The *Arabidopsis thaliana* Clock

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Abstract A combination of forward and reverse genetic approaches together with transcriptome-scale gene expression analyses have allowed the elaboration of a model for the *Arabidopsis thaliana* circadian clock. The working model largely conforms to the expected negative feedback loop model that has emerged from studies in other model systems. Although a core loop has emerged, it is clear that additional components remain to be identified and that the workings of the Arabidopsis clock have been established only in outline. Similarly, the details of resetting by light and temperature are only incompletely known. In contrast, the mechanism of photoperiodic induction of flowering is known in considerable detail and is consistent with the external coincidence model.

Key words *Arabidopsis thaliana,* biological clocks, circadian oscillator, circadian rhythm, cryptochrome, entrainment, light regulation, photoperiodism, phytochrome, temperature sensing

Plants were the first organisms for which the observation of a circadian rhythm was published (de Mairan, 1729). You would think that with a 200-year head start, more would be known on the plant circadian system. And in part it is true: We do know a lot about rhythms in many, many different plant species. The molecular study of plant clocks began in 1985 with the observation that the mRNA abundance of the light-harvesting chlorophyll *a/b*-binding protein genes (*LHCB*) of peas oscillated with a circadian rhythm (Kloppstech, 1985). However, Arabidopsis did not emerge as a clock system until the 1990s (McClung and Kay, 1994), and mutational analysis did not yield putative clock genes until 1995 (Millar et al., 1995a, 1995b), long after the Drosophila *period* and Neurospora *frequency* mutants had been identified (Feldman and Hoyle, 1973; Konopka and Benzer, 1971) and the corresponding genes cloned (Bargiello et al., 1984; McClung et al., 1989; Reddy et al., 1984). With the establishment of Arabidopsis as a model plant system and the use of luciferase as a noninvasive reporter gene (Millar et al., 1992), plant clock research has almost caught up with the other systems. The paradigm of a negative feedback loop has held in the Arabidopsis clock, although many of the molecular details remain to be elucidated.

FINDING A CLOCK GENE: WORKING UPSTREAM TO THE CLOCK FROM AN OUTPUT RHYTHM

Many plant genes are under the control of the clock. In fact, as much as 6% of the genome may be under

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clock control, as determined by microarray experiments using an Affymetrix chip that includes about 8000 of the estimated 30,000 Arabidopsis genes (Harmer et al., 2000). The most extensively studied clock-regulated gene in Arabidopsis is the *LHCB* gene encoding the light-harvesting chlorophyll *a/b*-binding protein. *LHCB* transcription is induced by light and shows a circadian pattern of expression with a peak in the middle of the subjective day (Millar and Kay, 1991). The red-light photoreceptors, the phytochromes, mediate the light induction of *LHCB* through a motif in the *LHCB* promoter. The same region of the *LHCB* promoter is also required for circadian expression, and attempts to separate the 2 activities have failed (Anderson et al., 1994). Minimal promoter fragments necessary and sufficient for light and circadian regulation of *LHCB* were identified (Carré and Kay, 1995), and Tobin's group identified a protein with affinity to this promoter fragment. They originally described a DNA-binding activity in plant cell extracts with affinity for the light-responsive element of the *LHCB* promoter and designated this activity CA-1 because of its preference for cytosine- and adenine-rich sequences (Kenigsbuch and Tobin, 1995; Sun et al., 1993). Using the same DNA fragment that was the target of CA-1 in vitro, they set out to clone the *CA-1* gene. The screening of an Arabidopsis expression library with the DNA fragment yielded clones that encoded one protein with specific CA-1 binding activity. The corresponding protein was named CCA1, originally meaning clone of CA-1 (Wang et al., 1997), but renamed CIRCADIAN CLOCK ASSOCIATED 1. CCA1 is an unusual DNA-binding protein in that it recognizes an asymmetric DNA sequence (the CCA1-binding site [CBS] AAAATCT or the evening element [EE] AAATATCT) and has only one Myb-like domain for DNA binding. Other plant and mammalian Myb proteins have several such domains and bind DNA as dimers (Romero et al., 1998).

Northern blot analysis first demonstrated that the expression of *CCA1* itself was under the control of the clock and that overexpression of *CCA1* resulted in the repression of the endogenous gene (Wang and Tobin, 1998). Strikingly, the expression of the clock-regulated genes *CCA1*, *LHCB*, *CCR2*, and *CAT3* was arrhythmic (Wang and Tobin, 1998). This provided a rare example of the isolation of a clock-associated protein through the analysis of the promoter of an output gene. In an accompanying article, a gene encoding a single Myb domain protein closely related to *CCA1* and named *LATE ELONGATED HYPOCOTYL* (*LHY*; Schaffer et al., 1998) was described. The *lhy-1* mutant was identified on the basis of late-flowering and long-hypocotyl phenotypes from a population of lines generated by random transposition of the maize Ds transposon. The Ds transposon can drive high levels of expression of genes adjacent to the insertion site by the strong outwardly directed Cauliflower Mosaic Virus 35S promoter. In this case, the transposon was inserted upstream of the *LHY* gene and caused constitutive overexpression (Schaffer et al., 1998). The overexpression of LHY had essentially the same effects on the clock as CCA1 overexpression: arrhythmicity in leaf movement, *LHCB* transcription, and *CCR2* expression (Schaffer et al., 1998). Overexpression of CCA1, like that of LHY, was also responsible for a long-hypocotyl and late-flowering phenotype. Because CCA1 was originally isolated as a protein binding to the light-responsive element of the *LHCB* promoter, light induction of *CCA1* expression was determined. *CCA1* message is induced by red and far-red lights, indicating a possible role for CCA1 in red-light-induced phase resetting of the clock (Wang and Tobin, 1998).

The isolation of *CCA1* is probably 1 of the 2 examples now available of the isolation of a clock component through the characterization of the activities binding to a clock output gene. The second example is the mammalian REV-ERBα (Preitner et al., 2002). Of course, one could argue that the discovery of the involvement of CCA1 in the clock was accidental and happened only because the light- and clock-responsive elements of the *LHCB* promoter overlap. Probably part of the success of finding CCA1 stemmed from the fact that it can bind to DNA as a monomer and not necessarily as a heteromultimer. The requirement for accessory proteins would have made the isolation of CCA1 more difficult.

The observed arrhythmicity in plants overexpressing either CCA1 or LHY suggested them as putative clock components. Certainly, overexpression of FRQ in Neurospora and PER or TIM in Drosophila results in a similar arrhythmicity (Aronson et al., 1994; Suri et al., 1999; Zeng et al., 1994). Null alleles of *frq*, *per*, and *tim* are also arrhythmic: what about *cca1* and *lhy* loss-of-function alleles? Plants homozygous for the likely null *cca1-1* allele, generated through insertion of a heterologous T-DNA, are viable with no gross morphological defects. The effect of the loss of CCA1 on

the clock is a shorter period in most rhythms, including leaf movement and *LHCB*, *CAT2*, *CCA1*, and *LHY* expression, as well as possibly a leading phase for the expression of the evening gene *CCR2* (Green and Tobin, 1999). Loss-of-function alleles of *LHY* also result in a short period of the same rhythms. However, neither single loss of function is arrhythmic, possibly because of partial redundancy between the 2 genes (Mizoguchi et al., 2002). Indeed, double-mutant analysis revealed that CCA1 and LHY are both important for proper clock function, as the double mutant turns arrhythmic after release from entrainment (Alabadí et al., 2002; Mizoguchi et al., 2002). The arrhythmicity is not immediate, suggesting that other *CCA1*-related genes partially compensate for the loss of both CCA1 and LHY. This functional redundancy is similar to that encountered in mammals, with multiple *PER* and *CRY* genes playing functionally redundant roles (Panda et al., 2002).

CLOSING THE LOOP THROUGH FORWARD GENETICS

CCA1 and *LHY* encode 2 DNA-binding proteins, and their transcription rates as well as mRNA and protein abundances exhibit a circadian rhythm with a peak near dawn. Based on the interconnected feedback loop models from other organisms, one more element is required to close the loop and would be expected to show peak accumulation in the evening, 12 h later than *CCA1* and *LHY* would. A mutant screen conducted in the Kay lab identified this missing link, although historically the mutant was identified years before CCA1 and LHY. Mutations at the *TIMING OF CAB EXPRESSION 1* (*TOC1*) locus result in a short period of all rhythms tested (Más et al., 2003a; Millar et al., 1995a; Somers et al., 1998). The cloning of TOC1 revealed a protein with homology to bacterial 2-component response regulators, although the protein lacks the conserved aspartic acid that normally receives the phosphoryl group from the associated kinase (Hwang et al., 2002; Strayer et al., 2000). Hence, *TOC1* is also known as *PSEUDO-RESPONSE REGULATOR 1* (*PRR1*). The expression of *TOC1* is under clock control and peaks in the evening, around ZT 12 (Strayer et al., 2000).

The link between CCA1 and LHY came about with the analysis of the *TOC1* promoter and closed the loop of the Arabidopsis clock. The search for a sequence within the *TOC1* promoter that was necessary and sufficient for the clock-regulated evening phase of an *LUC* reporter gene identified a DNA fragment of ~200 bp (Alabadí et al., 2001). This fragment of the *TOC1* promoter contained the motif AAATATCT, also known as the Evening Element (EE; Harmer et al., 2000). The EE was found in many clock-regulated genes showing a peak at dusk and was necessary and sufficient for both proper circadian expression and proper evening phasing of an *LUC* reporter gene (Harmer et al., 2000). In particular, the *CCR2* promoter, a well-studied clock-regulated gene peaking in the evening, has an EE within its promoter, and mutation or deletion of the EE results in arrhythmicity of the LUC reporter construct (Harmer et al., 2000). The same mutations in the EE of the *TOC1* promoter similarly cause arrhythmic expression of the LUC reporter, demonstrating the importance of the site for clock regulation and evening phasing. CCA1 and LHY proteins can bind to the EE of the *CCR2* and *TOC1* promoters in vitro (Alabadí et al., 2001; Harmer et al., 2000). Because the expression levels of *TOC1* are low in plants overexpressing CCA1 or LHY, and because of the direct binding of CCA1 and LHY to the *TOC1* promoter, a model was proposed whereby the 2 dawn transcription factors bind to and repress expression from the *TOC1* promoter. Later during the day, as CCA1 and LHY abundance declines, repression of *TOC1* is alleviated, and *TOC1* transcript and protein accumulate. The expression levels of *CCA1* and *LHY* are much lower in a strong mutant allele of *TOC1*, arguing that TOC1 is a positive regulator of the expression of *CCA1* and *LHY*, thereby closing the loop (Alabadí et al., 2001).

A simple model of the Arabidopsis clock is therefore composed of 3 proteins, forming an interconnected feedback loop (Fig. 1). CCA1 and LHY are induced by light and peak in the morning, when they repress their own expression, as well as that of *TOC1*. CCA1 and LHY are the functional equivalent of the Drosophila PER-TIM complex, which represses the activity of the CYC-dCLK complex. On degradation of CCA1 and LHY by an unidentified mechanism, TOC1 expression increases and induces expression of *CCA1* and *LHY*. TOC1 does not have an obvious DNA-binding motif and may therefore interact with other transcription factor(s) to mediate this induction. Curiously, transcription of *CCA1* and *LHY* does not increase immediately on accumulation of TOC1. Instead, CCA1 and LHY only start to accumulate when TOC1 has reached its peak levels. This lag in the response of the 2 transcription factors to TOC1 suggests

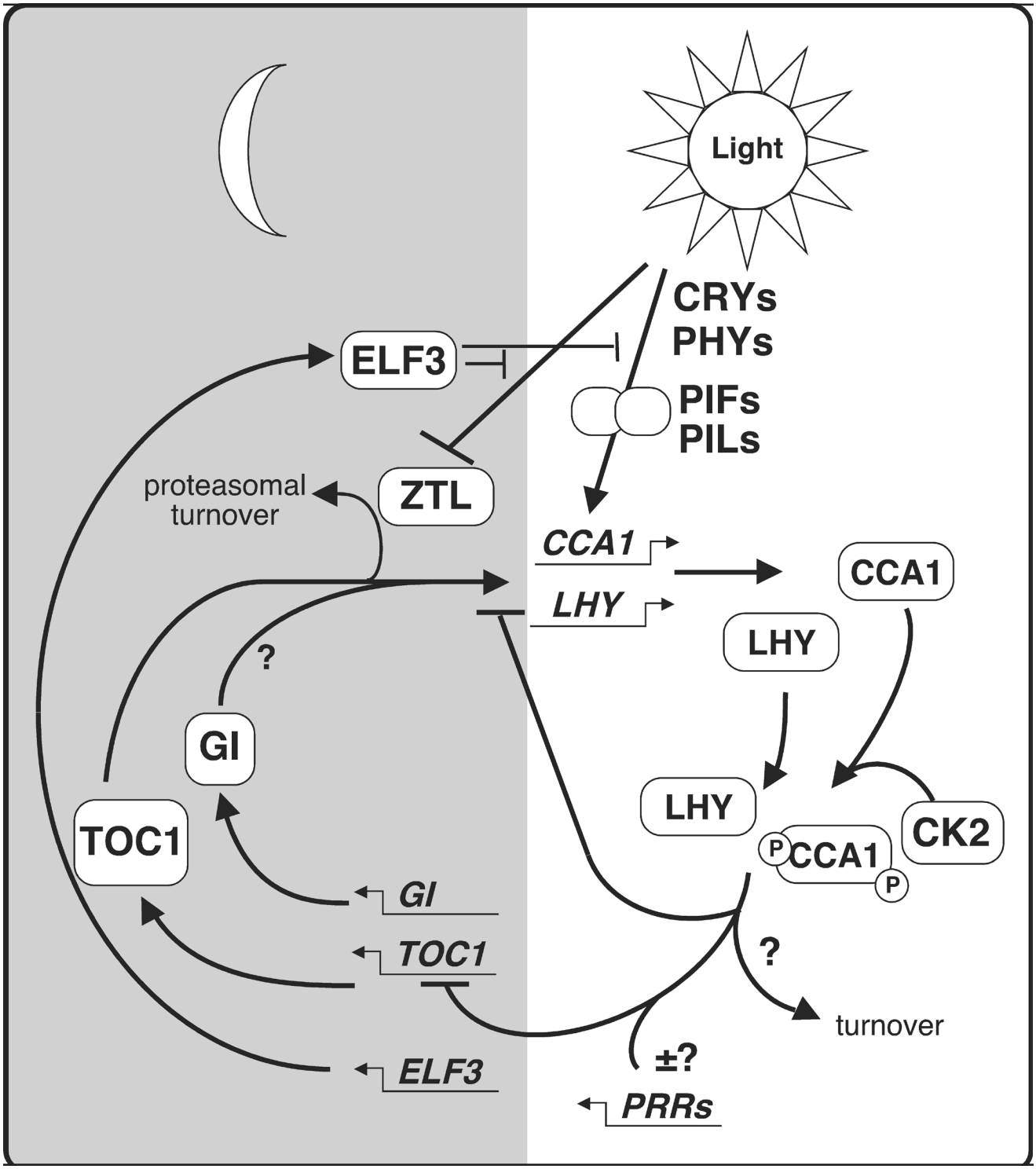

Figure 1. A model of the *Arabidopsis thaliana* oscillator. Light perceived by the PHYs and CRYs induces the expression of 2 transcription factors, *CCA1* and *LHY*. *CCA1* and *LHY* mRNA abundance peaks shortly after dawn. CCA1 requires phosphorylation by CK2 prior to binding to DNA. *PRR9*, *PRR7*, *PRR5*, and *PRR3* show clock-regulated mRNA abundances, peaking in that sequence at 2-h intervals throughout the day. A role of CCA1 and LHY in their regulation has not been established. One known target of the repressive activity of CCA1 and LHY is *TOC1*, with the result that *TOC1* (*PRR1*) mRNA abundance peaks around dusk, following the turnover of CCA1 and LHY proteins. TOC1 then feeds back onto *CCA1* and *LHY* and induces their expression for the next cycle. TOC1 may require a DNA-binding protein as a cofactor, as it is not predicted to directly bind to DNA. GI is necessary for high-level expression from the *CCA1* and *LHY* promoters and may be one TOC1 cofactor. TOC1 degradation is mediated by the F-box protein ZTL, whose activity is negatively regulated by light. CCA1 and LHY also negatively regulate their own promoters, possibly directly but possibly indirectly via TOC1. Light resetting may involve induction of CCA1 and LHY, possibly mediated through phytochrome and cryptochrome photoreceptors and PIF and PIF-like (PIL) transcription factors. *ELF3* mRNA also shows circadian oscillations, peaking after dusk; ELF3 protein is a negative regulator of light signaling to the clock.

that other factor(s) may be critical for the proper induction of CCA1 and LHY in response to TOC1.

A FEW LIMITATIONS OF THE PROPOSED MODEL

This proposed model of the Arabidopsis clock is based on our understanding of the oscillators of other clock model organisms and tries very hard to accommodate the available data. Although this model does not explain all aspects of clock function, it establishes a basic system to build on. In the following discussion, we will attempt to reconcile available data with this model, but some limitations of the model will become evident.

First, it is not understood why the transcription rates of *CCA1* and *LHY* do not increase earlier during the night. The model posits that TOC1 acts as a positive regulator of their expression, and yet the *CCA1* and *LHY* message only starts to accumulate when TOC1 has reached its peak levels. Analysis of protein levels by western blots for CCA1 and LHY reveals a narrow window during which the protein is detected, starting 2 h before subjective dawn and lasting until 4 h after dawn (JY Kim et al., 2003; Wang and Tobin, 1998). However, it should be noted that longer exposures of the blots were not reported, and it is therefore possible that some CCA1 and LHY protein remains present outside of this narrow window. Initial reports suggested that CCA1 and LHY could repress their own expression, as seen by the low levels of endogenous message in overexpressing lines (Schaffer et al., 1998; Wang and Tobin, 1998). Two plausible mechanisms come to mind. First, CCA1 and LHY may directly repress their own expression. An increase in LHY translation around dawn is accompanied by a decrease in *LHY* message, consistent with a negative role of LHY on its own expression (JY Kim et al., 2003). However, the overexpression of the 2 transcription factors will also result in the repression of *TOC1*, itself a positive regulator of CCA1 and LHY (Alabadí et al., 2001). In this scenario, overexpression of CCA1 and LHY will result, indirectly, in the repression of their own expression. Distinction between these 2 possibilities could be attempted via monitoring, with luciferase fusions, the expression of the clock genes in response to pulses of CCA1, LHY, or TOC1. If CCA1 and LHY do in fact directly repress their own expression, then they may maintain this repression even in the presence of the newly translated TOC1. Only on complete turnover of CCA1 and LHY will TOC1 be able to induce their expression. Alternatively, if the effect on their own expression is indirect and through TOC1, then a pulse of TOC1 expression would be predicted to induce *CCA1* and *LHY*, even when they are being overexpressed. However, it is also possible that overexpression of TOC1 is insufficient to induce CCA1 and LHY and that additional factors are required. Consistent with this, overexpression of TOC1 does not result in the induction of CCA1 and LHY but instead decreases the amplitude of their rhythms (Makino et al., 2002).

A second limit of the model is the current view of light resetting. A simple and attractive model proposed that the induction of CCA1 and LHY was mediated through the bHLH transcription factor Phytochrome Interacting Factor 3 (PIF3; Martínez-García et al., 2000). Light is perceived by the phytochrome red-light photoreceptors, which then activate PIF3 bound to the promoters of CCA1 and LHY. One would thus expect that a loss of function in PIF3 would have quite a strong phenotype, but no effects on period, phase, or entrainment can be detected in *pif3* loss-of-function (through T-DNA insertion) plants (PAS and CRM, unpublished) or in plants expressing an antisense *PIF3* message (Oda et al., 2004). Potential redundancy among the 162 bHLH proteins in the Arabidopsis genome (Toledo-Ortiz et al., 2003) might explain the apparent lack of phenotype for *pif3* single-loss-of-function plants, and only multiple-loss-of-function plants will define the role of the *PIF3* gene family.

Evidence is also accumulating for multiple oscillators, yet their molecular underpinnings are not known. Several output genes, for instance, *LHCB*, *CAT3*, *CHS*, and *PHYB*, have distinct free-running periods at the same light fluence, suggesting differential regulation of these genes (Hall et al., 2002; Michael et al., 2003a; Thain et al., 2002). In particular, Arabidopsis expresses 2 different circadian clocks that can be distinguished by their sensitivity to temperature. The oscillator that regulates *LHCB* expression responds preferentially to LD versus temperature cycles and fails to respond to the temperature step associated with release from stratification, whereas the oscillator that regulates *CAT3* expression responds preferentially to temperature versus LD cycles and entrains to the release from stratification (Michael et al., 2003a).

The spatial expression pattern of these genes is partially overlapping, raising the exciting possibility that multiple oscillators reside in the same cell.

POSTTRANSLATIONAL MODIFICATION PLAYS CRITICAL ROLES

Additional factors ensure that the clock runs properly and contribute to a more complex model (Fig. 1). For instance, binding of CCA1 to its target sequences requires prior phosphorylation of the protein and involves casein kinase II (Sugano et al., 1998, 1999). CCA1 interacts with the CKB3 regulatory subunit of CK2 (Sugano et al., 1998) in vitro, and its overexpression in plants leads to a short-period phenotype. In vitro, both CCA1 and LHY can be phosphorylated by CK2, although there is to date no evidence of in vivo phosphorylation for LHY (JY Kim et al., 2003). Visual comparison of the traces for *cca1-1* and the CKB3-ox plants suggests that overexpression of CKB3 causes a period shortening that is more pronounced than seen in the *cca1-1* mutant. One might expect that overexpression of CKB3 would result in a partial gain of function for CCA1, as it would be able to bind DNA constitutively. Instead, the CKB3-ox plants are more similar to and exhibit a more extreme phenotype than do the CCA1 loss-of-function plants. The apparent discrepancy between the requirement of CCA1 phosphorylation and the clock phenotype displayed by CKB3-ox plants may invoke a deregulated degradation rate for CCA1. Many proteins need to be phosphorylated prior to their degradation via the proteasome. Certainly in other systems, phosphorylation of a clock component is a prerequisite for its degradation via the proteasome (He et al., 2003; Ko et al., 2002). The elucidation of the pathway leading to CCA1 degradation will be very important to better place CK2 within the Arabidopsis clock.

PROTEASOMAL PROTEIN DEGRADATION OF TOC1 IS CRITICAL FOR CLOCK FUNCTION

In happy contrast with CCA1 and LHY, the mechanism underlying the daily degradation of TOC1 is known. TOC1 interacts with the F-box protein ZEITLUPE (ZTL; Más et al., 2003b; Somers et al., 2000). *ztl* was identified, in the same screen as *toc1*, as a long-period mutant. The *ztl-1* and *ztl-2* mutants are affected in the period length of numerous rhythms, including *LHCB* and *CCR2* transcription, cotyledon movement, and CO_2 assimilation (Dodd et al., 2004; Somers et al., 2000, 2004). In addition to the F-box domain, the ZTL protein is composed of an LOV domain and 6 kelch repeats (Somers et al., 2000). The LOV domain is very similar to those of WHITE COLLAR–1 and the Arabidopsis phototropins and suggests that ZTL may act as a blue-light photoreceptor (He et al., 2002). The kelch repeats form a potential protein-protein interaction domain.

That the null *ztl* mutants are not completely arrhythmic again raises the question of potential redundancy among related genes. *ZTL* is part of a 3-gene family, the other 2 members being *LKP2* and *FKF1* (Imaizumi et al., 2003; Schultz et al., 2001). *FKF1* mRNA oscillates, but overexpression of *FKF1* does not affect the transcription of *LHCB*, placing FKF1 along an output pathway (Imaizumi et al., 2003). Loss-of-function alleles for *LKP2* have no clock phenotypes (Jarillo et al., 2002), but overexpression of LKP2 and ZTL has very similar phenotypes, including long hypocotyls in red light, late flowering, and arrhythmicity for *LHCB* transcription (Schultz et al., 2001; Somers et al., 2004). ZTL may be able to fully compensate for the loss of LKP2. A definitive role for LKP2 in the clock will await the generation of *ztl lkp2* double mutants.

The mRNA abundance of the *ZTL* gene is not clock regulated, but ZTL protein levels are (WY Kim et al., 2003). Peak protein abundance is seen around dusk, while trough levels are reached around dawn (WY Kim et al., 2003). The rate of proteasome-mediated degradation of ZTL varies during the course of the day: ZTL is more stable at dusk, around its peak value, and is more rapidly degraded at dawn when it reaches its trough. F-box proteins provide specificity to proteasomal degradation pathways by specific interaction with targets for degradation. In this case, the interaction of ZTL with TOC1 recruits TOC1 for proteasomal degradation. Mutations that fall within the kelch repeats of ZTL abrogate ZTL-TOC1 interactions. In a *ztl* mutant, protein levels of TOC1 are elevated and only weakly rhythmic, demonstrating that ZTL is critical for degradation of TOC1. One puzzling result, however, is the repression of the degradation of TOC1 by light. Because ZTL is an LOV domain–containing protein, one might expect light-induced protein degradation; however, it appears that light

blocks the action of ZTL on TOC1. If this effect is direct, ZTL would be the first putative photoreceptor whose function is repressed by light.

OTHER PUTATIVE CLOCK COMPONENTS

Another component in close association with the circadian clock is GIGANTEA (GI; Fowler et al., 1999; Park et al., 1999). GI is a nuclear protein with no obvious functional domains (Huq et al., 2000). In *gi* mutants, the expression of *CCA1* and *LHY* is much reduced relative to wild type (Park et al., 1999). Both *GI* mRNA and protein are clock regulated, with peaks around dusk (Putterill et al., 2002). The timing of GI accumulation would be consistent with a role in promoting high-level expression of *TOC1*, which also peaks around that time (Alabadí et al., 2001). Lower levels of *TOC1* in *gi* mutants would result in lower levels of *CCA1* and *LHY*, as has been observed (Park et al., 1999). Alternatively, GI may interact with TOC1 for the induction of CCA1 and LHY. Because neither TOC1 nor GI has DNA-binding domains or transcriptional activation domains, it is possible that GI or TOC1 may recruit a number of accessory factors necessary for transcription of *CCA1* and *LHY*. In the yeast 2-hybrid system, TOC1 can interact with the transcription factors PIF3 (Más et al., 2003a) and ABSCISIC ACID INSENSITIVE 3 (ABI3; Kurup et al., 2000). However, a T-DNA insertion allele of *PIF3* has no circadian phenotype, indicating that PIF3 by itself is not required for proper clock function (PAS and CRM, unpublished). A circadian phenotype for *abi3* mutants has yet to be established.

Mutations in *EARLY FLOWERING 3* (*ELF3*; Hicks et al., 2001) cause arrhythmicity in the light but not in the dark (Hicks et al., 1996). In addition, plants overexpressing ELF3 display a weaker acute response to light, suggesting that ELF3 acts as a negative regulator of light signaling. Release from entrainment experiments showed that the clock in *elf3* mutants is stopped at dusk, when the ELF3 protein normally starts to accumulate (McWatters et al., 2000). Similar experiments showed that a mutation at the *TIME FOR COFFEE* (*TIC*) locus stops the clock in the morning (Hall et al., 2003). The *elf3 tic* double mutant is completely arrhythmic, demonstrating that both genes are important for the maintenance of strong amplitude in the clock. The molecular characterization of *TIC* will be very important to decipher the respective roles of TIC and ELF3 in light input to the clock.

Output rhythms for at least some clock-controlled genes are under the same transcriptional control as are *CCA1*, *LHY*, and *TOC1*. For instance, mutations in the EE found in the *CCR2* and *CAT3* promoters suppress the rhythmic expression from these promoters (Harmer et al., 2000; Michael and McClung, 2002). Similar mutation of the CBS of the *LHCB* promoter also results in arrhythmicity (Andersson et al., 1999). One obvious question is what determines the phase of the rhythm. The phase of the clock-regulated gene *PRR7* is very close to that of *CAT3*, yet *PRR7* has a CBS within its promoter, whereas *CAT3* has an EE (PAS and CRM, unpublished; Michael and McClung, 2002). Similarly, the *PRR9* gene peaks around the same time as *LHCB* does, yet the *PRR9* promoter contains an EE whereas the *LHCB* promoter contains a CBS (PAS and CRM, unpublished; Andersson et al., 1999; Harmer and Kay, 2003). Clearly, the simple presence or absence of a CBS or EE is insufficient to predict phase. In addition, it is becoming difficult to predict the exact role, positive or negative, of the binding of CCA1 and LHY on a promoter. Overexpression studies indicate that *LHCB* and *CAT3* mRNA levels are higher in CCA1-ox plants compared to wild type, suggesting that CCA1 acts as a positive regulator of the expression of the 2 genes (Wang and Tobin, 1998). The same overexpressing lines show lower levels of *CCA1*, *LHY*, *TOC1*, and *PRR9* mRNAs, consistent with the repressive role of CCA1 and LHY on *TOC1* and *PRR9* expression and on their own expression (Alabadí et al., 2001; Harmer and Kay, 2003). In short, genes with a CBS can be either induced (*LHCB*) or repressed (*CCA1*, *LHY*) by CCA1 and LHY, while genes with an EE in their promoters can be repressed (*PRR9*) or induced (*CAT3*, *TOC1*). The lack of a clear picture might argue that the CBS and the EE are not the whole story and that the context of the promoter is very important as well, as with the E-box in Drosophila (Munoz and Baler, 2003).

A detailed analysis of the circadian phenotypes displayed by the *toc1-2* mutant again raises the question of potential redundancy among related genes. The *cca1 lhy* double mutant is arrhythmic, and the model of the Arabidopsis clock posits *TOC1* as a target of CCA1 and LHY. It would therefore be expected that the *toc1-2* mutant, which is a strong allele, would be arrhythmic under the same conditions. However, the *toc1-2* mutant is arrhythmic in red light and in the dark but is rhythmic in blue and white lights. In addition,

temperature entrainment is not affected in the *toc1-2* mutant, although it does exhibit the short-period characteristic of the mutant phenotype when released into continuous conditions (PAS and CRM, unpublished). Nevertheless, overexpression of TOC1 leads to arrhythmicity in the light and strongly suggests that TOC1 is a central clock component (Más et al., 2003a). It is therefore possible that the function of TOC1 may be partially filled by other genes and that these genes are responsible for the oscillations detected in blue light and in response to temperature entrainment.

As in the cases of CCA1 and ZTL, TOC1 is the founding member of a small family of 5 pseudo-response regulators (Matsushika et al., 2000). Like *TOC1*, the other 4 *PRR* genes lack the conserved aspartic acid found in classical response regulators and so are unlikely to function via a conventional phospho-relay. All 5 genes are rhythmically expressed. The 1st gene, *PRR9*, shows maximum accumulation of its mRNA in the middle of the subjective day and is induced by red light through phytochromes (Ito et al., 2003). Following *PRR9*, *PRR7*, *PRR5*, *PRR3*, and, finally, *PRR1/TOC1*, expression peaks in succession at ~2-h intervals (Matsushika et al., 2000). T-DNA insertion alleles of each *PRR* gene implicate them in circadian regulation. Mutations in *PRR3* and *PRR5* shorten the period of cotyledon movement, while mutations in *PRR7* cause a period lengthening. Interestingly, mutations in *PRR9* do not affect period length but instead affect the phase of cotyledon movement (Michael et al., 2003b). However, Eriksson et al (2003) found that loss of *PRR9* resulted in lengthened period of several rhythms. The circadian phenotypes of the single *prr* mutants are modest (period alterations of 1-1.5 h) compared to the period shortening (3-4 h) seen in *toc1-2* mutants. Redundancy among the 4 *PRRs* may partially account for this. In a phylogenetic tree, *PRR3* and *PRR7* cluster together, while *PRR5* and *PRR9* are found on another branch (Matsushika et al., 2000). However, the addictive phenotype of the *prr5prr9* double mutant indicates they are not redundant (Eriksson et al., 2003), which suggests that redundancy among the *PRRs* may not be deduced solely based on sequence similarity. Overexpression of TOC1 alone results in arrhythmicity, while overexpression of PRR3, PRR5, or PRR9 only modestly affects period length, phase, or amplitude of the rhythms (Matsushika et al., 2002; Sato et al., 2002). Additional characterization of the remaining members of the PRR family will await multiple loss of function and overexpression studies.

PLANTS, THE CIRCADIAN CLOCK, AND PHOTOPERIODISM

The adaptive significance of the circadian clock stems from its ability to specifically regulate the expression of key genes at the times of the day when they are most needed. From microarray analysis, about 6% of the Arabidopsis genome is under clock regulation, and important pathways for the life of the plant are coregulated (Harmer et al., 2000).

One aspect of plant physiology that is greatly influenced by the clock is the response to photoperiod. Only in Arabidopsis has a mechanism for photoperiodic sensing been described at the molecular level, and it is centered on the regulation of the gene *CONSTANS* (*CO*) by the clock (Searle and Coupland, 2004; Yanovsky and Kay, 2003). One of the best-studied manifestations of photoperiodism is the flowering response. Arabidopsis is a facultative long-day plant, meaning that long days accelerate flowering, although the plant will eventually flower in short days. Genetic analysis has identified more than 80 genes important in the flowering process. A subset of these genes specifically affects the promotion of flowering in long days and defines the so-called long-day (photoperiodic) pathway. Mutants lacking the clock components *CCA1*, *LHY*, and *TOC1* show altered flowering time, providing strong genetic proof of the central role of the circadian clock in photoperiodism. Mutants affected in a second class of genes (e.g., *PHYA*, *PHYB*, *CRY2*, *ELF3*) that function in the light signal transduction pathways leading into the clock also display altered flowering time. Most interesting, however, is a third class represented by *CO*. CO positively regulates expression of the flowering inducers FLOWERING LOCUS T and SUPPRESSOR OF CONSTANS OVEREXPRESSION 1. *CO* expression peaks around ZT 16 in long days (16L:8D) and around ZT 20 in short days (8L:16D). The timing of the peak in *CO* mRNA relative to the timing of lights-off is very important, as it dictates how much CO protein will accumulate at the end of the light part of the day (Imaizumi et al., 2003; Roden et al., 2002; Suárez-López et al., 2001; Yanovsky and Kay, 2002). Under short-day conditions, very little CO protein accumulates, as it is degraded via the proteasome by an unidentified darkness-specific factor (Valverde et al., 2004). Under long days, however, the levels of CO protein are much higher because the blue-light photoreceptors CRY1 and CRY2 and far-red-light photoreceptor PHYA stabilize CO and allow it to accumu-

late and induce its downstream targets, which will lead to flowering. This represents by far the best description of a photoperiod-sensing mechanism and is consistent with an external coincidence model (Searle and Coupland, 2004; Yanovsky and Kay, 2003). Flowering will only occur once the expression of *CO* and the phase of the LD cycles from the environment coincide, allowing accumulation of CO protein, which in turn activates the floral inducers.

CONCLUDING REMARKS

The Arabidopsis circadian system has emerged as another example of a negative feedback loop. Consistent with other clock systems, posttranscriptional regulation by phosphorylation and proteasomal degradation play essential roles. The identification of TOC1, a pseudo-response regulator, as one component of the loop suggests that a mechanism distinct from those seen in the other model systems, perhaps more related to cyanobacterial clocks, must be in place in plants. Although the core loop largely is in view, there is still time to add more complexity.

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REFERENCES

Alabadí D, Oyama T, Yanovsky MJ, Harmon FG, Más P, and Kay SA (2001) Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. Science 293:880-883.

Alabadí D, Yanovsky MJ, Más P, Harmer SL, and Kay SA (2002) Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. Curr Biol 12:757-761.

Anderson SL, Teakle GR, Martino-Catt SJ, and Kay SA (1994) Circadian clock– and phytochrome-regulated transcription is conferred by a 78 bp *cis*-acting domain of the *Arabidopsis CAB2* promoter. Plant J 6:457-470.

Andersson CR, Harmer SL, Schultz TF, and Kay SA (1999) The Reveille (RVE) family of DNA binding proteins and the circadian clock [abstract 1-1]. 10th International Conference on Arabidopsis Research; 4-8 July 1999; Australia, University of Melbourne.

Aronson BD, Johnson KA, Loros JJ, and Dunlap JC (1994) Negative feedback defining a circadian clock: Autoregulation of the clock gene *frequency*. Science 263:1578-1584.

Bargiello T, Jackson F, and Young M (1984) Restoration of circadian behavioural rhythms by gene transfer in Drosophila. Nature 312:752-754.

Carré IA and Kay SA (1995) Multiple DNA-protein complexes at a circadian-regulated promoter element. Plant Cell 7:2039-2051.

de Mairan J (1729) Observation botanique. Hist Acad Roy Sci 35-36.

Dodd AN, Parkinson K, and Webb AAR (2004) Independent circadian regulation of assimilation and stomatal conductance in the *ztl-1* mutant of *Arabidopsis*. New Phytol 162:63-70.

Eriksson ME, Hanano S, Southern MM, Hall A, and Millar AJ (2003) Response regulator homologues have complementary, light-dependent functions in the *Arabidopsis* circadian clock. Planta 218:159-162.

Feldman JF and Hoyle M (1973) Isolation of circadian clock mutants of *Neurospora crassa*. Genetics 75:605-613.

Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, and Putterill J (1999) *GIGANTEA*: A circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several membrane-spanning domains. EMBO J 18:4679-4688.

Green RM and Tobin EM (1999) Loss of the circadian clock-associated protein 1 in *Arabidopsis* results in altered clock-regulated gene expression. Proc Natl Acad Sci U S A 96:4176-4179.

Hall A, Bastow RM, Davis SJ, Hanano S, McWatters HG, Hibberd V, Doyle MR, Sung S, Halliday KJ, Amasino RM, et al. (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, and Millar AJ (2002) Distinct regulation of *CAB* and *PHYB* gene expression by similar circadian clocks. Plant J 32:529-537.

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, and Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110-2113.

Harmer SL and Kay SA (2003) *Cis*- and *trans*-acting factors controlling clock-regulated gene expression [abstract 428]. 14th International Conference on Arabidopsis Research; http://www.arabidopsis.org/news/events.jsp.

He Q, Cheng P, Yang Y, He Q, Yu H, and Liu Y (2003) FWD1-mediated degradation of FREQUENCY in *Neurospora* establishes a conserved mechanism for circadian clock regulation. EMBO J 22:4421-4430.

He Q, Cheng P, Yang Y, Wang L, Gardner KH, and Liu Y (2002) White Collar–1, a DNA binding transcription factor and a light sensor. Science 297:840-843.

Hicks KA, Albertson TM, and Wagner DR (2001) *EARLY FLOWERING 3* encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. Plant Cell 13:1281-1292.

Hicks KA, Millar AJ, Carré IA, Somers DE, Straume M, Meeks-Wagner DR, and Kay SA (1996) Conditional cir-

cadian dysfunction of the *Arabidopsis early-flowering 3* mutant. Science 274:790-792.

Huq E, Tepperman JM, and Quail PH (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. Proc Natl Acad Sci U S A 97:9654-9658.

Hwang I, Chen H-C, and Sheen J (2002) Two-component signal transduction pathways in Arabidopsis. Plant Physiol 129:500-515.

Imaizumi T, Tran HG, Swartz TE, Briggs WR, and Kay SA (2003) FKF1 is essential for photoperiodic-specific light signalling in *Arabidopsis*. Nature 426:302-306.

Ito S, Matsushika A, Yamada H, Sato S, Kato T, Tabata S, Yamashino T, and Mizuno T (2003) Characterization of the APRR9 pseudo-response regulator belonging to the APRR1/TOC1 quintet in *Arabidopsis thaliana*. Plant Cell Physiol 44:1237–1245.

Jarillo JA, Capel J, Alonso JM, Ecker JR, Martinez-Zapater JM, and Cashmore AR (2002) Understanding the roles of the ZTL/ADO1 and ZTL-like proteins (LKP2/ADO2 and FKF1/ADO3) in the control of the circadian rhythms in Arabidopsis [abstract 9-01]. 13th International Conference on Arabidopsis Research; 28 June to 2 July 2002; Seville, Spain.

Kenigsbuch D and Tobin EM (1995) A region of the *Arabidopsis Lhcb1*3* promoter that binds to CA-1 activity is essential for high expression and phytochrome regulation. Plant Physiol 108:1023-1027.

Kim JY, Song HR, Taylor BL, and Carré IA (2003) Light-regulated translation mediates gated induction of the *Arabidopsis* clock protein LHY. EMBO J 22:935-944.

Kim WY, Geng R, and Somers DE (2003) Circadian phase-specific degradation of the F-box protein ZTL is mediated by the proteasome. Proc Natl Acad Sci U S A 100:4933-4938.

Kloppstech K (1985) Diurnal and circadian rhythmicity in the expression of light-induced nuclear messenger RNAs. Planta 165:502-506.

Ko HW, Jiang J, and Edery I (2002) Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. Nature 420:673-678.

Konopka R and Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68:2112-2116.

Kurup S, Jones HD, and Holdsworth MJ (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J 21:143-155.

Makino S, Matsushika A, Kojima M, Yamashino T, and Mizuno T (2002) The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: I. Characterization with APRR1-overexpressing plants. Plant Cell Physiol 43:58-69.

Martínez-García JF, Huq E, and Quail PH (2000) Direct targeting of light signals to a promoter element-bound transcription factor. Science 288:859-863.

Más P, Alabadí D, Yanovsky MJ, Oyama T, and Kay SA (2003a) Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. Plant Cell 15:223-236.

Más P, Kim WY, Somers DE, and Kay SA (2003b) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature 426:567-570.

Matsushika A, Imamura A, Yamashino T, and Mizuno T (2002) Aberrant expression of the light-inducible and circadian-regulated *APRR9* gene belonging to the circadian-associated APRR1/TOC1 quintet results in the phenotype of early flowering in *Arabidopsis thaliana*. Plant Cell Physiol 43:833-843.

Matsushika A, Makino S, Kojima M, and 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.

McClung CR, Fox BA, and Dunlap JC (1989) The *Neurospora* clock gene *frequency* shares a sequence element with the *Drosophila* clock gene *period*. Nature 339:558-562.

McClung CR and Kay SA (1994) Circadian rhythms in *Arabidopsis thaliana*. In *Arabidopsis*, Meyerowitz EM and Somerville CR, eds, pp 615-637. Plainview, NY: Cold Spring Harbor Laboratory Press.

McWatters HG, Bastow RM, Hall A, and Millar AJ (2000) The *ELF3 zeitnehmer* regulates light signalling to the circadian clock. Nature 408:716-720.

Michael TP and McClung CR (2002) Phase-specific circadian clock regulatory elements in *Arabidopsis thaliana*. Plant Physiol 130:627-638.

Michael TP, Salomé PA, and McClung CR (2003a) Two *Arabidopsis* circadian oscillators can be distinguished by differential temperature sensitivity. Proc Natl Acad Sci U S A 100:6878-6883.

Michael TP, Salomé PA, Yu HJ, Spencer TR, Sharp EL, Alonso JM, Ecker JR, and McClung CR (2003b) Enhanced fitness conferred by naturally occurring variation in the circadian clock. Science 302:1049-1053.

Millar AJ, Carré IA, Strayer CA, Chua NH, and Kay SA (1995a) Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science 267:1161-1163.

Millar AJ and Kay SA (1991) Circadian control of *cab* gene transcription and mRNA accumulation in *Arabidopsis*. Plant Cell 3:541-550.

Millar AJ, Short SR, Chua NH, and Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell 4:1075-1087.

Millar AJ, Straume M, Chory J, Chua NH, and Kay SA (1995b) The regulation of circadian period by phototransduction pathways in *Arabidopsis*. Science 267:1163-1166.

Mizoguchi T, Wheatley K, Hanzawa Y, Wright L, Mizoguchi M, Song HR, Carré IA, and Coupland G (2002) *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. Devel Cell 2:629-641.

Munoz E and Baler R (2003) The circadian E-box: When perfect is not good enough. Chronobiol Int 20:371-388.

Oda A, Fujiwara S, Kamada H, Coupland G, and Mizoguchi T (2004) Antisense suppression of the *Arabidopsis PIF3* gene does not affect circadian rhythms but causes early flowering and increases *FT* expression. FEBS Lett 557:259-264.

Panda S, Hogenesch JB, and Kay SA (2002) Circadian rhythms from flies to human. Nature 417:329-335.

Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, and Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. Science 285:1579-1582.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, and Schibler U (2002) The orphan nuclear receptor REV-ERB controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251-260.

Putterill J, Milich R, and David K (2002) The circadian regulated *GIGANTEA* gene and photoperiodic flowering [abstract 9-29]. 14th International Conference on Arabidopsis Research; http://www.arabidopsis.org/news/events.jsp.

Reddy P, Zehring WA, Wheeler DA, Pirotta V, Hadfield C, Hall JC, and Rosbach M (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell 38:701-710.

Roden LC, Song HR, Jackson S, Morris K, and Carré IA (2002) Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. Proc Natl Acad Sci U S A 99:13313-13318.

Romero I, Fuertes A, Benito MJ, Malpica JM, Leyva A, and Paz-Ares J (1998) More than 80 *R2R3-MYB* regulatory genes in the genome of *Arabidopsis thaliana*. Plant J 14: 273-284.

Sato E, Nakamichi N, Yamashino T, and Mizuno T (2002) Aberrant expression of the *Arabidopsis* circadian-regulated *APRR5* gene belonging to the APRR1/TOC1 quintet results in early flowering and hypersensitiveness to light in early photomorphogenesis. Plant Cell Physiol 43:1374-1385.

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J, Carré IA, and Coupland G (1998) *LATE ELONGATED HYPOCOTYL*, an *Arabidopsis* gene encoding a MYB transcription factor, regulates circadian rhythmicity and photoperiodic responses. Cell 93:1219-1229.

Schultz TF, Kiyosue T, Yanovsky M, Wada M, and Kay SA (2001) A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell 13:2659-2670.

Searle I and Coupland G (2004) Induction of flowering by seasonal changes in photoperiod. EMBO J 23:1217–1222.

Somers DE, Kim WY, and Geng R (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell 16:769-782.

Somers DE, Schultz TF, Milnamow M, and Kay SA (2000) ZEITLUPE encodes a novel clock-associated PAS protein from *Arabidopsis*. Cell 101:319–329.

Somers DE, Webb AAR, Pearson M, and Kay SA (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development 125:485-494.

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Más P, Panda S, Kreps JA, and Kay SA (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. Science 289:768-771.

Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, and Coupland G (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature 410:1116-1120.

Sugano S, Andronis C, Green RM, Wang ZY, and Tobin EM (1998) Protein kinase CK2 interacts with and phosphorylates the *Arabidopsis* circadian clock-associated 1 protein. Proc Natl Acad Sci U S A 95:11020-11025.

Sugano S, Andronis C, Ong MS, Green RM, and Tobin EM (1999) The protein kinase CK2 is involved in regulation of circadian rhythms in *Arabidopsis*. Proc Natl Acad Sci U S A 96:12362-12366.

Sun L, Doxsee RA, Harel E, and Tobin EM (1993) CA-1, a novel phosphoprotein, interacts with the promoter of the *cab140* gene in *Arabidopsis* and is undetectable in *det1* mutant seedlings. Plant Cell 5:109-121.

Suri V, Lanjuin A, and Rosbash M (1999) TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock. EMBO J 18:675-686.

Thain SC, Murtas G, Lynn JR, McGrath RB, and Millar AJ (2002) The circadian clock that controls gene expression in Arabidopsis is tissue specific. Plant Physiol 130:102-110.

Toledo-Ortiz G, Huq E, and Quail PH (2003) The Arabidopsis basic/helix-loop-helix transcription factor family. Plant Cell 15:1749-1770.

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, and Coupland G (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science 303:1003-1006.

Wang ZY, Kenigsbuch D, Sun L, Harel E, Ong MS, and Tobin EM (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis *Lhcb* gene. Plant Cell 9:491-507.

Wang ZY and Tobin EM (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. Cell 93:1207-1217.

Yanovsky MJ and Kay SA (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. Nature 419:308-312.

Yanovsky MJ and Kay SA (2003) Living by the calendar: How plants know when to flower. Nat Rev Mol Cell Biol 4:265-276.

Zeng H, Hardin PE, and Rosbash M (1994) Constitutive overexpression of the *Drosophila* period protein inhibits *period* mRNA cycling. EMBO J 13:3590-3598.