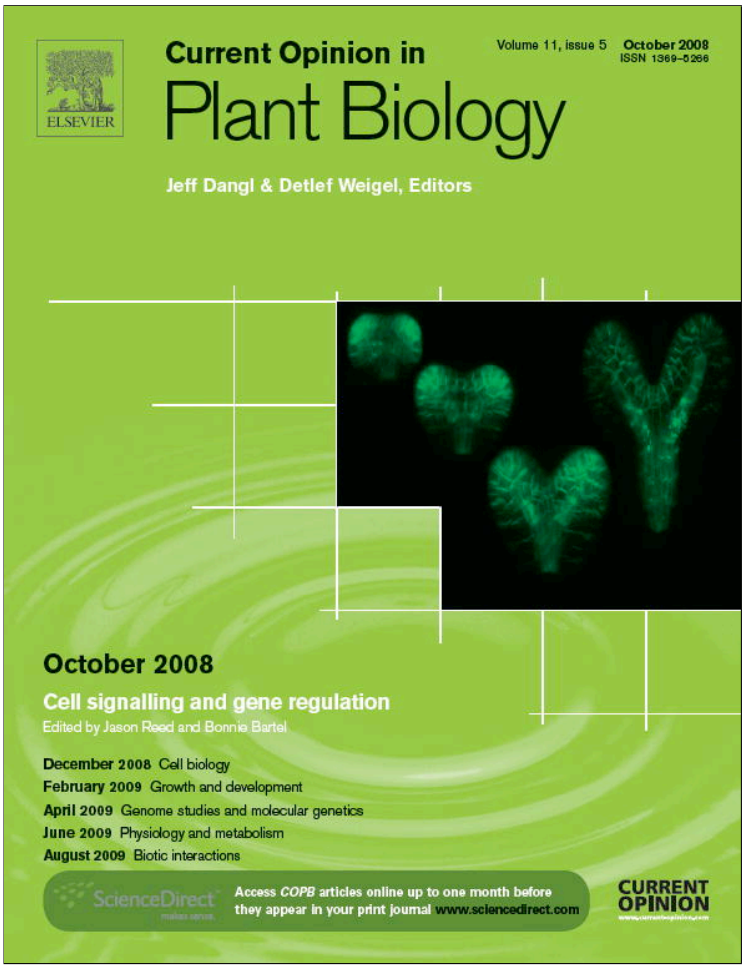

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Comes a time

C Robertson McClung

The circadian clock is a selfsustaining oscillator with an endogenous period of ~24 hours. The *Arabidopsis* clock is composed of a set of interlocking negative feedback loops entailing transcriptional, post-transcriptional, and post-translational, particularly regulated proteolysis, control. Clock control of the transcriptome is widespread; up to 90% of the transcriptome cycles in at least one condition in seedlings exposed to a variety of environmental cycles. Clock control extends to the metabolome, though diurnal oscillations in enzyme activities and metabolites are less dramatic than oscillations in cognate transcripts. Metabolites, including organic nitrogen intermediates, feed back to modulate clock function, consistent with the view of the circadian clock as a key integrator of metabolic signals to coordinate metabolism and physiology with the environment.

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Current Opinion in Plant Biology 2008, **11**:514–520

This review comes from a themed issue on
Cell Signalling and Gene Regulation
Edited by Jason Reed and Bonnie Bartel

Available online 3rd August 2008

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2008.06.010

Introduction

Circadian rhythms are endogenous rhythms with a period that closely approximates the 24-hour period of the rotation of the earth. Crucial defining properties of circadian rhythms include an endogenous, selfsustaining period of 24 hours, entrainment to the environmental period (often by light or temperature cues), and temperature compensation [1]. An underlying premise to the study of circadian rhythms has been that the circadian clock allows an organism to coordinate its biology with its temporal environment subjects and thus enhances evolutionary fitness. This premise has now been experimentally verified in several organisms, including *Arabidopsis*, where it has been shown that net photosynthesis is greatest when the endogenous circadian period matches the environmental period, even in mutants where the circadian period diverges substantially from 24 hours [2].

There was a time when a review of the current research in plant circadian rhythms for Current Opinions could be comprehensive. Comes a time when the volume of research simply exceeds the word limit of this type of review. Plant circadian research has soared past this threshold, which is tremendously exciting but necessitates an apology to those whose work is omitted (but certainly not overlooked). Comes a time also when the old ways of gene-at-a-time analysis must be augmented with systems-level analysis. I have attempted to highlight this transition and to emphasize some of the most exciting areas in plant clocks research, but there is much more worthy of your attention. Comprehensive reviews of the plant circadian system are available [3–5].

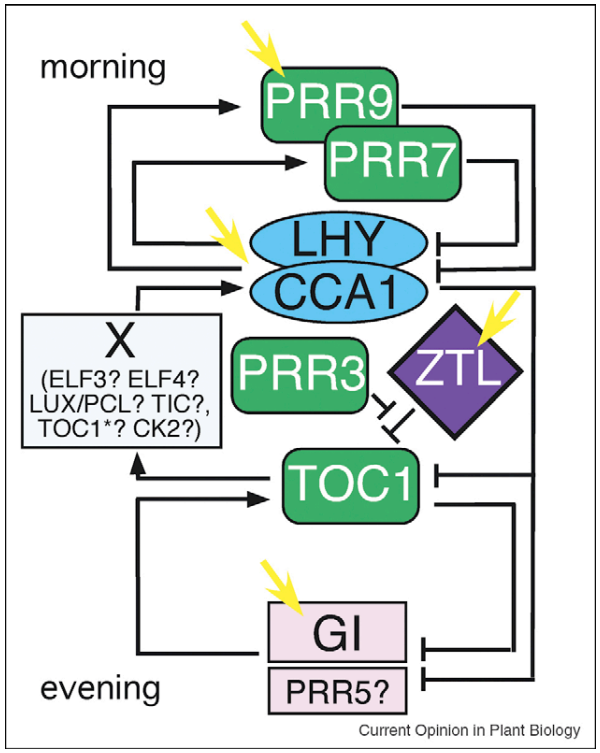
The *Arabidopsis* oscillator

Oscillations arise from negative feedback loops that include a time delay. All eukaryotic circadian oscillators studied to date are based on multiple interlocked negative feedback loops [1]. Mathematical analysis suggests that the increased complexity associated with multiple interlocked loops increases flexibility, which enhances robust entrainment and temperature compensation [6]. Current models of the *Arabidopsis* circadian clock (Figure 1) postulate multiple interlocked feedback loops [7••,8••]. A pair of single Myb-domain transcription

Glossary

Period: The duration of one cycle.
Phase: The time of a consistent marker (e.g. peak or trough) on the circadian oscillation.
Amplitude: One-half the peak to trough difference.
Temperature compensation: The period is relatively invariant across a range of physiologically relevant temperature, rather than simply shortening with increasing temperature as might be expected from a biochemical reaction.
Clock gene: A gene whose function is required for sustained endogenous circadian oscillation with wild-type period and phase.
Output gene: A clock-regulated gene whose function is not required for sustained endogenous circadian oscillation with wild-type period and phase.
Free-run: Conditions of constant light and temperature, allowing the endogenous nature of the circadian oscillation to be evident.
Entrainment: The process by which an environmental cycle, such as light–dark, regulates the period and phase of the circadian clock.
Photocycle: An entraining cycle of light and dark.
Thermocycle: An entraining cycle of warm and cool temperatures.
Waveform: The shape of a circadian oscillation, including the timing and rate of both accumulation and decline and duration of peak and trough.
Slave oscillator: An oscillator with circadian period but which is not selfsustaining, but which requires continual input from a selfsustaining circadian oscillation to maintain oscillation.
Gating: Limiting a response to a defined portion of the circadian cycle.

Figure 1



Simplified cartoon outlining the architecture of the *Arabidopsis* clock. Yellow arrows indicate light regulation and indicate sites of entrainment by light. An activated form of TOC1, indicated as TOC1*, is one possible component of X. Casein kinase 2 (CK2) phosphorylates CCA1 and may also be a component of X.

factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), plays central roles in two loops. In one loop, CCA1 and LHY repress the expression of the Pseudo-Response Regulator (PRR) gene *TIMING OF CAB EXPRESSION1* (*TOC1*), also known as *PRR1*. TOC1 closes the first loop by inducing *CCA1* and *LHY* transcription for the next cycle. Proper regulation of *CCA1* and *LHY* requires other evening-expressed clock genes, including EARLY FLOWERING4 (*ELF4*) [9,10], which encodes a protein of unknown function, and *LUX ARRHYTHMO* (*LUX*)/*PHYTOCLOCK1* (*PCL1*), which encodes a Myb-domain transcription factor [11,12]. *TIME FOR COFFEE* (*TIC*) encodes a nuclear protein of unknown function necessary for proper *LHY*, but not *CCA1*, expression [13]. In a second loop, two *TOC1*-related genes, *PRR7* and *PRR9*, are induced by and subsequently repress *CCA1* and *LHY* [14–16]. In a third loop, GIGANTEA (GI) and, possibly, PRR5 are positive regulators of *TOC1* [7]. *GI* itself is negatively regulated by both CCA1/LHY and TOC1, though the mechanism by which CCA1 and LHY influence *GI* expression remains unclear. The PRR7/PRR9 and GI loops have been termed 'morning' and 'evening' oscillators, by analogy to clock architecture in mice and fruit flies, and may permit accurate tracking of dawn and dusk, respectively [7••].

PRR7 and PRR9 are important for temperature entrainment of the clock, but have not yet been tested for roles in temperature compensation [16]. FLOWERING LOCUS C (FLC) is important for period maintenance at high temperature and contributes to natural variation in temperature compensation [17]. Although current mathematical models of the *Arabidopsis* clock posit that CCA1 and LHY are equivalent and interchangeable for the sake of simplification [7••,8••], their relative importance in maintaining clock function varies according to ambient temperature and they are therefore also implicated in temperature compensation [18•].

A combination of forward and reverse genetic approaches continues to uncover new loci necessary for proper clock function. Loss of function of *FIONA1* (*FIO1*), which encodes a novel nuclear protein of unknown function, lengthens period and alters seedling growth and photoperiodic flowering [19]. Constitutive expression of *CIRCADIAN1* (*CIR1*), which encodes a Myb transcription factor related to CCA1 and LHY, shortens period length [20]. Genetic analysis is being used to probe the complexity of interactions among various clock components. In particular, the biochemical function of the PRRs, with the exception of PRR3 (see below), remains unknown. It is clear that the Pseudo-Receiver (PsR) domain is crucial for function, as overexpression of only the PsR domain of PRR5 confers long period [21]. Genetic analysis of mutants with lesions affecting combinations of *PRRs* and other clock genes reveals complex relationships (e.g. [15,22–24,25•]). The sheer number of potential combinations argues for a rational approach, informed by modeling, to identify specific hypotheses and mutant combinations for experimentation [7••]. One excellent example of this was the incorporation of *GI* into the evening loop, which was suggested through modeling and tested with a *gi cca1 lhy* triple mutant [7••]. A second example is the suggestion that *PRR5* might also participate in this evening loop, which could possibly be tested through analysis of a *gi prr5* double mutant [7••]. The three-loop structure of the current model emphasizes the crucial importance of component X, the activator(s) of CCA1 and LHY in the central TOC1/CCA1/LHY loop [7••,8••], which in turn provides impetus for the study of *CCA1* and *LHY* transcription and activation.

A number of signaling pathways are implicated in clock function. For example, cyclic adenosine diphosphate ribose (cADPR) signaling has been shown to modulate clock function [26•]. Loss of function of LIGHT INSENSITIVE PERIOD 1 (LIP1), a plant-specific atypical small GTPase, shortens period, renders period length nearly insensitive to light intensity, and alters the

response of clock phase to light pulses [27]. The clock governs an oscillation in cytosolic free Ca^{2+} that is uncoupled from the oscillation in *LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN* (*LHCB*) expression by the *toc1-1* but not by the *toc1-2* mutation, providing new evidence in support of multiple oscillators [28].

Circadian regulation of the transcriptome

It has long been known that the circadian clock controls the transcription of many genes, including both output genes, genes not required to generate the oscillation itself, and some, but not all, clock genes, genes required for wild-type oscillator function. Initial microarray analyses indicated that 10–15% of the *Arabidopsis* transcriptome shows circadian oscillation in abundance during free run in constant conditions following entrainment to photocycles [17,29,30,31••]. Clock-regulated transcripts were enriched in a set of transcripts with short half-lives, suggesting that transcript stability might obscure transcriptional oscillations [32]. Moreover, enhancer trapping [33] suggested that clock control of transcription was more widespread than was captured in the initial transcriptome studies. Recently, a comprehensive investigation of plants grown under a variety of thermocycles, photocycles, and free-run conditions has shown that ~90% of the *Arabidopsis* transcriptome cycles in at least one condition [34••]!

Mechanistically, how are rhythmic induction and repression of transcription mediated? The details of circadian transcription in plants are not yet fully described. cis-Regulatory elements associated with phase-specific expression have been defined [34••], but in very few cases have the cognate DNA-binding proteins been identified and characterized. The best — though still incompletely — characterized example is *TOC1*. Recently it has been established that rhythmic transcription of *TOC1* is correlated with binding of chromatin remodeling factors to the *TOC1* promoter and histone H3 acetylation, associated with open chromatin structure [35•]. Rhythmic binding of CCA1 (and presumably LHY) to the evening element (EE) antagonizes histone H3 acetylation at the *TOC1* promoter [35•]. Pharmacological inhibition of histone deacetylation alters the waveform of *TOC1* mRNA abundance [35•], but the responsible histone deacetylase(s) is/are not known. In mammals, CLOCK has been established to have histone deacetylase activity [36].

Post-transcriptional regulation

Post-transcriptional and post-translational regulation plays crucial roles in clocks of plants, as in other taxa [1]. Oscillations in transcript abundance can originate through transcriptional regulation, but the clock regulates the degradation of a subset of transcripts via the downstream instability determinant (DST) pathway [37]. Recently it has been shown that light regulates the stability of the *CCA1* transcript, offering a new route for light input to set clock phase [38]. Alternative splicing has been implicated in the slave oscillator involving *At*GRP7, which autoregulates its expression by influencing alternative splicing of its own pre-mRNA. Mutation of the AtGRP7 RNA recognition motif abolishes autoregulation as well as regulation of downstream targets, including *AtGRP8* [39].

Post-translational regulation

The temporally regulated proteasomal degradation of specific clock proteins is necessary for progression through the oscillation. The stability of a number of plant clock proteins, including GI [40], LHY [41], CASEIN KINASE 2 BETA 4 (CKB4) [42], PRR7 [43•], and PRR9 [44] is clock-regulated. Most is known about the TOC1 protein, which peaks in abundance at dusk and must be turned over for the cycle to proceed. What TOC1 does at a molecular level remains enigmatic. TOC1 is a positive regulator of *CCA1* and *LHY* transcription, though TOC1 lacks demonstrated DNA-binding activity and so must act indirectly, possibly through interactions with transcription factors such as ELF4 [9,10] and LUX/PCL1 [11,12]. Period is sensitive to TOC1 abundance; reduced TOC1 shortens and elevated TOC1 lengthens period. An E3 ubiquitin ligase SCF complex including the F-box protein ZEITLUPE (ZTL) [45] is crucial for clock-regulated proteasomal degradation of TOC1 [46]. Consistent with its role in TOC1 degradation, the effects of reduction or increase in ZTL abundance are period lengthening and shortening, respectively [47]. ZTL also targets PRR5 for proteasomal degradation through direct interaction with the PsR domain of PRR5 [48•]. ZTL is a large protein with two recognized functional motifs in addition to the F box. The Kelch repeats of ZTL are necessary for interaction with TOC1. PRR3 binds directly to TOC1, which perturbs the interaction of TOC1 with ZTL and, hence, stabilizes TOC1 [49•]. As PRR3 expression is limited to the vasculature, this emphasizes the potential for spatially restricted modulation of clock function.

ZTL possesses a LOV (light, oxygen, voltage) domain capable of flavin binding and implicated in blue-light photochemistry. The LOV domain is responsible for the interaction of ZTL with GI, which stabilizes ZTL [50••]. Because GI abundance cycles, driven by rhythmic *GI* transcription, this interaction provides a molecular explanation for the rhythm in ZTL protein abundance despite a conspicuous lack of cycling in *ZTL* transcript abundance. The ZTL–GI interaction is dramatically enhanced by blue light and this enhancement is abolished by mutational disruption of LOV domain photochemistry; thus, ZTL is a blue-light photoreceptor that mediates direct light input into the clock [50••].

Circadian regulation of growth and reproduction

Circadian regulation of plant physiology and development is widespread [5]. One mechanism by which the clock attains such broad influence is through modulation of signaling pathways. One dramatic example of this is the regulation of auxin signal transduction; the clock controls sensitivity to auxin and thereby modulates both transcriptional and growth responses to this hormone [31••]. This observation offers a mechanism to effect circadian regulation of multiple aspects of plant growth and development, potentially including tropisms and organ formation. There is accumulating evidence for crosstalk among multiple hormone signaling pathways in growth and development, and clock function is modulated by several phytohormones, including abscisic acid, brassinosteroids, and cytokinin [51,52], though not by auxin [31].

Many environmental responses are temporally modulated (gated) by the circadian clock (reviewed by [53]). The clock gates responses to a number of abiotic stresses, such as cold temperature [54], light-quality modulation of cold acclimation is also gated by the clock [55]. The clock may also regulate responses, including stresses mediated through abscisic acid and methyl jasmonate [56•].

Does the pervasive nature of clock regulation of growth, physiology, and environmental responsiveness extend throughout the life of the plant? Circadian oscillation in gene expression is detected in both light-grown and etiolated seedlings within a day or so of seed hydration, which provides an important signal to synchronize the clocks both within a seedling and among a population of seedlings [57]. It will be interesting to explore clock function both very late in life (is the clock important during senescence?) and at the very beginning, during fertilization, embryogenesis, and seed maturation.

Circadian studies have focused on rhythms in constant conditions to emphasize the endogenous nature of the clock, yet plants are normally exposed to diurnal cycles and considerable insight can be gained by studying these more biologically relevant diurnal conditions. An emerging theme is that the coincidence of clock-controlled internal cycles with external environmental cycles allows coordination of plant processes with the environment. For example, hypocotyl elongation has been known for some time to be clock-regulated [3–5], but recent work in diurnal conditions has revealed the underlying mechanism [58••]. Two basic helix–loop–helix transcription factors, PHYTOCHROME INTERACTING FACTOR4 (PIF4) and PIF5, are positive regulators of hypocotyl elongation. Transcript abundance of *PIF4* and *PIF5* is regulated by the clock, accumulating before dawn, and protein stability is negatively regulated by light. The coincidence of high-transcript levels (internal cycle) and protein stabilization in the dark (external cycle) allows growth promotion at the end of the night [58••].

The photoperiodic pathway of flower induction offers a second example that illustrates this theme of coincidence of external and internal cycles. FLAVIN-BINDING, KELCH REPEATS F-BOX1 (FKF1), a close relative of ZTL, regulates the accumulation of CONSTANS (CO), a crucial inducer of flowering. Blue light perceived via the FKF1-LOV domain stimulates the interaction of FKF1 with GI, analogous to the ZTL–GI interaction described above. The FKF1–GI complex forms on the *CO* promoter and binds to and mediates the degradation of CYCLING DOF FACTOR1 (CDF1), a transcriptional repressor of *CO* [59], to allow daytime *CO* transcription [60••]. Interestingly, *CDF1* expression is markedly derepressed in the *toc1-2 prr5-11* double mutant, consistent with the late-flowering phenotype of the double mutant and suggestive that these two clock genes encode repressors of *CDF1* [25•]. Similar genetic analysis of double and triple mutants suggests that PRR5, PRR7, and PRR9 stimulate flowering through repression of CDF1 [61]. CO protein is stabilized in the light and thus accumulates in long but not in short days [62]. SUPPRESSOR of PHYA-105 (SPA1) interacts with CO and is implicated in its degradation [63,64].

Systems biology

As described above, circadian control of the transcriptome is widespread and influences many metabolic pathways [17,29,30,31••,34••]. In both carbon and nitrogen metabolism, many metabolite–transcript correlations are detected, though changes in enzyme activities and metabolite levels are less dramatic than might be predicted from the large observed changes in transcript abundance [65•]. These observations suggest important feedback by metabolite levels on clock-regulated gene expression [65•]. Retrograde signaling from the chloroplast (see review by Fernández and Strand in this issue) is implicated in the modulation of circadian function by mutations in *CHLOROPLAST RNA BINDING* (*CRB*), which alter amplitude and waveform, but not period length, of CCA1 and LHY expression [66]. CCA1, in addition to its role in the clock, is a key regulator of a subnetwork of organic nitrogen responsive genes (see review by Vidal and Gutiérrez in this issue), including key nitrogen assimilatory genes [67•]. *CCA1* expression responds to nitrogen status and pulses of either inorganic or organic forms of nitrogen shifts clock phase [67•]. This is consistent with an emerging view of the clock as a crucial integrator of metabolic inputs, allowing temporal coordination of metabolism.

Conclusions

Simple models cannot adequately describe the complex network of circadian control of plant physiology and metabolism. Clock control is pervasive. In addition, the

emerging view is that the clock is sensitive to a wide variety of internal metabolic and hormonal signals, as well as to environmental signals. Systems biology and mathematical modeling are increasingly important in capturing the subtle modulation of clock function necessary to coordinate and optimize metabolism, growth, and development in an oscillating environment.

Acknowledgements

I thank Neil Young (Reprise Records, 1978) for the title. My work on circadian rhythms is supported by grants from the National Science Foundation (MCB-0343887 and IOB-0517111) and from the United States–Israel Binational Science Foundation (#2005223).

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