Circadian clock signaling in *Arabidopsis thaliana*: from gene expression to physiology and development

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ABSTRACT  The daily rotation of the earth on its axis leads to predictable periodic fluctuations of environmental conditions. Accordingly, most organisms have evolved an internal timing mechanism, the circadian clock, which is able to recognize these 24-hour rhythmic oscillations. In plants, the temporal synchronization of physiology with the environment is essential for successful plant growth and development. The intimate connection between light signaling pathways and the circadian oscillator allows the anticipation of the environmental transitions and the measurement of day-length as an indicator of changing seasons. In recent years, significant advances have been made in the genetic and molecular dissection of the plant circadian system, mostly in *Arabidopsis thaliana*. The overall plant clock organization is highly complex; the system seems to include several input pathways, tightly regulated central oscillators and a myriad of outputs. The molecular cloning and characterization of a number of clock components has greatly improved our view of the plant central oscillator and additional players will most likely come into place very soon. Molecular mechanisms underlying circadian clock function are also beginning to be characterized. The emerging model relies on negative feedback loops at the core of the oscillator. Additional levels of post-transcriptional and post-translational regulation also contribute to the generation and maintenance of the rhythms. Globally, these studies have shed new light on how the clock coordinates plant physiology and development with the daily and seasonal environmental cycles.

KEY WORDS: biological clock, circadian rhythms, *Arabidopsis thaliana*

The biological clock entails a 24-hour rhythm on biochemical and physiological processes such that they occur at specific, advantageous times of the day-night cycle (Dunlap, 1999, Young and Kay, 2001). The circadian movements of leaves and flowers, the fragrance emission, the stomatal opening, hypocotyl expansion or the photoperiodic control of flowering time are some examples of processes tightly regulated by the plant clockwork (Barak *et al.*, 2000, McClung, 2001). Underlying all these physiological rhythms are endogenous circadian oscillations of gene expression. Indeed, genomic approaches have identified in *Arabidopsis* hundreds of genes under clock control, with peaks of expression at all phases of the day/night cycle (Harmer *et al.*, 2000, Schaffer *et al.*, 2001). Underlying all these physiological rhythms are endogenous circadian oscillations of gene expression. Indeed, genomic approaches have identified in *Arabidopsis* hundreds of genes under clock control, with peaks of expression at all phases of the day/night cycle (Harmer *et al.*, 2000, Schaffer *et al.*, 2001). Although the rhythmic oscillations persist in constant conditions (i.e. in the absence of environmental transitions) the circadian clock does not run in isolation from the environment. The clock includes a resetting mechanism by which it is synchronized each day to the correct time (Devlin and Kay, 2001). The presence of an endogenous timing system provides an adaptive advantage, enabling the anticipation of the environmental transitions and the temporal coordination of physiological events to occur at specific phase relationships with the environment (Johnson, 2001). Thus, the circadian clock can be considered as an internal processor of environmental signals (such as light and temperature) that coordinates the appropriate timing of metabolic and developmental activities in the plant (Harmer *et al.*, 2001, McClung, 2001).

Classically, the circadian system has been divided into three main components: the *input pathways* involved in the perception and transmission of environmental signals to synchronize the *central oscillator* or pacemaker that generates and maintains rhythmicity through multiple *output pathways* that connect the oscillator to physiology and metabolism (Figure 1). Clearly, this is an oversimplified conceptual model of the clock. Several lines of evidence reveal the existence of a far more complicated circadian system, with output elements modulating the pace of the oscillator and input elements being themselves tightly controlled by the clock. The existence of different free-running periods in the expression of diverse clock outputs is indicative of separate
The core players in the *Arabidopsis* circadian system

The identification of molecular components in the circadian system was facilitated by the use of plants expressing the promoter of the clock-controlled gene *LHCb* or *CAB* (light-harvesting chlorophyll a/b protein) fused to the firefly *LUCIFERASE* (*LUC*). The *CAB* promoter drives a robust rhythm of bioluminescence that can be monitored with a photon-counting video camera (Millar et al., 1995). Mutagenesis of these transgenic plants followed by bioluminescence analysis of abnormal CAB rhythms resulted in the isolation of a number of circadian period mutants. One of these mutants, the *timing of CAB expression 1* (*toc1-1*), displayed a shorter than wild-type circadian period not only for *CAB:LUC* expression but also for other circadian outputs under a wide range of temperature and light conditions (Millar et al., 1995). The *toc1-1* mutant plants also exhibited a day-length-insensitive, early-flowering phenotype, under cycles of 24 h, but not under 21 h environmental cycles (which match the endogenous period of this mutant (Strayer et al., 2000). This indicates that the photoperiodic insensitivity is due to the improper functioning of the clock caused by the *toc1-1* mutation. Cloning of the gene revealed that TOC1 encoded a nuclear protein containing a receiver domain similar to the one found in plant response regulators (Strayer et al., 2000). However, the conserved phospho-accepting aspartate residues present in *bona fide* response regulators is missing in TOC1 (also denominated pseudo-response regulator), suggesting that it does not function in a canonical phosphor-relay mechanism (Mizuno, 2004). In addition, TOC1 contains a distinctive COOH-terminal motif (CCT motif) which is conserved within the CONSTANS (CO) family of plant transcription factors (Strayer et al., 2000). Analysis of TOC1 expression revealed that the mRNA rhythmically cycled and participated in a negative feedback loop mechanism to control its own expression (Strayer et al., 2000). Together, these observations led to the idea that TOC1 was an important component of the core of the oscillator, rather than part of the light input pathway to the clock. However, a detailed characterization of TOC1 function using RNAi plants and a strong allele of *TOC1* (*toc1-2*) provided evidence of unpredicted roles for TOC1 in the control of circadian and photo-morphogenic responses (Más et al., 2003a). These studies showed that silencing of the *TOC1* gene caused arrhythmia in constant darkness and in various intensities of red light. In addition, *TOC1* RNAi and *toc1-2* mutant plants displayed an important reduction in sensitivity to red and far-red light in the control of hypocotyl elongation whereas increments in *TOC1* gene dosage clearly enhanced light sensitivity (Más et al., 2003a). Thus, based on these studies, it was postulated a new role for TOC1 in the integration of light signals from phytochromes to clock outputs, controlling circadian gene expression and other light-dependent developmental processes in the plant.

Two transcription factors, CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), with a single MYB-like domain, have also been closely associated with the plant circadian system (Schaffer et al., 1998, Wang and Tobin, 1998). CCA1 was identified as a binding factor to a region of the promoter of *Arabidopsis LHCb* gene. These initial studies indicated that CCA1 protein was an important element in the functioning of the phytochrome signal transduction leading to increased transcription of the *LHCb* gene (Wang et al., 1997). Later studies also revealed an additional role of CCA1 in the circadian clockwork. Loss of function *cca1* mutants displayed a shorter than wild-type period of genes expressed at different circadian times while CCA1 constitutive over-expression clearly disrupted rhythmicity in various clock outputs including hypocotyl elongation, leaf movements and circadian gene expression (Green and Tobin, 1999, Wang and Tobin, 1998).

The *lhy* mutation was caused by the insertion of a transposon (Ds) within the 5′ untranslated region (5′UTR) of the *LHY* gene, causing its over-expression. Studies of these LHY overexpressing plants as well as loss of function *lhy* mutants showed clear alterations in circadian rhythmicity for leaf move-
ment and for the circadian regulation of gene expression. Both CCA1 and LHY transcripts rhythmically oscillate, with a peak of expression early in the morning, shortly after dawn. The rhythms persist in constant conditions (constant light and constant darkness) indicating that both genes are under circadian control (Schaffer et al., 1998, Wang and Tobin, 1998). The circadian expression of the LHY and CCA1 transcripts is abolished in lhy1 and CCA1-overexpressing (CCA1-ox) plants, suggesting that the rhythmic expression of LHY and CCA1 is required for normal circadian function. Interestingly, CCA1 and LHY regulated their own and each other’s expression. Over-expressing plants of either gene exhibited arrhythmic expression of the endogenous LHY and CCA1 transcripts. Furthermore, the expression of both endogenous genes was repressed to levels similar to trough in wild-type plants. These results suggested that LHY and CCA1 might function redundantly as components of a negative feedback loop (Schaffer et al., 1998, Wang and Tobin, 1998). Indeed, recent analysis comparing phenotypes in single and double mutant lhy-cca1 plants indicated that the LHY and CCA1 genes are partially redundant and that they are required for maintenance of circadian rhythmicity in Arabidopsis (Alabadi et al., 2002, Mizoguchi et al., 2002).

More recently, another player has joined the clockwork team, in very close association with the circadian oscillator. ELF4 (EARLY-FLOWERING 4) which encodes a protein of 111 amino acids without predictable protein domains, was identified by its involvement in photoperiodic perception and circadian regulation (Doyle et al., 2002). Analysis of plants containing a T-DNA insertion in the ELF4 gene revealed an early flowering phenotype in short photoperiods while under long-day conditions, the mutant plants flowered at about the same time as wild-type (Doyle et al., 2002). The elf4 mutation reduced CCA1 expression and affected the rhythms of clock-controlled genes expressed at different circadian times. Their expression rapidly became arrhythmic under constant conditions, but individual seedlings were transiently rhythmic with highly variable periods. These results suggest a role for ELF4 in maintaining the accuracy of the rhythms. Consistent with the early flowering phenotype in short days, the elf4 mutation increased expression of CO, a gene that has a relevant role in floral induction (see below).

Synchronization with external time: light input to the clock

The earliest experimental evidence of the existence of an internal time-keeping mechanism came with the observations that the daily leaf movement of Mimosa plants continued even when plants were placed in constant darkness. These observations demonstrated that the environmental changes were not required to trigger the rhythmic response and revealed one of the main properties characterizing circadian rhythmicity: its ability to persist in the absence of environmental cues. In constant conditions, the period of the rhythms is approximately, but not exactly 24-hours. To generate an accurate 24-hours period, the endogenous oscillator must be synchronized with the external, environmental time (Johnson, 2001). Environmental transitions between dawn and dusk help to adjust the endogenous period of the clock to exactly match the 24-hour period that we find in nature (Devlin and Kay, 2001). As light is an essential environmental signal in the synchronization of the clock with the outside world, a long-standing goal in the plant circadian field has been the identification of the components in the signal transduction pathway responsible for resetting the clock. Studies of photoreceptor-deficient mutants crossed into the CAB::LUC plants provided evidence that two classes of photoreceptors, phytochromes (PHY) and cryptochromes (CRY), participated in the light-driven entrainment of the Arabidopsis clock (Somers et al., 1998a). Four of the five phytochromes identified in Arabidopsis (PHYA, PHYB, PHYD and PHYE) act additively in the red-light input to the clock while Arabidopsis CRYPTOCHROME 1 (CRY1) acts as a clock photoreceptor for high and low fluences of blue light and both CRY1 and CRY2 act redundantly at intermediate fluences of blue light (Devlin and Kay, 2000, Somers et al., 1998a). Mutations in cry2 were also shown to affect entrainment in white light conditions (Más et al., 2000) suggesting an interaction of CRY2 with the phytochrome signaling pathway. Indeed, using Fluorescent Resonance Energy Transfer (FRET) microscopy, PHYB and CRY2 were shown to interact in vivo, co-localizing in nuclear speckles in response to light treatments (Más et al., 2000). Surprisingly, cryptochrome mutant plants also showed altered entrainment under red light suggesting that CRY1 is required for PHYA signaling to the clock in both red and blue light (Devlin and Kay, 2000). Quadruple mutants lacking phyA, phyB, cry1 and cry2 maintained rhythmicity (Yanovsky et al., 2000), indicating that additional components participate in the light input pathway to reset the clock. Some possible candidates to perform this function are the gene family ZEITLUPE (ZTL), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) and LOV, KELCH PROTEIN 2 (LKP2). The genes encode proteins that contain six kelch repeats as well as a LOV and an F-box domain. The LOV domain is highly similar to the one found in the blue light photoreceptors NPH1 (Arabidopsis) and WHITE COLLAR 1 (Neurospora). The F-box is a motif found in proteins that act as adapters and bring specific substrates to ubiquitin protein ligase subunits for degradation (Craig and Tyers, 1999). The unique combination of these domains in the ZTL family of proteins suggests that they might mediate light-dependent protein degradation of critical clock components. Indeed, a recent report has shown TOC1 as one of the ZTL substrates (see below and Más et al., 2003b). Analysis of ztl mutant plants revealed a long period circadian phenotype that was dependent on light intensity (Somers et al., 2000) while over-expression of LKP2 resulted in arrhythmic expression of several clock-controlled genes (Schultz et al., 2001). The fkt1 mutant was initially identified by its late flowering phenotype (Nelson et al., 2000) and more recently, its role in the photoperiodic control of CO expression has been described (Imaizumi et al., 2003). The possible function of these proteins in photo-entrainment might result from their direct interaction with photoreceptors. Indeed, ZTL has been shown to interact in vitro with PHYB and CRY1 (Jarillo et al., 2001) although it remains to be determined the biological relevance of these interactions in vivo.

The central oscillator can be also entrained by temperature (warm/cold) cycles. This feature has been used to analyze whether circadian mutants affect specifically light signaling
input to the clock (Millar, 2004). A detailed phase response
curve to temperature was recently published (Michael et al.,
2003a) and new evidence suggest that the light- and tempera-
ture-sensitive systems in plants might be connected, since
photoreceptor signaling pathways can be temperature sensi-
tive (Halliday and Whitelam, 2003, Mazzella et al., 2000). In
spite of these new advances, we are far from understanding the
mechanisms of clock entrainment by temperature cues.

Additional genes: searching for functional roles

ELF3 (EARLY FLOWERING 3) is an important component of
the light signaling pathway to the clock. Initial studies showed
that elf3 mutants displayed arrhythmic clock output expression
in continuous light whereas rhythmicity was retained in con-
stant darkness, suggesting a defect in the light input pathway to
the clock (Hicks et al., 1996). In addition, the elf3 mutant plants
did not discriminate different photoperiods, flowering as early
in short days as in long days. In wild-type plants, CAF induction
is rhythmically repressed by the clock during the night, allowing
a higher induction during the light period of the day (Millar and
Kay, 1996). The circadian \textit{gating} of elf3, the circadian regulation of leaf movement and transcription
oscillations, suggesting a connection of GI with the light input to the
clock (Fowler et al., 1999, Park et al., 1999). Rhythmic expres-
sion of GI transcript was shown to be altered in gi mutants,
suggesting that GI could form a feedback loop required for
normal clock function (Fowler et al., 1999, Park et al., 1999). In
plants over-expressing CCA1 or LHY, the circadian expression of
GI was disrupted and reciprocally, the absence of GI caused a
reduction in the CCA1 and LHY expression. The identification
of interacting partners of GI might help to clarify GI function in
this puzzled network. Recently, a report showed the interaction
of SPINDLY (SPY), a negative regulator of gibberellin, with
GI (Tseng et al., 2004). The report describes their involvement
in light responses, flowering and rhythms in cotyledon move-
ments. A further gi allele was identified in a screen for alter-
ations in hypocotyl elongation under red light (Huq et al., 2000).
These studies revealed that GI was also associated with PHYB
function although the correlation of GI roles in photo-morpho-
genesis and in the light input to the clock remains to be
eclucidated.

Transcriptional feedback loops

Knowledge of circadian clock mechanisms in \textit{Arabidopsis thaliana} has been aided by the formulation of circadian function
in other circadian systems. The general mechanism of the
clockwork seems to be conserved among organisms and it is
based on negative feedback loops at the core of the oscillator
(Dunlap, 1999, Young and Kay, 2001). This common theme, at
its simplest, involves positive and negative components that
mutually regulate their rhythmic abundance and/or activity. The
oscillatory expression and regulation of clock components
generates circadian rhythms that are translated to multiple
clock outputs. While this mechanism has been firmly estab-
lished in \textit{D. melanogaster}, mammals and \textit{Neurospora crassa}
(Harmer et al., 2001), we are just beginning to ascertain clock
mechanisms in higher plants (Figure 2). In \textit{Arabidopsis}, the first
working model for the plant clockwork came with the observa-
tion of a reciprocal regulation between CCA1/LHY and TOC1
(Alabadí et al., 2001). The two MYB proteins (LHY and CCA1)
negatively regulated TOC1 expression that in turn positively
induced LHY/CCA1. According to this model, the MYB tran-
scription factors CCA1 and LHY might function as negative elements within this transcriptional loop, with a similar role to
the one described for PERIOD (PER) and TIMELESS (TIM) in
the circadian system of \textit{Drosophila}, or FREQUENCY (FRQ) in the
\textit{Neurospora} clock. TOC1 might work as a positive compo-
nent, similar to the clock components CYCLE (CYC) and
CLOCK (CLK) in \textit{Drosophila}, or WHITE COLLAR 1 and 2 (WC1
and WC2) in the circadian system of \textit{Neurospora}. The repres-
sion of TOC1 might take place by the direct binding of CCA1/
LHY proteins to a region in the TOC1 promoter (denominated
Decreasing CCA1/LHY levels throughout the day causes a
relieved in that repression, resulting in rising levels of TOC1
mRNA. The positive action of TOC1 on CCA1 and LHY
expression thereby reinitiate the oscillatory cycle. The TOC1
activation of CCA1/LHY is probably indirect because at least
three other components also positively participate in CCA1/
Additional levels of complexity are added to this regulatory loop since CCA1/LHY as well as TOC1 are members of multi-gene families. Several homologues of CCA1/LHY (denominated REVEILLES) have been shown to circadianly oscillate with a peak of expression around dawn (Andersson et al., 1999). These additional MYB proteins could form homo- and hetero-interactions among them that could contribute to the oscillatory loop. TOC1 is also a member of a gene family composed of 4 more components (denominated pseudo-response regulators, PRR 3, 5, 7 and 9). Phenotypic analysis of mutant and over-expressing plants of individuals PRR has revealed alterations of period, phase and/or amplitude in some overt rhythms, changes in flowering time and affected sensitivity to red-light control of hypocotyl elongation (Mizuno, 2004). PRR transcripts accumulate rhythmically in the order PRR9-PRR7-PRR5-PRR3-TOC1 with peak levels after dawn from 2 h (PRR9) to 10 h (TOC1, also known as PRR1) (Matsushika et al., 2000, Strayer et al., 2000). It is suggested that PRR participate in the light signaling within the circadian clock but are not required for rhythm generation (Mizuno, 2004).

Several studies have reported different circadian periods in various output genes suggesting regulation by multiple oscillators (Hall et al., 2002, Michael et al., 2003a, Thain et al., 2002). Based on their sensitivity to temperature, it was postulated the presence of two circadian clocks differentially controlling CAB and CAT3 expression in Arabidopsis (Michael et al., 2003a). The partial overlapping expression of these genes opens up the possibility of the co-existence of at least two oscillators in the same cell although further experiments will be required to unequivocally confirm this hypothesis.

Post-translational regulation of the plant clockwork

Protein phosphorylation has been shown to be necessary in the functioning of the circadian clock in Drosophila, Neurospora and in humans. Various studies reveal that casein kinases (CK1 and CK2) are the main protein kinases involved in the phosphorylation of essential clock components (Dunlap, 2004, Edery, 1999, Loros and Dunlap, 2001, Toh et al., 2001). In Arabidopsis, the protein kinase CK2 was shown to phosphorylate in vitro the clock-associated protein CCA1 (Sugano et al., 1998). Over-expression of a CK2 regulatory subunit (denominated CKB3) affected the regulation of circadian rhythmicity, shortening the period of expression of several output genes (Sugano et al., 1999). By examining the effects of a constitutively-expressing CCA1 mutant that could not be phosphorylated by CK2, it was demonstrated that CCA1 phosphorylation by CK2 is important for the normal functioning of the Arabidopsis circadian clock (Daniel et al., 2004).

Detailed studies of LHY over-expressing plants showed that in constant light conditions, circadian rhythmicity was abolished although rhythmic expression was preserved under light-dark conditions. The Arabidopsis circadian clock

Fig. 2. Current molecular model of circadian clock signaling in the Arabidopsis. Light signals are perceived by a set of multiple photoreceptors, including phytochromes (PHYs) and cryptochromes (CRYs), that participate in the resetting of the clock. Light effects on the clock are also modulated by the action of both positive (GI) and negative light-signaling components (ELF3). At the core of the oscillator, a feedback loop generates rhythmicity by the reciprocal regulation between CCA1/LHY and TOC1/ELF4. CCA1 phosphorylation by CK2 is important for the normal functioning of the Arabidopsis circadian clock. The ZTL family (ZTL, FKF1 and LKP2) mediates between photoreceptors and the circadian clock. The targeted degradation of TOC1 protein by ZTL is essential in the control of circadian period by the clock. In the scheme, some of the genes and the physiological/metabolic processes regulated by the clock are represented: photosynthetic processes (CAB), lipid metabolism (CHS, CHALCONE SYNTHASE), cell elongation (EXP, EXPANSIN) and flowering-time regulation (CO). The transcription of CO is regulated by FKF1 and GI, whose transcription in turn is under the control of the circadian clock. The clock-controlled regulation of gene expression enables plants to anticipate and adapt to periodic changes in the environment. The gray shaded circle represents the feedback loop that is central for clock function in Arabidopsis; dotted arrows indicate gene expression regulated by the clock.
cycles (Kim et al., 2003a). The rhythmic oscillations were correlated with light-induced regulation of LHY translation, causing high amplitude changes in LHY protein levels. The authors suggested that both translational induction and transcriptional repression of LHY at dawn contribute to the robustness and accuracy of the circadian oscillations.

Recent genetic and molecular evidence have also revealed that a precise post-translational regulation of the TOC1 protein is essential for clock function in Arabidopsis. The F-box protein ZEITLUPE (ZTL) was shown to be involved in the targeted degradation of TOC1 (Más et al., 2003b). In these studies, TOC1’s physical interaction with ZTL was abolished by the ztl-1 mutation resulting in constitutive levels of TOC1 protein expression. The proteosome-mediated degradation of TOC1 protein occurs mainly during the dark period and requires a functional ZTL. It was demonstrated that the TOC1-ZTL interaction was important in the control of TOC1 protein stability and responsible for the accurate regulation of circadian period by the clock (Más et al., 2003b). The abundance of ZTL protein is also regulated by the proteosome. ZTL is more rapidly degraded at dawn when the protein reaches trough levels (Kim et al., 2003b). Post-translational regulation of protein stability through the proteosome pathway has been shown to be essential in clock function in the mammalian system and in Drosophila (Grima et al., 2002, Ko et al., 2002, Yagita et al., 2002). Together, these results suggest that tightly regulated protein degradation by the proteosome might be a conserved aspect in the regulation of the eukaryotic circadian clocks.

Clock control of output gene expression

Global analysis of clock-controlled gene expression is a useful tool for identifying new components of the circadian system and finding co-expressed genes integrated into common metabolic or physiologic pathways. The use of oligonucleotide microarrays has provided new insights into global transcription networks regulated by the Arabidopsis clock (Harmer et al., 2000, Schaffer et al., 2001). Clustering based on functional features of these genes has revealed how the clock enables plants to anticipate and adapt to the daily and seasonal fluctuations of light and temperature. A large set of photosynthetic genes were shown to be under clock control. All these genes exhibited a co-regulated expression, peaking around midday. Nine genes involved in energetically demanding processes such as nitrogen assimilation were expressed early in the day, when energy levels are increased by light harvesting. The expression of 23 genes encoding enzymes involved in the biosynthesis of photo-protective pigments was shown to peak coordinately before dawn. This pattern of expression might help to protect plants from the damaging effects of UV-B light. Another cluster of coordinately expressed genes was represented by a group involved in lipid modification which has been shown to oscillate and correlate with chilling tolerance. These genes have a peak of expression when most needed, just before dusk. These results illustrate the importance of the clock in compartmentalizing metabolic events to occur rhythmically at most advantageous time of the day (Harmer et al., 2000, Schaffer et al., 2001). The current challenge is the follow-up of all these data to unravel the clock-controlled gene regulatory networks and their connection to physiology and development.

Microarray experiments combined with computational analysis have also helped to identify common motifs in the upstream regions of co-regulated genes (Harmer et al., 2000, Schaffer et al., 2001). A nine base-pair sequence, denominated EVENING ELEMENT (EE) was found to be over-represented in a cluster of genes, whose expression peaked at the end of the day. Mutational analysis confirmed the importance of the EE in the oscillatory expression of COLD, CIRCADIAN RHYTHM 2 (CCR2) and in CATALASE 3 (CAT3) (Harmer et al., 2000, Michael and McClung, 2002). A region of the CCR2 promoter containing the EE was shown to confer circadian rhythmicity to the β-GLUCORONIDASE reporter gene. The sequence of the EE (AAAAATATCT) is identical to the one recognized in the TOC1 promoter by the transcriptional repressors CCA1 and LHY. The sequence is also highly similar to the one present in the CAB promoter where CCA1 binds acting as a positive regulator. These results suggest that CCA1 and LHY might control the expression of genes with opposite phases.

Circadian regulation of plant growth

The regulation of hypocotyl length is controlled by environmental signals (light and temperature) as well as by endogenous signaling pathways (e.g. gibberellic acid, brassinosteroids, ethylene, abscisic acid, cytokinins). In constant light conditions, the pattern of hypocotyl growth displays rhythmic pauses near subjective dawn with a rapid elongation at subjective dusk. This rhythmicity is entrained by light-dark cycles and its period was shown to be shortened in the toc1-1 mutant, indicating that it is controlled by the circadian clock. TOC1 RNAi, toc1-2 mutants and mutants that over-express either CCA1 or LHY exhibit long hypocotyl phenotypes. Mutations in several other clock-associated components (such as ZTL, FKF1, ELF3, ELF4, GI, SRR1) also cause both circadian and hypocotyl-length phenotypes. The
altered hypocotyl length in these clock mutants was shown to be
due to severe circadian defects on the regulation of cell expansion
(Dowson-Day and Millar, 1999). Interestingly, the correlation
between the clock and hypocotyl length is not absolute; the
toc1-1 mutant exhibits a short circadian period but does not have a
hypocotyl phenotype. The fact that toc1-1 mutant plants shorten
multiple circadian outputs but still have wild-type hypocotyl length
suggests that hypocotyl elongation during seedling de-etiolation
could be controlled by different routes, dependent and indepen-
dent of the clock. toc1-1 represents a mutation with affected clock
function but with normal light-mediated de-etiolation response
independent of the clock.

The role of the clock in the regulation of hypocotyl growth has
been proposed to be based on the "gating" of the light signaling
pathways in a similar way to the gated induction of CAB expres-
sion. When the gate is closed, the light signaling pathways are
inhibited, even in the presence of light. This repression leads to
the growth of hypocotyls. When the gate is open, the clock allows
the light input signals, inhibiting hypocotyl elongation. As
described above, ELF3 has an essential function in controlling this
gating mechanism. Accordingly, loss-of-function elf3 mutant
alleles result in a loss of rhythmic hypocotyl growth.

Photoperiodic regulation of flowering time: a role for
the circadian clock

A crucial step of the plant life cycle is the transition from a
vegetative stage to a reproductive mode (Mouradov et al., 2002,
Searle and Coupland, 2004). Initiation of flowering occurs as a
response to a number of environmental signals, including sea-
sonal changes in day-length (Hayama and Coupland, 2004). The
role of the circadian clock in the photoperiodism is illustrated by
the fact that several mutants identified in Arabidopsis on the basis
of their defective photoperiodic regulation of flowering also dis-
play altered circadian rhythms (e.g. ELF3, Hicks et al., 1996; LHY,
Schaffer et al., 1998; Gl, Fowler et al., 1999, Park et al., 1999;
ELF4, Doyle et al., 2002). Mutants originally isolated for their
circadian defects, such as TOC1 (Millar et al., 1995, Somers et al.,
1998b, Strayer et al., 2000) and ZTL (Somers et al., 2000), also
showed a reduced sensitivity to day-length. Recently, an impor-
tant progress has been made in the molecular understanding the
circadian clock function in the photoperiodic control of flowering
time. These studies set up the basis for a molecular understand-
ing of the interactions between the circadian clock and the
developmental control of flowering (Hayama and Coupland, 2004,
Yanovsky and Kay, 2003). One of the key genes mediating the
transition to flowering in Arabidopsis is CONSTANS (CO)
(Putterill et al., 1995). Analysis of CO expression reveals a
rhythmic oscillation of both transcript and protein (Hayama and
Coupland, 2003). An early flowering phenotype is observed in CO
over-expressing plants although no circadian phenotypes are
identified, indicating that CO is a clock output and mediates
between the circadian system and initiation of flowering (Hayama
and Coupland, 2003). The gene encodes a nuclear protein that
contains a CCT motif (also found in TOC1 and in CO-like genes)
and two B-box type zinc-finger domains, believed to mediate
protein-protein interaction. Under long days, the peak of CO
expression is at the end of the day and during the night. A different
pattern is observed under short-day conditions with a majority of
CO expression during the night (Figure 3). The coincidence of
higher levels of CO with the light period has been proposed to be
essential for the induction of flowering in long-days (Suárez-
López et al., 2001). The mechanism of CO action seems to be
related with its involvement in FLOWERING LOCUS T (FT)
activation. FT is an essential gene triggering flowering in
Arabidopsis and its expression has been tightly linked to the
presence of active CO (Kardailsky et al., 1999, Kobayashi et al.,
1999, Samach et al., 2000). The importance of CO phase of
expression has been demonstrated in the toc1-1 mutant (with a
short circadian period of 21 h). CO expression in this mutant is
phase-shifted such that expression occurs during the light period
in both long- and short-days. This pattern of CO expression
results in a photoperiodic-insensitive early flowering. However,
the photoperiodic control is restored when the toc1-1 mutant
plants are grown under light-dark cycles that match toc1-1 endog-
ous period of 21 hours (Yanovsky and Kay, 2002). These
studies reveal the importance of the clock in controlling the phase
of CO expression. The coincidence of light with the FT activation
by CO is a key event in the photoperiodic control of flowering time
(Roden et al., 2002, Yanovsky and Kay, 2003) and two photore-
ceptors, PHYA andCRY2, have been shown to be involved in this
process (Yanovsky and Kay, 2002).

Recent studies have also provided new insights into the
mechanism that generates the diurnal pattern of CO transcrip-
tion. In the fKF1 mutant, the high levels of CO mRNA are strongly
reduced and the daytime CO peak is abolished. Under long-days,
FKF1 protein levels exhibit a diurnal pattern with a peak in the late
day. However, under short-days the peak protein expression
occurs during the early to mid nighttime, with low FKF1 protein
levels during the day (Iizumizumi et al., 2003). A model was
proposed suggesting that under long-days, high levels of FKF1
protein and its direct activation by light occur simultaneously,
generating the daytime peak of CO mRNA.

Analysis of CO over-expressing plants revealed that CO pro-
tein accumulates under continuous white light, whereas CO
levels are strongly reduced in constant darkness (Valverde et al.,
2004). The dark-dependent instability of CO protein is due to an
active degradation of CO protein by the proteosome. Further-
more, CO protein accumulates to high levels in continuous blue
and far-red light, while the protein is rapidly degraded under
constant red light. Under long-day conditions, CO protein shows
a strong peak at the end of the day, whereas under short-days the
protein displays a much weaker peak of expression at the early
nighttime (Valverde et al., 2004). The regulation of CO protein
abundance during the day seems to be mediated by PHYB, which
decreases CO protein levels early in the morning. In contrast,
PHYA andCRYs stabilize CO protein at the end of the day (Figure
3). These studies led to the conclusion that PHYB promotes CO
protein degradation and antagonizes the action of PHYA and
CRYs in the morning (Hayama and Coupland, 2004).

Gene homologous to CO and FT have been identified in many
species suggesting a conservation in the components of the
Arabidopsis photoperiod pathway (Liu et al., 2001a, Yano et al.,
2000, Kojima et al., 2002, Griffiths et al., 2003). Molecular and
mechanistic studies in other plant species might help us to
understand how the diversity in photoperiodic pathways was
generated during evolution of different photoperiodic responses
in plants.
Perspectives

The precise control of gene expression by the circadian clock modulates the rhythmicity of physiological and developmental processes that are essential for plant survival. The clock is able to precisely recognize the time of the day in the light/dark cycle, allowing the anticipation of the environmental transitions. Elegant experiments performed in bacteria (Ouyang et al., 1998) and more recently in plants (Green et al., 2002, Michael et al., 2003b) have shown that this anticipation provides an adaptive advantage and increases the fitness of the organisms. The Arabidopsis circadian oscillator might be comprised by multiple interlocked feedback loops that generate and maintain circadian rhythmicity through a dynamic signaling network. Identifying clock components, their different levels of regulation and the interaction among them will be crucial in understanding mechanisms of action and influences of the clock on all circadianly-regulated processes. The first steps are already taken. New tools and experimental approaches will help to assemble the pieces into place and solve the intricate puzzle of the Arabidopsis circadian clock.

Acknowledgments

I thank T. Stratmann for critical reading of the manuscript. Our work on circadian rhythms is supported by grants (BIO2004-02144) from Spanish MEC and (FP6-012239) from EC.

References


