environmental changes set the clock to local time. Among these changes are alterations in temperature and, most prominently, light. A suite of chromoproteins including five phytochrome and two cryptochrome-photoreceptors continuously monitor fluctuations in light quality and quantity across the day and initiate a web of signalling chains. These impact on the oscillator components, resulting in a change of the clock's phase so that it is in synchrony with the external world. Progress in understanding

of light input to the clock and of putative targets for light entrainment in *Arabidopsis* has been reviewed recently (Fankhauser & Staiger 2002; Hayama & Coupland 2004; Millar 2004; Salome & McClung 2005a).

Less well understood is how periodic temperature changes accomplish synchronisation of the endogenous clock. Lately, PRR7 and PRR9, two pseudo-response regulators, have been identified that convey entrainment by thermocycles to the clock (see below) (Salome & McClung 2005b).

### **Molecular cogs of the *Arabidopsis* clock**

The first *Arabidopsis* proteins that were found to be not only controlled by the circadian clock but also causally involved in the generation of circadian rhythms were CCA1 (CIRCADIAN CLOCK ASSOCIATED) and LHY (LATE ELONGATED HYPOCOTYL) (Schaffer et al. 1998; Wang & Tobin 1998).

CCA1 is a transcription factor with a single Myb domain that was originally identified as recognising the *CAB3* (CHLOROPHYLL A/B BINDING PROTEIN) promoter (Wang et al. 1997). *CCA1* mRNA and protein show high-amplitude rhythms with peaks around dawn. In transgenic plants constitutively over-expressing CCA1 (CCA1-ox plants), oscillations of the endogenous *CCA1* mRNA are repressed. Because a CCA1- $\beta$ -glucuronidase reporter gene is also downregulated in CCA1-ox plants, CCA1 appears to negatively autoregulate its own expression at the transcriptional level, reminiscent of clock components in *Drosophila* or mammals (Wang & Tobin 1998). Furthermore, oscillations of the clock output genes *CAB*, *ATGRP7* (*Arabidopsis thaliana* GLYCINE RICH RNA BINDING PROTEIN)/*CCR2* (COLD AND CIRCADIAN REGULATED) and *CAT3* (CATALASE 3) were also damped, pointing to a prominent role of CCA1 in the generation of circadian rhythmicity.

A related single Myb-type DNA-binding protein was identified in the *lhy-1* (*late elongated hypocotyl*) mutant that is late-flowering and has elongated hypocotyls (Schaffer et al. 1998). This phenotype is caused by insertion of the maize Ds-transposable element that leads to LHY over-expression driven by the Cauliflower Mosaic Virus promoter within the transposon. As in CCA-ox plants, leaf movement and *CAB* transcript rhythms are abolished in the *lhy-1* mutant. Moreover, the endogenous *LHY* transcript and the *CCA1* transcript are arrhythmic in *lhy-1* and, when LHY translation rises early in the day, abundance of the *LHY* mRNA falls (Kim et al. 2003).

Thus, CCA1 and LHY negatively autoregulate their expression and at the same time reciprocally regulate each other; accumulation of CCA1 and LHY protein above a certain threshold during the day limits their further accumulation. This scenario resembles the circadian oscillators from known systems. If CCA1 and LHY were essential non-redundant components of a core oscillator, null mutants should be arrhythmic. However, CCA1 knockout plants and LHY loss of function plants have a short period in leaf movement rhythms and transcript oscillations (Green & Tobin 1999; Mizoguchi et al. 2002). Only when both genes are inactivated is arrhythmicity observed, and then only during extended times in free-run conditions (Alabadi et al. 2002; Mizoguchi et al. 2002). These observations suggest that CCA1 and LHY are partially redundant components close to or within the core clockwork, and that additional components can maintain rhythmicity to some degree in *cca1 lhy1* double-mutants.

For self-sustained oscillations of CCA1 and LHY, in addition to the negative elements, at least one positive element needs to rhythmically reactivate *CCA1* and *LHY* expression. Such an activator appeared when TOC1 (TIMING OF *CAB* EXPRESSION) was identified

molecularly (Strayer et al. 2000). The *toc1* mutant was, in fact, the first mutant with a period defect identified in *Arabidopsis* by virtue of aberrant CAB::LUC rhythms with a 21-h period (Millar et al. 1995). TOC1 is the founding member of a family of so-called pseudo-response regulators, PRR1 (see below). The *TOC1* mRNA oscillates in antiphase to *CCA1* and *LHY* (Figure 1A). In a strong *toc1* allele, *CCA1* and *LHY* transcripts are down-regulated, implicating a positive effect of TOC1 on *CCA1* and *LHY* expression (Alabadi et al. 2001). In turn, *CCA1*-ox plants and *lhy1* mutants have a reduced *TOC1* mRNA level. The observation that recombinant *CCA1* and *LHY* can bind to the conserved EE (evening element; AAATATCT) in the *TOC1* promoter suggested that *CCA1* and *LHY* inhibit *TOC1* expression by direct interaction with its promoter (Alabadi et al. 2001). Depletion of the *CCA1* and *LHY* repressors during the course of the day would allow reaccumulation of TOC1, which then would reactivate *CCA1* and *LHY* expression (Figure 1B). This final step is not entirely understood as yet: TOC1 does not feature an obvious DNA-binding module and no DNA-binding activity has been found *in vitro*. Furthermore, it takes several hours from the peak in TOC1 until *CCA1* and *LHY* reaccumulate. Thus, TOC1 may influence *CCA1* and *LHY* transcription indirectly and/or in combination with other proteins. Several components identified in mutants with aberrant circadian rhythms are expressed early in the night and thus would be able to team up with TOC1. Also, in the absence of functional TOC1, other components may partially take over TOC1's function: loss of TOC1 function leads to arrhythmia in the dark and in red light only but still allows short period rhythms in white and blue light (Mas et al. 2003a).

## Post-translational regulation of clock protein activity

### Targeted degradation

Any protein that cycles between high and low concentrations within one day, like components of the core clockwork, needs to have a short lifetime. *Drosophila* TIMELESS was the first clock protein for which daily variation in the rate of degradation through the proteasome was shown (Naidoo et al. 1999). In *Arabidopsis*, *ZTL* (ZEITLUPE) has been shown to channel

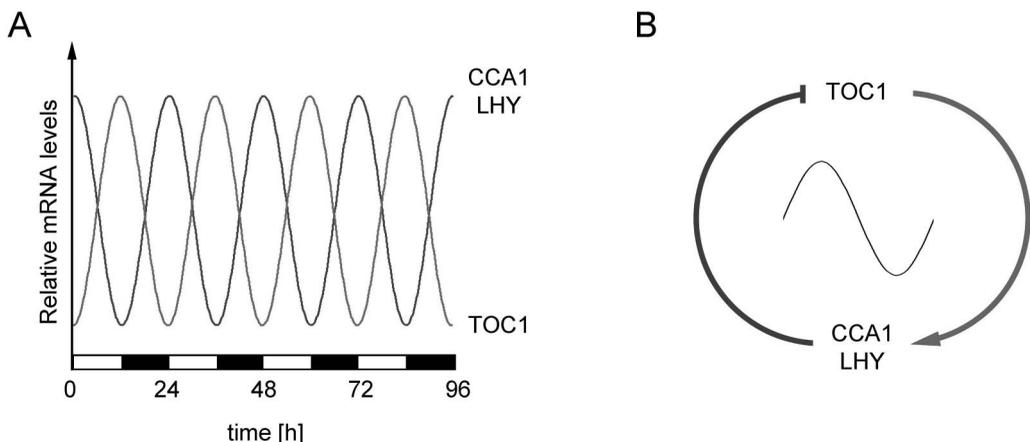


Figure 1. (A) The *Arabidopsis* clock components *CCA1* and *LHY* cycle in antiphase to *TOC1*. (B) A minimal core clockwork in *Arabidopsis* based on mutual regulation of *CCA1/LHY* and *TOC1* (Alabadi et al. 2001).

substrates into the proteasome pathway for degradation (Han et al. 2004; Somers et al. 2000). ZTL was originally identified in the same screen as TOC1 on the basis of long period LHC::LUC oscillations (*toc7*) (Millar et al. 1995). ZTL features an unusual combination of a LOV domain, of an F-box motif and of six kelch repeats implicated in protein–protein interaction. LOV (light, oxygen, voltage) domains are specialised PAS (PER, ARNT, SIM) domains that serve to sense environmental factors. The ZTL LOV domain is related to the LOV domain of the *Arabidopsis* phototropin blue/UV-A light receptor which has a tightly bound FMN (flavin mononucleotide) group involved in photosensing (Briggs et al. 2001). The F-box and kelch repeats bring target proteins into a larger Skp1-Cullin-F-box (SCF) complex for ubiquitination and subsequent proteasome-dependent degradation (Han et al. 2004).

Although the *ZTL* mRNA remains constant across the day, the *ZTL* protein oscillates with a dusk peak and a dawn trough (Kim et al. 2003). This can be attributed to a faster breakdown around dawn and a higher stability around dusk.

How does rhythmic *ZTL* protein impact on the period of the oscillator? Changes in abundance of TOC1 protein during the day are accomplished not only by alternating activation and repression of *TOC1* transcription but, additionally, through regulated protein degradation. *ZTL* interacts with TOC1 both in the yeast two-hybrid system and *in planta* (Mas et al. 2003b). Moreover, the degradation of TOC1 is inhibited by proteasome inhibitors and is impaired in *ztl* mutants. These data implicate *ZTL* function in the time-of-day specific degradation of TOC1 through the ubiquitin–proteasome pathway. Surprisingly, TOC1 is preferentially degraded in the dark and stabilised in the light. This suggests that light absorption by the LOV domain may negatively affect *ZTL* function. Alternatively, *ZTL* may not be the sole factor responsible for timed breakdown of TOC1.

### *Phosphorylation*

Whether steady-state abundance of CCA1 and LHY proteins also depends substantially on timed breakdown is not yet known. CCA1 has been shown to interact with a new type of regulatory subunit of the protein kinase CK2 (CASEIN KINASE) (Sugano et al. 1998). This CKB3 subunit has so far been found only in *Arabidopsis* and, when over-expressed in transgenic plants, leads to short-period oscillations of *CCA1*, *LHY* and several output genes. CK2 phosphorylates CCA1 *in vitro*. To test whether CCA1 phosphorylation is of relevance *in vivo*, a mutant form of CCA1 was over-expressed that had several serine residues changed into alanine and thus could not be phosphorylated (Daniel et al. 2004). These changes abrogated the effects on transcript oscillations, hypocotyl elongation and flowering time seen in CCA1-ox plants. Thus, CCA1 phosphorylation appears to be crucial for correct functioning of the *Arabidopsis* clock. CCA1 binding to the *CAB* promoter *in vitro* is promoted by CCA1 phosphorylation through CK2 (Sugano et al. 1998). It is not known yet whether this has a function *in vivo* and whether interaction of CCA1 with other proteins, its degradation or its subcellular localisation are influenced by CK2 phosphorylation.

### *Poly(ADP-ribosyl)ation*

A post-translational modification that so far has been observed mostly for nuclear proteins in response to DNA damage has been implicated in the function of the *Arabidopsis* clockwork. Poly(ADP-ribosyl)ation is catalysed by poly(ADP-ribose) polymerase (PARP) using NAD<sup>+</sup> as a substrate, and the poly(ADP-ribose) polymers in turn are catabolised by poly(ADP-ribose) glycohydrolase (PARG). A long period mutant, *tej*, was found to be defective in the

enzyme Poly(ADP-Ribose) glycohydrolase (Panda et al. 2002). All of the roughly 450 transcripts that have been shown by microarray analysis to undergo circadian oscillations in *Arabidopsis* seedlings cycle with a long period in *tej* (Harmer et al. 2000; Panda et al. 2002). Because this long period is independent of light quality and quantity and appears also in DD, TEJ presumably operates close to the core clockwork rather than in clock input.

The defect in PARG in *tej* mutant seedlings results in an extremely elevated level of poly(ADP-ribose) polymers. Interestingly, administering the PARP inhibitor 3-aminobenzamide cures the long period phenotype in *tej* mutants and causes a short period in wild-type seedlings, indicating that poly(ADP-ribose) levels indeed influence the pace of the clock. Although its targets are unknown, one may envisage that TEJ catalyses post-translational poly(ADP-ribosyl)ation of clock component(s) and thus may alter the period.

**Additional components of the circadian system**

During recent years, numerous proteins associated with circadian functions in *Arabidopsis* have been identified (Barak et al. 2000; Eriksson & Millar 2003; Hayama & Coupland 2004; Salome & McClung 2004; Staiger et al. 2006; Young & Kay 2001). Some of them are part of small multigene families comprising proteins with known functions in the clockwork, suggesting at least partial redundancy. Others have been identified based on their role in light signalling or photoperiodic flower induction. Their exact mode of action within the circadian system still has to be resolved (Figure 2).

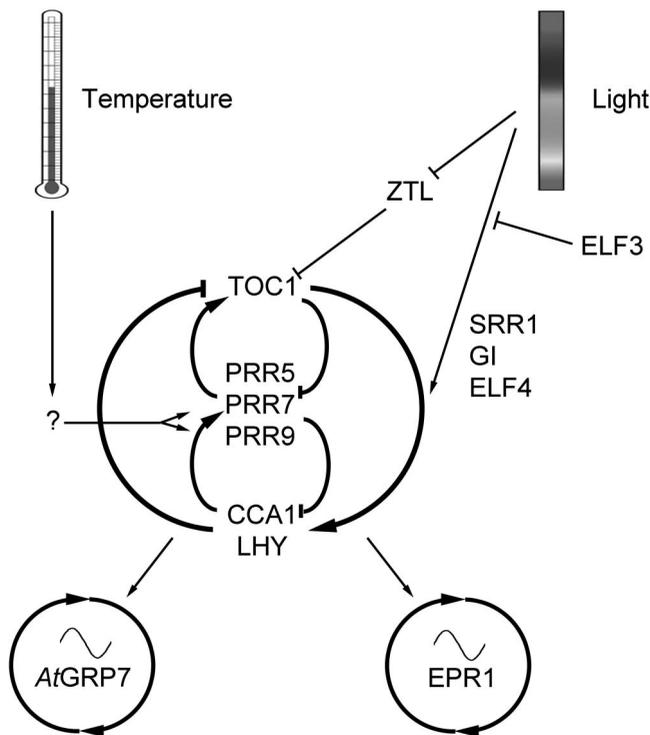


Figure 2. Extended scheme of the *Arabidopsis* circadian system including proteins that are known to affect the function of the core clockwork (Salome & McClung 2004; Mizuno & Nakamichi 2005). Details are given in the text.

*FKF1 and LKP2, members of the ZTL gene family*

ZTL is the founding member of a small gene family comprising FKF1 (FLAVIN BINDING KELCH F BOX) and LKP2 (LOV KELCH PROTEIN). FKF1 was isolated from a late-flowering mutant that also showed alterations in rhythmic gene expression (Nelson et al. 2000). In contrast to *ZTL*, the *FKF1* mRNA undergoes circadian oscillations. In transgenic plants with high constitutive FKF1 levels, *CAB* is still rhythmic. Thus FKF1 is, presumably, only an output of the clock. On the contrary, LKP2 over-expression leads to arrhythmic *CAB* levels (Schultz et al. 2001). However, LKP2 mutants have no clock phenotype suggesting some redundancy among the *ZTL* family (Jarrillo et al. 2001). Like *ZTL*, LKP2 interacts with TOC1/PRR1 and it also binds to PRR5, but not PRR3, PRR7 and PRR9 (see below) in the yeast two-hybrid system, although the functional relevance of these interactions in planta have not yet been established (Yasuhara et al. 2004).

*ELF3*

ELF3 (EARLY FLOWERING) was identified in an early-flowering mutant that shows arrhythmic outputs in continuous light while it retains rhythmicity in continuous dark (Hicks et al. 1996). This conditional arrhythmicity has been attributed to deregulated light input to the oscillator, as *elf3* mutants show impaired gating of the acute response of *CAB* to light (strong induction during subjective day and weak induction during subjective night in wild-type). Also, in plants with constitutively elevated ELF3 levels, the acute light response of *CAB* is attenuated (McWatters et al. 2000; Covington et al. 2001).

*TIC*

The *tic* (TIME FOR COFFEE) mutant was identified on the basis of a very-low-amplitude *CAB::LUC* rhythm in LL and it also shows aberrant *CCR2::LUC* rhythms in DD (Hall et al. 2003). The requirement for TIC to maintain oscillations of the clock components *CCA1*, *LHY*, and *TOC1* highlights the importance of TIC for clock function. Notably, *elf3 tic* double-mutants are completely arrhythmic. So far, TIC has not been identified molecularly.

*ELF4*

Mutants defective in the *ELF4* (EARLY FLOWERING) locus flower earlier than wild-type plants under short-day conditions and thus are impaired in photoperiodic timekeeping (Doyle et al. 2002). In individual *elf4* seedlings, the period of *CAB::LUC* rhythms varies to a large extent in constant conditions before the seedlings become arrhythmic, indicating that ELF4 is required for keeping the clock running accurately. Furthermore, ELF4 is required for *CCA1* expression, as *CAA1::LUC* levels are low and arrhythmic both in LL and DD (Doyle et al. 2002). As ELF4 has no known domains, the molecular mechanism of its action is not clear yet.

*GIGANTEA*

The *gigantea* mutant, *gi*, was originally identified by George Redei on the basis of its extremely delayed onset of flowering and concomitant production of large “gigantic” leaves (Redei 1962). *Gi* mutants later were shown to be affected also in transcript oscillations and leaf movement rhythms (Fowler et al. 1999; Park et al. 1999). The period-shortening effect of *gi-1*

depends on light intensity, suggesting that GI mediates light input to the circadian oscillator. In line with this, GIGANTEA was also identified in a screen for reduced seedling de-etiolation in red light and thus has been implicated in phyB signalling (Huq et al. 2000). On the other hand, *GI* itself cycles and thus also is a clock output, suggesting that GI is part of a feedback loop required for the clockwork to maintain period and amplitude. *TOC1* mRNA levels are reduced in the *gi* mutant. As GI reaches its highest level around dusk, it may be a positive regulator of *TOC1*. The reduced *TOC1* levels in turn would result in lower expression of *CCA1* and *LHY*, as, indeed, is observed in *gi* (Park et al. 1999).

GIGANTEA was also recovered as an interaction partner of the SPY (SPINDLY) protein that negatively regulates gibberellin (GA) signalling (Tseng et al. 2004). SPY, like GI, can localise to the nucleus. It is an enzyme that modifies serine or threonine residues with O-linked  $\beta$ -*N*-acetylglucosamine. This modification is known to affect half-life and localisation of the target proteins. Apart from its roles in GA-dependent and light-dependent hypocotyl elongation, and photoperiodic and GA-dependent flower induction, SPY also has a function in the circadian system; this is because several *spy* alleles lengthen the free-running period of cotyledon movement rhythms, which is shortened in SPY over-expressors (Tseng et al. 2004). It will be interesting to determine whether protein GlcNAcylation may be involved in these circadian phenotypes, and so indicate that yet another type of post-translational regulation appears in the clock system.

### *SRR1*

A further component important for clock function was identified in a mutant with reduced inhibition of hypocotyl elongation, predominantly in red light. The *srr1* (*sensitivity to red light reduced*) mutant flowers almost as early in short days as in long days and thus is more or less insensitive to photoperiod (Staiger et al. 2003a). Interestingly, in the *srr1* mutant, mRNA oscillations of the clock genes *TOC1*, *LHY*, and *CCA1* as well as of *Gi* and the output genes *CAB*, *CAT3*, and *ATGRP7/CCR2*, also have a short period in continuous light. Under entrainment conditions these transcripts show an advanced phase, as expected for a short-period circadian oscillation. *ATGRP7/CCR2* oscillations are also impaired in continuous darkness, indicating that SRR1 not only functions in light signalling but also is important for clock function in the dark. Oscillations of *CAT3* damp out and remain at high levels in continuous darkness as in wild-type plants (Staiger et al. 2003a). Because this, somewhat unexpected, constitutive high *CAT3* level has previously been shown to depend on phyA and cry1 in the wild-type (Zhong et al. 1997), this is consistent with a role for SRR1 in red light signal transduction downstream of phyB.

The domain structure of SRR1 does not give a clue as to its function. Interestingly, however, related sequences have been found in genomes of other eukaryotes including yeast, *Drosophila*, mouse and human (Staiger et al. 2003a). So far, apart from the cryptochrome photoreceptors, plant proteins implicated in the generation of rhythmicity are not present in animals. It will be interesting to determine whether SRR1 related proteins are connected to the circadian system in these organisms.

### *The PRR1/PRR3/PRR5/PRR7/PRR9 quintet of pseudo-response regulators*

Two-component systems are widespread mechanisms in bacteria for sensing stress factors in the environment, transducing this information within the cell and eliciting an appropriate response, mostly at the level of transcription of target genes. A histidine kinase (HK) monitors

specific signals, resulting in autophosphorylation of a histidine residue (Figure 3A). The phosphoryl group is then transferred onto an aspartic acid residue of a cognate response regulator (RR).

Recently, two-component systems have also been found in higher plants. For example, in *Arabidopsis* the HK ETR1 serves as receptor for the gaseous hormone ethylene and AHK2, AHK3 and AHK4/CRE1 sense the phytohormone cytokinin (Chang et al. 1993; Inoue et al. 2001). In keeping with the prokaryotic strategy, cognate response regulators have been identified. Notably, in the *Arabidopsis* genome, several RR-like proteins have been disclosed that have an N-terminal receiver-like domain and C-terminal CCT domain, originally found in CONSTANS, constans-like protein and TOC1 (Figure 3B). Because the receiver-like domain contains a glutamate in the place of the conserved phospho-accepting aspartate, it is designated a pseudo-receiver domain and the proteins accordingly are termed PRRs (pseudo-response regulators) (Makino et al. 2000). *PRR9*, *PRR7*, *PRR5*, *PRR3*, and *PRR1* (which is identical to TOC1) transcripts oscillate and sequentially reach their peaks at about 2-h intervals starting from dawn (Matsushika et al. 2000).

Considering the crucial function of both of the two closely related CCA1 and LHY proteins for clock function, it was of interest whether *PRR3*, *PRR5*, *PRR7* and *PRR9* would play a function similar to *PRR1/TOC1* in the circadian system. Analysis of single null mutants *prr3*, *prr5*, *prr7* and *prr9* uncovered subtle effects on rhythms. *Prr3* and *prr5* confer a short period (Eriksson et al. 2003; Nakamichi et al. 2005; Salome & McClung 2005b). *Prr7* confers a low-amplitude long period at best (Kaczorowski & Quail 2003; Farre et al. 2005; Nakamichi et al. 2005; Salome & McClung 2005b). *Prr9* confers a long period in clock gene expression but a lagging phase in cotyledon movement (Eriksson et al. 2003; Michael et al. 2003b; Nakamichi et al. 2005; Salome & McClung 2005b). Together, these data suggest redundancy among the individual family members. Indeed, a recent double-mutant analysis revealed that *prr5 prr7* retained rhythmicity with a very short free-running period in LL and an advanced phase in LD. *Prr7 prr9* shows an unusually long period in LL and a delayed phase in LD

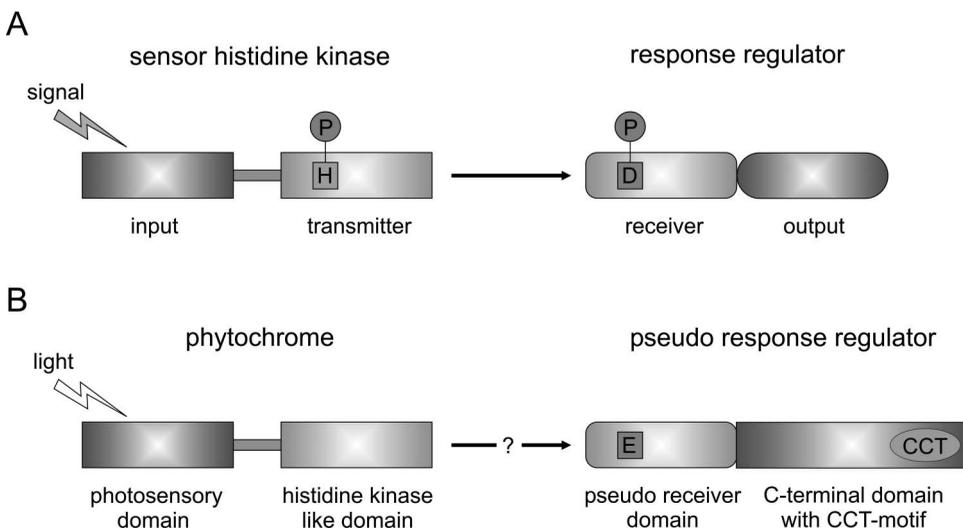


Figure 3. (A) Sensor histidine kinase and response regulator of prokaryotic two-component systems. (B) Domain structure of *Arabidopsis* pseudo-response regulators and phytochromes with sequences related to sensory histidine kinases.

(Nakamichi et al. 2005; Salome & McClung 2005b). Thus, although PRR7 only has a minor effect on rhythmicity by itself, it enhances the effects of either PRR5 or PRR9, this effect becoming apparent following loss of PRR5 or PRR9 function.

When assayed under different light intensities, *prp7 prp9* double-mutants still display a long period. Thus, light input that provokes fluence-dependent shortening of free-running periods in wild-type plants is impaired (Farre et al. 2005). In addition to their involvement in light input, a role for PRR7 and PRR9 close to the core clockwork is underlined by the observation that, in *prp7 prp9* double-mutants, CCR2::LUC and CCA1::LUC rhythms rapidly damp out in constant darkness (Farre et al. 2005; Salome & McClung 2005b). *Prp5 prp9* double-mutants have a free-running period like the wild-type, indicating that the opposite effects of PRR5 and PRR9 are additive and nullified in the double-mutant (Eriksson et al. 2003; Nakamichi et al. 2005). Notably, in *prp5 prp7 prp9* triple-mutants, the clock is almost non-functional, underlining the importance of the three components for clock function.

What is the relationship between the core clock components and the additional *PRR* family members? In the *prp5 prp7 prp9* triple-mutant, *CCA1* is constitutively high whereas *TOC1* is constitutively low, suggesting that PRR5, PRR7 and PRR9 have a negative effect on *CCA1* and a positive effect on *TOC1* (Nakamichi et al. 2005). On the other hand, *PRR9* mRNA levels are strongly reduced and *PRR7* mRNA levels are weakly reduced in *cca1 lhy-R* mutants. Furthermore, *CCA1* and *LHY* over-expression leads to higher overall *PRR7* and *PRR9* levels, suggesting that *CCA1* and *LHY* contribute to the activation of *PRR7* and *PRR9* (Farre et al. 2005). Consistent with the idea that *CCA1* directly regulates their transcription, recombinant *CCA1* binds to a *CCA1* binding site (CBS) in the *PRR7* promoter and to an EE in the *PRR9* promoter.

The opposite effect is observed in plants over-expressing *TOC1/PRR1*, where *PRR9* is repressed and *PRR3*, *PRR5* and *PRR7* rhythms are strongly damped (Mizuno 2004).

Based on the information on the expression of the *PRR* family members and the known clock components in the mutant combinations available, a model has been proposed to incorporate PRR5, PRR7 and PRR9 into the basic framework of the *TOC1/PRR1-CCA1-LHY* oscillator (Figure 2) (Mizuno & Nakamichi 2005). In the basic loop model, a day in the life of an *Arabidopsis* plant would start with *TOC1* promoting *CCA1* and *LHY* transcription, presumably by indirect means. Accumulating levels of *CCA1* and *LHY* during the day in turn lead to *TOC1* repression by binding to its promoter (Alabadi et al. 2001). In the extended model by Mizuno and Nakamishi, PRR5, PRR7 and PRR9 are mediators of additional *TOC1* effects on *CCA1* and *LHY* and *CCA1/LHY* effects on *TOC1*, respectively (Mizuno & Nakamichi 2005).

On the one hand, *PRR5*, *PRR7* and *PRR9* are linked to *CCA1* and *LHY* in a feedback circuit in which PRR5, PRR7 and PRR9 negatively affect *CCA1* and *LHY* expression and, in turn, *CCA1* and *LHY* positively affect PRR5, PRR7 and PRR9. On the other hand, PRR5, PRR7 and PRR9 are linked to *TOC1/PRR1* in another feedback circuit, in which PRR5, PRR7 and PRR9 positively affect *TOC1* expression whereas *TOC1* negatively affects PRR5, PRR7 and PRR9. Both PRR loops are thus interdigitated with the basic *TOC1/PRR1-CCA1-LHY* loop. These interlocked feedback loops may help to fine-tune the oscillator under different conditions.

PRR5 and PRR7 have also been implicated in temperature entrainment (Salome & McClung 2005b). The *prp7* mutant and *prp9* mutant are impaired in the entrainment to thermocycles and the *prp7 prp9* double-mutant is unable to reset the clock in response to temperature pulses, and fails to maintain oscillations after entrainment to thermoperiods. This would be consistent with a role for PRR7 and PRR9 in the temperature input pathway. Because PRR7 and PRR9 also have been shown to mediate light input and to operate close to

the core clockwork, it seems more likely that they are elements of the clockwork that mediate responses to temperature.

The molecular mechanisms underlying the function of PRRs remain to be determined. They can be directed to the nucleus through the nuclear localisation signal within the CTT domain (Makino et al. 2000; Strayer et al. 2000). So far, no evidence for a DNA binding activity has been described. The lack of the conserved aspartic acid residue in the pseudo-receiver domain precludes a classic His-Asp-phospho relay where phosphorylation of the receiver leads to conformational changes and initiates downstream signalling. Such conformational changes may also be caused by protein–protein interaction instead of phosphoryl transfer.

Interestingly, an atypical receiver domain is also found in the CikA (CIRCADIAN INPUT KINASE) protein that mediates environmental input to the cyanobacterial clock (see below) (Ditty et al. 2003). This pseudo-receiver domain is located at the C-terminus of the CikA histidine kinase domain and also lacks the conserved aspartic acid residue; thus it may not be involved in a canonical phospho-relay mechanism. It has been suggested that CikA may receive environmental stimuli through interaction with sensor molecules, e.g. bacteriophytochromes, and communicate this information through interaction with response regulators (Ditty et al. 2003).

Are there candidate partner molecules for signalling through the PRRs? Notably, the plant phytochrome photoreceptors show sequences related to the sensory histidine kinase of bacterial phytochromes, however, they do not phosphorylate histidine but serine/threonine residues (Figure 3B). One may speculate whether signalling through these pseudo-HKs and the PRRs and other, as yet, unidentified partners may be involved in clock-input regulation.

Evidently, despite considerable progress in the characterisation of individual components involved in clock function, much remains to be learned about how they cooperate to make up a functional clockwork. Moreover, additional factors contributing to clock function may be found among the mutants identified in a high throughput screening for aberrant rhythms of LUC bioluminescence, for example (Onai et al. 2004). Furthermore, individuals with altered flowering time that appear in the increasing populations of T-DNA-tagged plants will continue to be a rich source of mutants changed with regard to photoperiodic timekeeping and circadian rhythmicity (Doyle et al. 2002).

### **How many clocks in one plant?**

Physiological processes in diverse plant organs have been shown to be under the control of an endogenous timekeeper, as they show a 24-h rhythm that persists in isolated organs. For example, rhythmic opening and closure of petals through an underlying rhythm in cell elongation continues in isolated *Kalanchoë* flowers (Engelmann & Johnsson 1977), and guard cells in epidermal peels of *Vicia faba* show rhythmic opening and closure, suggesting that they harbor individual clocks (Gorton et al. 1989).

More recently, transgenic plants harbouring the LUC reporter protein driven by clock-controlled promoters revealed rhythms in individual plant organs that persisted in explants (Thain et al. 2002). These molecular markers will be extremely useful to answer the question whether identical clocks control rhythms in individual parts of the plant or whether the plant has different clocks, maybe within one tissue or even one cell.

For example, differences in the free-running period between CAB::LUC oscillations and PHYB::LUC oscillations in one study and between CAB::LUC oscillations and CAT3::LUC oscillations in another have been recorded (Hall et al. 2002; Michael et al. 2003a). This kind of finding indicates that the underlying clocks could, theoretically, be different. Moreover, the

clock regulating *CAB* and the one regulating *CAT3* exhibit differential sensitivity to temperature cues (Michael et al. 2003a).

Presently, it is not known to what extent these clocks which drive rhythms with different free-running periods are actually made up of different components. Mutations in the same clock proteins have been shown to affect rhythms with different free-running periods. For example, the *toc1* mutation shortens the period of LHC::LHC oscillations to 21 h and of rhythmic leaf movement to 23.3 h. The *ztl* mutation lengthens the period of stomatal conductance by 5 h and that of CO<sub>2</sub> assimilation by 3.5 h (Millar et al. 1995; Dodd et al. 2004). Although this suggests that both TOC1 and ZTL are active in distinct clocks, these clocks need not necessarily comprise entirely different components. Rather, factors specific for a given cell type or tissue may convey distinct features upon otherwise identical clockworks.

### Control of clock output genes

A global analysis of transcript oscillations in *Arabidopsis* seedlings revealed that as much as 6% of the genome is under control of the circadian clock, including gene products involved in photosynthetic light absorption, photorespiration, carbon metabolism, lipid metabolism, cell elongation, transport, and synthesis of phenolic compounds involved in protection against UV light (Harmer et al. 2000; Schaffer et al. 2001). This implies that rhythmic control of output gene expression is a widespread means to convey circadian regulation on physiological, biochemical and developmental processes.

#### *Transcriptional regulation*

McClung's group determined, through random insertion of firefly luciferase driven by a minimal promoter, that about 36% of the tagged promoters exhibit a circadian transcription rate, with periods ranging from 22 h to 28 h (Michael & McClung 2003). Thus, clock control at the level of transcription is even more prevalent as evident from genome-wide transcript profiling. Additional regulation at the post-transcriptional level altering the half-life of rhythmically expressed transcripts may be widespread, resulting in non-oscillating mRNA at constant levels.

The identification of the same cis-acting elements within promoters of clock genes and output genes indicates that clock proteins, in addition to autoregulation within the core clockwork, may also exert control of downstream targets by direct binding (Staiger & Apel 1999; Harmer et al. 2000; Alabadi et al. 2001).

Among the cycling transcripts are several transcription factors that can establish a network of transcriptional regulation downstream from the core clockwork (Harmer et al. 2000). Apart from direct control of output genes, clock proteins can initiate transcriptional cascades that provide means to synchronise entire pathways.

#### *Post-transcriptional regulation*

The observation that rhythmic expression of many *Arabidopsis* genes does not manifest itself in circadian mRNA oscillations highlights the importance of regulation at the post-transcriptional level (Millar & Kay 1991; Michael & McClung 2002, 2003). Post-transcriptional control may affect the stability, splicing and transport of pre-mRNAs that are synthesised from clock-regulated promoters; this control is mediated by proteins that specifically bind to pre-mRNAs. An example of an RNA-binding protein that exerts post-transcriptional control within the circadian system is described in the next section.

### The clock-regulated RNA-binding protein *ATGRP7*, a slave oscillator

Transcripts encoding oscillating RNA-binding proteins in *Arabidopsis thaliana*, with a peak at the end of the day, have been described (van Nocker & Vierstra 1993; Carpenter et al. 1994; Heintzen et al. 1994). The oscillations of the *ATGRP7* mRNA are at least in part due to specific promoter activity that varies with time of day (Staiger & Apel 1999). A minimal clock-responsive element mediating a basal circadian oscillation, with peak abundance at the end of the day, is located within 112 base-pairs upstream from the transcription start site. Several lines of evidence indicate that this transcriptional regulation is exerted through the CCA1-LHY-TOC1 oscillator. In transgenic *Arabidopsis* plants over-expressing CCA1, the *ATGRP7* transcript shows irregular fluctuations with a reduced amplitude in continuous light, whereas over-expression of mutant CCA1 does not abolish *ATGRP7* rhythms (Wang & Tobin 1998; Daniel et al. 2004). In plants constitutively over-expressing LHY, *ATGRP7* expression is arrhythmic at trough levels in continuous light (Schaffer et al. 1998), indicating that *ATGRP7* is under negative control of the “morning” clock proteins CCA1 and LHY. The presence of sequence motifs with homology to the EE within the *ATGRP7* clock-responsive region suggests that CCA1, LHY or related factors may directly act on the promoter and repress its expression in the evening. In the *toc1* mutant, the period of *ATGRP7/CCR2* oscillations is shortened (Kreps & Simon 1997; Strayer et al. 2000). *ATGRP7*, therefore, additionally receives temporal input from the “evening” clock protein TOC1.

Transcriptional regulation accounts only in part for the observed *ATGRP7* mRNA oscillation. In transgenic plants over-expressing the *ATGRP7* protein from a constitutive promoter (*ATGRP7*-ox plants), the oscillation of the endogenous *ATGRP7* transcript is strongly depressed, indicating that the *ATGRP7* mRNA is under control by negative feedback. This autoregulation is not conferred by the promoter because, in *ATGRP7*-ox plants harbouring an *ATGRP7*- $\beta$ -glucuronidase reporter gene, oscillations of the reporter mRNA continue (Staiger & Apel 1999). Taken together, these observations suggest that the *ATGRP7* feedback loop functions downstream from the oscillator. It receives rhythmic input through transcriptional regulation and conserves the rhythm through negative autoregulation at the post-transcriptional level.

The residual oscillation of the endogenous *ATGRP7* transcript seen in the *ATGRP7*-ox plants is due to an alternatively spliced transcript that appears at the expense of the mRNA (Staiger et al. 2003b). The strongly reduced half-life of this alternatively spliced *ATGRP7* transcript species may account for its low steady-state abundance. This implicates a mechanism by which *ATGRP7* contributes to oscillations of its own transcript. In response to increasing *ATGRP7* protein accumulation during the circadian cycle, a shift in splice site selection occurs, favouring production of the alternatively spliced transcript that rapidly decays. Moreover, a premature stop codon prevents its translation into functional protein, so that *ATGRP7* protein levels decline. Recombinant *ATGRP7* protein specifically interacts with its own transcript *in vitro*, suggesting that the shift in splice site selection and downregulation *in vivo* may be initiated by binding of *ATGRP7* to its pre-mRNA. It is conceivable that post-translational modifications may also contribute to *ATGRP7* protein cycling.

Additionally, *ATGRP7* over-expression depresses the oscillations of *ATGRP8* encoding a related RNA-binding protein, indicating that *ATGRP7* regulates other clock-controlled transcripts apart from its own. Furthermore, comparison of transcript profiles between wild-type plants and *ATGRP7*-ox plants has revealed several transcripts with differences in steady-state abundance, suggesting that these are targets of *ATGRP7* regulation (our unpublished observations). Thus, the *ATGRP7* feedback loop may be a “slave” oscillator within the clock

output pathway (Figure 2). Due to its high-amplitude cycling and the negative autoregulation, it may prevent damping of the signal received by the main oscillator. Furthermore, depending on the half-lives of their components, such slave oscillators may also serve to phase rhythms to different times of the day.

### **A transcriptional feedback loop as slave oscillator: EPR1**

Another example of a molecular slave oscillator is based on transcriptional feedback regulation. A novel member of the protein family with one Myb domain like the clock proteins CCA1 and LHY, EPR1 (EARLY PHYTOCHROME RESPONSIVE), was isolated in a screen for genes rapidly induced by red light (Kuno et al. 2003). The transcript undergoes circadian oscillations with a peak a few hours later than CCA1. Constitutive over-expression of EPR1 represses accumulation of its own transcript. This result suggests that EPR1 protein is part of a negative feedback loop through which it regulates oscillations of its own transcript, presumably through interfering with transcriptional activation of its own promoter-like components of the core oscillator. In addition to the EPR1 transcript itself, CAB oscillations are perturbed in EPR1-ox plants, suggesting that EPR1 is a regulator of CAB. Transcripts of the oscillator components CCA1 and LHY are not affected, whereas EPR1 is arrhythmic in plants constitutively overexpressing CCA1 and LHY, indicating that EPR1 does not feedback on the central oscillators. Therefore the EPR1 feedback loop operates downstream of the CCA1-LHY-TOC1 oscillator and may confer its 24-h-rhythm upon output genes such as CAB (Figure 2).

### **Alternative timing mechanism of the cyanobacterial clock**

Inspection of the completed genome sequence revealed that *Arabidopsis* does not have homologues of clock components known from mammals, flies or fungi (*Arabidopsis* Genome Initiative 2000). This indicates that plant clocks evolved independently. However, the use of transcription–translation based feedback loops with a 24-h period for biological timekeeping seems to have been conserved between eukaryotic species.

Even so, the clock system of prokaryotic cyanobacteria is constructed according to a divergent principle. The discovery of circadian rhythms in cyanobacteria in 1986 abandoned a long-standing dogma that the generation of self-sustained 24-h rhythms is tied to the organisation of a eukaryotic cell (Grobelaar et al. 1986). Among physiological processes controlled by the cyanobacterial clock are photosynthesis, synthesis of carbohydrates, nitrogen fixation and cell division. Notably, these circadian rhythms continue in bacteria that divide more often than once per 24 h (Mori et al. 1996; Kondo et al. 1997). Using bioluminescence of a reporter driven by a rhythmic promoter, Leibler and colleagues uncovered an unexpected robustness of the oscillator system (Mihalcescu et al. 2004).

In recent years it has become increasingly clear that the cyanobacterial clock is not constructed according to the principle of the conventional transcription–translation based autoregulatory circuits (Ditty et al. 2003). Crucial for clock function in the model system *Synechococcus elongatus* PCC 7942 are the *kai* (Japanese for “cycle”) genes. The *kaiA* mRNA and *kaiBC* dicistronic mRNA accumulate with a circadian rhythm. The peak of KaiB and KaiC protein oscillations is delayed about 6 h relative to the *kaiBC* mRNA oscillation (Xu et al. 2000). KaiC negatively regulates its own expression whereas KaiA positively regulates *kaiBC* expression, generating a basic autoregulatory feedback loop for self-sustaining *kaiBC* oscillations (Figure 4) (Ishiura et al. 1998). This view had to be broadened when KaiC over-expression was found to enhance *kaiBC* promoter activity in a *KaiA* null-mutant, indicating

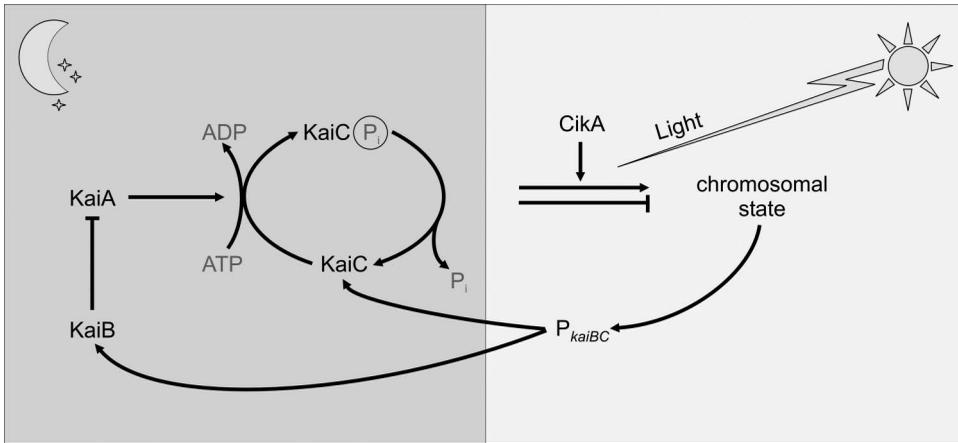


Figure 4. Model of the cyanobacterial KaiABC clock. Left: In the dark, a post-translational cycle of KaiC phosphorylation maintains rhythmicity without transcriptional and translational contribution. Right: in light–dark cycles, this minimal loop is extended by an influence of KaiC on chromosomal state which influences *kaiBC* promoter activity as part of a genome-wide influence on transcriptional activity (Tomita et al. 2005).

that KaiC is also a positive regulator of its own expression (Iwasaki et al. 2002). Thus the Kai clock may comprise a positive in addition to a negative feedback loop, as previously described for the clocks in mammals, flies and *Arabidopsis*.

The lack of an obvious DNA interaction domain makes it unlikely that KaiC binds specifically to the *kaiBC* promoter. KaiC consists of two similar domains that each have ATP-binding motifs, placing KaiC in the DNA-binding ATPase super-family that includes DNA helicases and recombinases (Iwasaki et al. 1999). This led to the hypothesis that KaiC may exert a more global control on transcription by targeting the super-helicity of chromosomal DNA (Mori & Johnson 2001). Indeed, upon inducible KaiC expression, more than 800 promoters were repressed in addition to the *kaiBC* promoter itself (Nakahira et al. 2004).

Recently, the idea has been put forward that rhythmic *kaiBC* transcription is dispensable for circadian oscillation and that post-translational regulation of KaiC plays a crucial role (Tomita et al. 2005). The KaiC protein oscillates between a phosphorylated and a dephosphorylated state due to its intrinsic kinase activity (Iwasaki et al. 2002). This autophosphorylation is essential for timekeeping, because mutations of the phosphorylation sites abolish circadian rhythms in *Synechococcus* (Nishiwaki et al. 2004; Xu et al. 2004). KaiA enhances autophosphorylation of KaiC, whereas KaiB inhibits the positive action of KaiA (Iwasaki et al. 2002; Williams et al. 2002; Kitayama et al. 2003).

To investigate clock function in the absence of transcriptional rhythms, Iwasaki and co-workers transferred *Synechococcus* cultures to continuous darkness; this led to a drastic reduction of *de novo* RNA and protein synthesis and metabolic activity (Tomita et al. 2005). *KaiA* and *kaiBC* mRNAs did not accumulate to detectable levels, and rhythms of KaiA, KaiB and KaiC proteins were no longer observed in constant dark. Instead, the proteins were present at a constant level. The phosphorylation state of KaiC protein, however, continued to oscillate, even in the presence of inhibitors of transcription and translation. The post-translational rhythm therefore does not require *kaiBC* transcription (Figure 4).

The photoautotrophic cyanobacteria were the endosymbiotic ancestors of chloroplasts. What fate did the Kai clock undergo? In completely sequenced plastid genomes no homologues of the kai clock gene were found (Ditty et al. 2003). In fact, evaluation of the *Arabidopsis* genome revealed that most genes of cyanobacterial origin have been relocated to

the nuclear genome. Thus, genes that were rhythmically expressed in the endosymbiont could acquire control by the host's clock during evolution, making an extra clock in plastids superfluous.

Working on the molecular principles that make the circadian clock work has been an exciting and fruitful area of research in recent years. However, many more secrets slumber beneath the surface, waiting for their discovery.

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## References

- Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA. 2001. Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. *Science* 293:880–883.
- Alabadi D, Yanovsky MJ, Más P, Harmer SL, Kay SA. 2002. Critical role for CCA1 and LHY in Maintaining circadian rhythmicity in *Arabidopsis*. *Curr Biol* 12:757–761.
- Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408:796–815.
- Barak S, Tobin EM, Green RM, Andronis C, Sugano S. 2000. All in good time: the *Arabidopsis* circadian clock. *Trends Plant Sci* 5:517–522.
- Briggs WR, Beck CF, Cashmore AR, Christie JM, Hughes J, Jarillo JA, Kagawa T, Kanegae H, Liscum E, Nagatani A, Okada K, Salomon M, Rudiger W, Sakai T, Takano M, Wada M, Watson JC. 2001. The phototropin family of photoreceptors. *Plant Cell* 13:993–997.
- Carpenter CD, Kreps JA, Simon AE. 1994. Genes encoding glycine-rich Arabidopsis thaliana proteins with RNA-binding motifs are influenced by cold treatment and an endogenous circadian rhythm. *Plant Physiol* 104:1015–1025.
- Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. 1993. Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science* 262:539–544.
- Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, Kay SA. 2001. *ELF3* modulates resetting of the circadian clock in Arabidopsis. *Plant Cell* 13:1305–1315.
- Daniel X, Sugano S, Tobin EM. 2004. CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in Arabidopsis. *Proc Natl Acad Sci USA* 101:3292–3297.
- Ditty JL, Williams SB, Golden SS. 2003. A cyanobacterial circadian timing mechanism. *Annu Rev Genet* 37:513–543.
- Dodd AN, Parkinson K, Webb AAR. 2004. Independent circadian regulation of assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*. *New Phytol* 162:63–70.
- Doyle MR, Davis SJ, Bastow RM, McWatters HG, Kozma-Bognar L, Nagy F, Millar AJ, Amasino R. 2002. The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* 419:74–75.
- Dunlap JC. 1999. Molecular bases for circadian clocks. *Cell* 96:271–290.
- Engelmann W, Johnsson A. 1977. Attenuation of the petal movement rhythm in *Kalanchoe* with light pulses. *Physiol Plant* 43:68–76.
- Eriksson ME, Hanano S, Southern MM, Hall A, Millar AJ. 2003. Response regulator homologues have complementary, light-dependent functions in the *Arabidopsis* circadian clock. *Planta* 218:159–162.
- Eriksson ME, Millar AJ. 2003. The circadian Clock. A plant's best friend in a spinning world. *Plant Physiol* 132:732–738.
- Fankhauser C, Staiger D. 2002. Photoreceptors in Arabidopsis thaliana: light perception, signal transduction and entrainment of the endogenous clock. *Planta* 216:1–16.
- Farre EM, Harmer SL, Harmon FG, Yanovsky MJ, Kay SA. 2005. Overlapping and Distinct Roles of PRR7 and PRR9 in the Arabidopsis Circadian Clock. *Curr Biol* 15:47–54.
- Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, Putterill J. 1999. GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. *EMBO J* 18:4679–4688.

- Gorton HL, Williams WE, Binns ME, Gemmill CN, Leheny EA, Shepherd AC. 1989. Circadian stomatal rhythms in epidermal peels from *Vicia faba*. *Plant Physiol* 90:1329–1334.
- Green RM, Tobin EM. 1999. Loss of the circadian clock-associated protein 1 in *Arabidopsis* results in altered clock-regulated gene expression. *Proc Natl Acad Sci USA* 96:4176–4179.
- Grobbelaar N, Huang T-C, Lin HY, Chow T-J. 1986. Dinitrogen-fixing endogenous rhythm in *Synechococcus* RF-1. *FEMS Microbiol Lett* 37:173–177.
- Hall A, Bastow RM, Davis SJ, Hanano S, McWatters HG, Hibberd V, Doyle MR, Sung S, Halliday KJ, Amasino RM, Millar AJ. 2003. The TIME FOR COFFEE gene maintains the amplitude and timing of *Arabidopsis* circadian clocks. *Plant Cell* 15:2719–2729.
- Hall A, Kozma-Bognar L, Bastow RM, Nagy F, Millar AJ. 2002. Distinct regulation of CAB and PHYB gene expression by similar circadian clocks. *Plant J* 32:529–537.
- Han L, Mason M, Risseuw EP, Crosby WL, Somers DE. 2004. Formation of an SCF complex is required for proper regulation of circadian timing. *Plant J* 40:291–301.
- Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290:2110–2113.
- Hayama R, Coupland G. 2004. The molecular basis of diversity in the photoperiodic flowering responses of *Arabidopsis* and rice. *Plant Physiol* 135:677–684.
- Heintzen C, Melzer S, Fischer R, Kappeler S, Apel K, Staiger D. 1994. A light- and temperature-entrained circadian clock controls expression of transcripts encoding nuclear proteins with homology to RNA-binding proteins in meristematic tissue. *Plant J* 5:799–813.
- Hicks KA, Millar AJ, Carre IA, Somers DE, Straume M, Meeks-Wagner DR, Kay SA. 1996. Conditional circadian dysfunction of the *Arabidopsis* early-flowering 3 mutant. *Science* 274:790–792.
- Huq E, Tepperman JM, Quail PH. 2000. GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. *Proc Natl Acad Sci USA* 97:9789–9794.
- Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification on CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409:1060–1063.
- Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A, Golden SS, Johnson CH, Kondo T. 1998. Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science* 281:1519–1523.
- Iwasaki H, Nishiwaki T, Kitayama Y, Nakajima M, Kondo T. 2002. KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc Natl Acad Sci USA* 99:15788–15793.
- Iwasaki H, Taniguchi Y, Ishiura M, Kondo T. 1999. Physical interactions among circadian clock proteins KaiA, KaiB and KaiC in cyanobacteria. *EMBO J* 18:1137–1145.
- Jarillo JA, Capel J, Tang RH, Yang HQ, Alonso JM, Ecker JR, Cashmore AR. 2001. An *Arabidopsis* circadian clock component interacts with both CRY1 and phyB. *Nature* 410:487–490.
- Kaczorowski KA, Quail PH. 2003. *Arabidopsis* PSEUDO-RESPONSE REGULATOR7 is a signaling intermediate in phytochrome-regulated seedling deetiolation and phasing of the circadian clock. *Plant Cell* 15:2654–2665.
- Kim J-Y, Song H-R, Taylor BL, Carré IA. 2003. Light-regulated translation mediates gated induction of the *Arabidopsis* clock protein LHY. *EMBO J* 22:935–944.
- Kim WY, Geng R, Somers DE. 2003. Circadian phase-specific degradation of the f-box protein ztl is mediated by the proteasome. *Proc Natl Acad Sci USA* 100:4933–4938.
- Kitayama Y, Iwasaki H, Nishiwaki T, Kondo T. 2003. KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. *Embo J* 22:2127–2134.
- Kondo T, Mori T, Lebedeva NV, Aoki S, Ishiura M, Golden SS. 1997. Circadian rhythms in rapidly dividing cyanobacteria. *Science* 275:224–227.
- Kreps JA, Simon AE. 1997. Environmental and genetic effects on circadian clock-regulated gene expression in *Arabidopsis*. *Plant Cell* 9:297–304.
- Kuno N, Moller SG, Shinomura T, Xu X, Chua N-H, Furuya M. 2003. The novel MYB protein EARLY-PHYTOCHROME-RESPONSIVE1 is a component of a slave circadian oscillator in *Arabidopsis*. *Plant Cell* 15:2476–2488.
- Makino S, Kiba T, Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Ueguchi C, Sugiyama T, Mizuno T. 2000. Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in *Arabidopsis thaliana*. *Plant Cell Physiol* 41:791–803.
- Mas P, Alabadi D, Yanovsky MJ, Oyama T, Kay SA. 2003a. Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. *Plant Cell* 15:223–236.
- Mas P, Kim WY, Somers DE, Kay SA. 2003b. Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. *Nature* 426:567–570.

- Matsushika A, Makino S, Kojima M, Mizuno T. 2000. Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in *Arabidopsis thaliana*: insight into the plant circadian clock. *Plant Cell Physiol* 41:1002–1012.
- McWatters HG, Bastow RM, Hall A, Millar AJ. 2000. The ELF3 zeitnehmer regulates light signalling to the circadian clock. *Nature* 408:716–720.
- Michael TP, McClung CR. 2002. Phase-specific circadian clock regulatory elements in *Arabidopsis*. *Plant Physiol* 130:627–638.
- Michael TP, McClung CR. 2003. Enhancer trapping reveals widespread circadian clock transcriptional control in *Arabidopsis*. *Plant Physiol* 132:629–639.
- Michael TP, Salome PA, McClung CR. 2003a. Two *Arabidopsis* circadian oscillators can be distinguished by differential temperature sensitivity. *Proc Natl Acad Sci USA* 100:6878–6883.
- Michael TP, Salome PA, Yu HJ, Spencer TR, Sharp EL, McPeck MA, Alonso JM, Ecker JR, McClung CR. 2003b. Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* 302:1049–1053.
- Mihalcescu I, Hsing W, Leibler S. 2004. Resilient circadian oscillator revealed in individual cyanobacteria. *Nature* 430:81–85.
- Millar AJ. 2004. Input signals to the plant circadian clock. *J Exp Bot* 55:277–283.
- Millar AJ, Carré IA, Strayer CS, Chua N-H, Kay S. 1995. Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science* 267:1161–1163.
- Millar AJ, Kay S. 1991. Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. *Plant Cell* 3:541–550.
- Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song H-R, Carré IA, Coupland G. 2002. *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev Cell* 2:629–641.
- Mizuno T. 2004. Plant response regulators implicated in signal transduction and circadian rhythm. *Curr Opin Plant Biol* 7:499–505.
- Mizuno T, Nakamichi N. 2005. Pseudo response regulators (PRRs) or true oscillator components (TOCs). *Plant Cell Physiol* 46:677–685.
- Mori T, Binder B, Johnson CH. 1996. Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proc Natl Acad Sci USA* 93:10183–10188.
- Mori T, Johnson CH. 2001. Circadian programming in cyanobacteria. *Semin Cell Dev Biol* 12:271–278.
- Naidoo N, Song W, Hunter-Ensor M, Sehgal A. 1999. A role for the proteasome in the light response of the timeless clock protein. *Science* 285:1737–1741.
- Nakahira Y, Katayama M, Miyashita H, Kutsuna S, Iwasaki H, Oyama T, Kondo T. 2004. Global gene repression by KaiC as a master process of prokaryotic circadian system. *Proc Natl Acad Sci USA* 101:881–885.
- Nakamichi N, Kita M, Ito S, Yamashino T, Mizuno T. 2005. Pseudo-response regulators, PRR9, PRR7, and PRR5, play together essential roles close to the circadian clock of *Arabidopsis thaliana*. *Plant Cell Physiology* 46:686–698.
- Nelson DC, Lasswell J, Rogg LE, Cohen MA, Bartel B. 2000. FKF1, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. *Cell* 101:331–340.
- Nishiwaki T, Satomi Y, Nakajima M, Lee C, Kiyohara R, Kageyama H, Kitayama Y, Temamoto M, Yamaguchi A, Hijikata A, Go M, Iwasaki H, Takao T, Kondo T. 2004. Role of KaiC phosphorylation in the circadian clock system of *Synechococcus elongatus* PCC 7942. *Proc Natl Acad Sci USA* 101:13927–13932.
- Onai K, Okamoto K, Nishimoto H, Morioka C, Hirano M, Kami-Ike N, Ishiura M. 2004. Large-scale screening of *Arabidopsis* circadian clock mutants by a high-throughput real-time bioluminescence monitoring system. *Plant J* 40:1–11.
- Panda S, Poirier GG, Kay SA. 2002. *tej* defines a role for poly(ADP-ribosyl)ation in establishing period length of the *Arabidopsis* circadian oscillator. *Dev Cell* 3:51–61.
- Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG. 1999. Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis* GIGANTEA gene. *Science* 285:1579–1582.
- Redei GP. 1962. Supervital mutants of *Arabidopsis*. *Genetics* 47:443–460.
- Salome PA, McClung CR. 2004. The *Arabidopsis thaliana* clock. *J Biol Rhythm* 19:425–435.
- Salome PA, McClung CR. 2005a. What makes the *Arabidopsis* clock tick on time? A review on entrainment. *Plant Cell Environ* 28:21–38.
- Salome PA, McClung CR. 2005b. PSEUDO-RESPONSE REGULATOR 7 and 9 are partially redundant genes essential for the temperature responsiveness of the *Arabidopsis* circadian clock. *Plant Cell* 17:791–803.
- Schaffer R, Landgraf J, Accerbi M, Simon V, Larson M, Wisman E. 2001. Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell* 13:113–123.

- Schaffer R, Ramsay N, Samach ACS, Putterill J, Carré IA, Coupland G. 1998. The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93:1219–1229.
- Schultz TF, Kiyosue T, Yanovsky M, Wada M, Kay SA. 2001. A role for LKP2 in the circadian clock of *Arabidopsis*. *Plant Cell* 13:2659–2670.
- Somers DE, Schultz TF, Milnamow M, Kay SA. 2000. ZEITLUPE encodes a novel clock-associated PAS protein from *Arabidopsis*. *Cell* 101:319–329.
- Staiger D, Apel K. 1999. Circadian clock-regulated expression of an RNA-binding protein in *Arabidopsis*: characterisation of a minimal promoter element. *Mol Genet Gen* 261:811–819.
- Staiger D, Allenbach L, Salathia N, Fiechter V, Davis SJ, Millar AJ, Chory J, Fankhauser C. 2003a. The *Arabidopsis* *SRR1* gene mediates phyB signaling and is required for normal circadian clock function. *Genes Dev* 17:256–268.
- Staiger D, Streitner C, Rudolf F, Huang X. 2006. Multiple and slave oscillators. In: Hall A, McWatters H, editors. *Endogenous plant rhythms*. Oxford: Blackwell Publishers. Chapter 3.
- Staiger D, Zecca L, Kirk DA, Apel K, Eckstein L. 2003b. The circadian clock regulated RNA-binding protein AtGRP7 autoregulates its expression by influencing alternative splicing of its own pre-mRNA. *Plant J* 33:361–371.
- Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA. 2000. Cloning of the *Arabidopsis* clock gene TOC1, an autoregulatory response regulator homolog. *Science* 289:768–771.
- Sugano S, Andronis C, Green RM, Wang Z-Y, Tobin EM. 1998. Protein kinase CK2 interacts with and phosphorylates the *Arabidopsis* circadian clock-associated gene 1 protein. *Proc Natl Acad Sci USA* 95:11020–11025.
- Thain SC, Murtas G, Lynn JR, McGrath RB, Millar AJ. 2002. The circadian clock that controls gene expression in *Arabidopsis* is tissue specific. *Plant Physiol* 130:102–110.
- Tomita J, Nakajima M, Kondo T, Iwasaki H. 2005. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* 307:251–254.
- Tseng TS, Salome PA, McClung CR, Olszewski NE. 2004. SPINDLY and GIGANTEA interact and act in *Arabidopsis thaliana* pathways involved in light responses, flowering, and rhythms in cotyledon movements. *Plant Cell* 16:1550–1563.
- van Nocker S, Vierstra RD. 1993. Two cDNAs from *Arabidopsis thaliana* encode putative RNA binding proteins containing glycine-rich domains. *Plant Mol Biol* 21:695–699.
- Wang ZY, Kenigsbuch D, Tobin EM. 1997. A *Myb*-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* *Lhcb* gene. *Plant Cell* 9:491–499.
- Wang ZY, Tobin EM. 1998. Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207–1217.
- Williams SB, Vakonakis I, Golden SS, LiWang AC. 2002. Structure and function from the circadian clock protein KaiA of *Synechococcus elongatus*: a potential clock input mechanism. *Proc Natl Acad Sci USA* 99:15357–15362.
- Xu Y, Mori T, Johnson CH. 2000. Circadian clock-protein expression in cyanobacteria: rhythms and phase setting. *EMBO J* 19:3349–3357.
- Xu Y, Mori T, Pattanayek R, Pattanayek S, Egli M, Johnson CH. 2004. Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses. *Proc Natl Acad Sci USA* 101:13933–13938.
- Yasuhara M, Mitsui S, Hirano H, Takanabe R, Tokioka Y, Ihara N, Komatsu A, Seki M, Shinozaki K, Kiyosue T. 2004. Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein 2) in *Arabidopsis*. *J Exp Bot* 55:2015–2027.
- Young MW, Kay SA. 2001. Time zones: a comparative genetics of circadian clocks. *Nat Rev Genet* 2:702–715.
- Zhong HH, Resnick AS, Straume M, McClung CR. 1997. Effects of synergistic signaling by phytochrome A and cryptochrome 1 on circadian clock-regulated catalase expression. *Plant Cell* 9:947–955.