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Network news: prime time for systems biology of the plant circadian clock

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Whole-transcriptome analyses have established that the plant circadian clock regulates virtually every plant biological process and most prominently hormonal and stress response pathways. Systems biology efforts have successfully modeled the plant central clock machinery and an iterative process of model refinement and experimental validation has contributed significantly to the current view of the central clock machinery. The challenge now is to connect this central clock to the output pathways for understanding how the plant circadian clock contributes to plant growth and fitness in a changing environment. Undoubtedly, systems approaches will be needed to integrate and model the vastly increased volume of experimental data in order to extract meaningful biological information. Thus, we have entered an era of systems modeling, experimental testing, and refinement. This approach, coupled with advances from the genetic and biochemical analyses of clock function, is accelerating our progress towards a comprehensive understanding of the plant circadian clock network.

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Introduction

Circadian clocks are nearly ubiquitous endogenous timers that play critical roles in the temporal organization of biological activities and in the coordination of those activities with daily environmental cycles. The accumulation of large quantities of experimental data is proving a challenge to all fields of biology. The growing need for interpretation, integration, and modeling these data is incentivizing biologists to move away from individual molecules towards a systems view of biological process. Circadian biology is no exception. Systems biology is a relatively new field in the biological sciences which aims to integrate the existing knowledge about biological components, build a model of the system *as a whole* and extract the unifying organizational principles that explain the form and function of living organisms [1]. System level models of biological processes that explain observed behaviors should be predictive and be used to derive testable hypotheses. Experimental work can then validate these hypotheses or provide new ways to refine the model. Iterating through the cycle of modeling, testing, and refinement is a hallmark of research in systems biology. Ultimately, understanding systems structure and dynamic principles will allow the design of new ones with desirable properties.

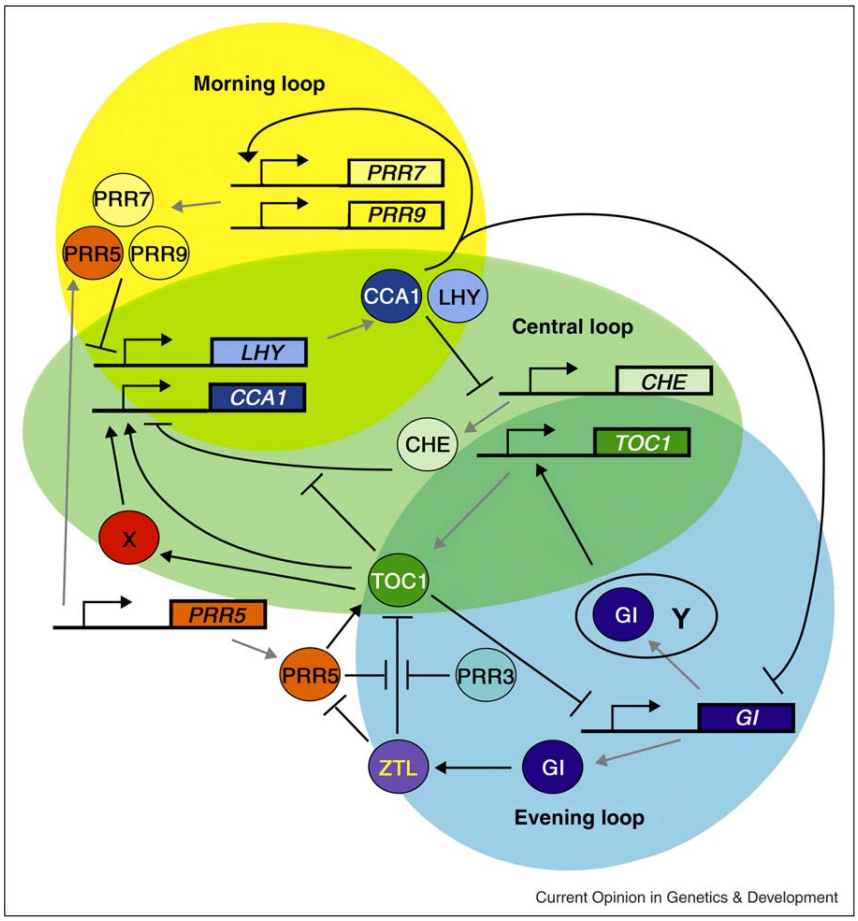
Circadian clocks consist of interlocked feedback loops

Detailed analysis of plant circadian clocks reveals an intricate network of molecular components that is responsible for rhythmic behaviors. At the heart of the clock lie multiple interlocked negative feedback loops, each loop involving transcriptional activation and repression (Figure 1). The central loop consists of two MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which repress expression of a pseudo-response regulator (*PRR*) gene, *TIMING OF CAB EXPRESSION 1* (*TOC1*). TOC1 is recruited to the *CCA1* and *LHY* promoters and activates their expression. Three additional PRRs (PRR5, 7, and 9) repress their activators, *CCA1* and *LHY*, to form a second interlocked 'morning loop'. TOC1 forms a third 'evening loop' through repression of a hypothetical component 'Y,' that includes GIGANTEA (GI), a positive regulator of TOC1. In this work we emphasize new studies, including systems biology approaches, which enhance our understanding of circadian network architecture, including advances in oscillator function and in how the clock orchestrates plant biological activities. These studies add to the number and complexity of oscillator loops and improve our understanding of the multiple levels of regulation that contribute to clock function. Comprehensive reviews of the plant circadian system should be consulted for a more complete vision of the plant clock [2–4].

In the beginning

When does circadian clock function begin? A number of years ago it was inferred that a clock is running from the time of imbibition in etiolated seedlings, based on circadian gating of the light-mediated acute induction of clock-regulated *CATALASE2* expression [5]. Consistent with this, the clock gates the promotion of germination by

Figure 1



A simplified working model of the Arabidopsis circadian clock. The Arabidopsis circadian clock consists of a series of interconnected feedback loops. In the central loop, CCA1 and LHY negatively regulate *TOC1* through direct promoter binding. TOC1 is a positive regulator of *CCA1* and *LHY*. This may involve an intermediate component, 'X,' which is a proposed transcriptional activator of *CCA1* and *LHY*. 'X' may include LUX/PCL and/or ELF4. CCA1 and CHE form a reciprocally negative loop. TOC1 binds to CHE, which blocks CHE's inhibition of *CCA1* expression. CCA1 and LHY also form the positive arm of the 'morning loop,' serving as positive regulators of *PRR9*, *PRR7*, and possibly *PRR5*. These three PRRs in turn negatively regulate *CCA1* and *LHY* through direct promoter binding. In the evening loop, CCA1 and LHY negatively regulate a hypothetical component, 'Y,' which is a positive regulator of *TOC1*. GI is a likely component of 'Y.' ZTL is a cytosolic F-box protein that binds to TOC1 and PRR5, targeting them for proteasomal degradation. PRR5 stabilizes TOC1 by facilitating its nuclear accumulation, which may also contribute to TOC1 activity. GI binds to ZTL in the light and stabilizes ZTL and thus this primary interaction is indicated as a positive arrow. However, the binding of GI to ZTL may have a negative effect on ZTL degradation of TOC1 and PRR5 by blocking their interaction with ZTL. PRR3 and PRR5 also stabilize TOC1 by blocking its interaction with ZTL. Genes are coded as rectangles and proteins are coded as circles. Regulatory interactions are in black.Modified from [3,4].

far-red light pulse administered following imbibition [6]. Clock-regulated rhythmic gene expression can be detected immediately following germination, and imbibition provides a signal sufficient to synchronize clocks within a population of seedlings [7]. Clock genes play critical roles even earlier, and are necessary both for the proper establishment of dormancy and for the proper response to dormancy breaking in seeds [8].

How many clocks? Revisiting tissue and organ-specific clocks

One often speaks of 'the clock,' but considerable data have accumulated to argue persuasively for tissue-specific and organ-specific variants. For example, PRR3 exhibits vascular expression where it serves to modulate TOC1 stability [9]. Two clocks, distinguished by their temperature responsiveness, were shown to regulate the morning-expressed *LIGHT HARVESTING CHLOROPHYLL a/b BINDING PROTEIN* (*LHCB/CAB*) and evening-expressed *CATALASE3* [10]. Different periods in shoot *LHCB/CAB* and root *CHALCONE SYNTHASE* rhythmic expression suggested that shoot and root clocks were distinct [11]. The clock in mature roots is a simplified one governed by the 'morning loop' of PRR7, PRR9, CCA1, and LHY, disconnected from the 'evening loop' because TOC1, although expressed, does not cycle [12]. Consistent with

this, *toc1* mutants do not show a shortened period in roots. However, when the 'morning loop' is disrupted in the *prr7 prr9* double mutant, *TOC1* cycling is restored [12] and apparently clock function is provided by the evening loop requiring TOC1. It would be interesting to test root clock function in the triple *prr7 prr9 toc1* mutant.

Entrainment

Circadian clocks are entrained to local time by a variety of input cues. The most important input cues are most obvious: light and temperature [13]. As mentioned above, imbibition also acts as a strong entraining cue [5,7]. More recently it has become apparent that the clock also monitors metabolites, such as organic nitrogen intermediates [14] or hormone levels, including cytokinin, brassinosteroid, and abscisic acid (ABA) [15,16,17•], and uses their status to modulate clock phase. Because these intermediates are themselves under clock control [2–4], these inputs represent feedback loops in which the clock monitors its own outputs to modulate the status of central oscillator components.

Mechanistic insights into oscillator function: loops within loops

One of the most important milestones in the analysis of plant circadian clocks was the identification of the feedback loop in which TOC1 induces CCA1 and LHY, which in turn represses TOC1 expression through direct binding to the TOC1 promoter [18]. However, the mechanism by which TOC1 positively regulates CCA1 and LHY expression remains enigmatic.

Chromatin immunoprecipitation (ChIP) shows that TOC1 binds to the CCA1 promoter [19••]. This same study identified a second protein, CCA1 Hiking Expedition (CHE), a TCP (TEOSINTE BRANCHED1, CYCLOIDEA AND PCF) transcription factor, as a regulator of *CCA1* expression via a large-scale yeast one-hybrid screen of a library of Arabidopsis transcription factors for activated transcription from the *CCA1* promoter [19••]. CHE binds to a canonical TCP binding site in the *CCA1* promoter *in vitro* and *in vivo*, although CHE does not bind to the *LHY* promoter. CHE is a negative regulator of *CCA1*; although loss of CHE function alone does not affect period, the *che lhy* double mutant has a shorter period than the *lhy* mutant, demonstrating redundancy of CHE and LHY in *CCA1* repression. CCA1 (and also LHY) binds to the *CHE* promoter both *in vitro* and *in vivo* to repress *CHE*. Thus, CHE and CCA1 form a novel reciprocally repressive feedback loop within the central oscillator.

Yeast two-hybrid experiments establish that CHE and TOC1 interact and CHE overexpression antagonizes the period lengthening resulting from TOC1 overexpression [19••,20]. This would be consistent with CHE blocking a direct transcriptional induction of *CCA1* by TOC1, but no data are available to support this direct induction by TOC1. Other components, including LUX ARRHYTHMO/PHYTOCLOCK1 (LUX/PCL) [21,22] and EARLY FLOWERING 4 (ELF4) [23,24], are positive regulators of CCA1 (and LHY), but this could be via indirect means. If these other players induce *CCA1*, it could be that TOC1 relieves CHE inhibition of *CCA1* expression, and thereby leads to *CCA1* induction indirectly. The details of *CCA1* transcriptional activation are slowly being revealed, but our understanding remains fragmentary.

CHE does not bind to the *LHY* promoter, emphasizing that these two close relatives are differently regulated. This difference might contribute to the differential expression of *CCA1* and *LHY* at low and high temperatures, which has been hypothesized to contribute to temperature compensation [25]. A recent study of the *LHY* promoter identified functionally important motifs, including a G-box and a CArG-like sequence to which FLC is recruited [26]. This may explain the known effects of FLC on circadian period [27]. However, the rhythmic expression of the *LHY* promoter is redundantly specified and none of the mutations tested abolished rhythmicity. As noted above, both LUX/PCL1 and ELF4 are positive regulators of *LHY* [21–24].

PRR7 and PRR9 are negative regulators of *CCA1* and *LHY* expression [28]. Recently, Nakamichi and colleagues [29••] established that *CCA1* and *LHY* are direct targets of PRR5 through induction of a PRR5-GR-CFP fusion protein in the presence of the translational inhibitor, cycloheximide. Both *CCA1* and *LHY* mRNAs decreased immediately, making it clear that this regulation is direct. PRR5, PRR7 and PRR9 all have transcriptional repressor activity when targeted to a *LUC* reporter gene. This is relevant *in vivo* because PRR5, PRR7 and PRR9 can all be detected in the promoter regions of *CCA1* and *LHY* through ChIP assays. Peak binding of the three proteins occurs sequentially from PRR9 in early morning through PRR7 in the mid-light period to PRR5 in late afternoon/early night. The sequential expression patterns of the three PRR proteins extends the temporal window over which *CCA1* and *LHY* expression is repressed, offering a mechanistic explanation for their partially redundant function shown through genetic analysis of loss of function mutations [28,30–33].

The targeting of TOC1 to the *CCA1* promoter [19••] and of PRR5, PRR7, and PRR9 to the *CCA1* and *LHY* promoters [29••] raises the interesting question as to whether these proteins can bind directly to DNA or whether they require interaction with a known DNA-binding protein, as they have not been thought to possess a DNA-binding motif. The PRR proteins share two motifs, an N-terminal Pseudo-Receiver Domain related to the Receiver Domain found in two-component signaling response regulators, and a C-terminal CCT (named for CONSTANS [CO], CONSTANS-LIKE and TOC1) domain, which

was thought to function in protein–protein interactions. Recently it has been established that CO is recruited to a novel element in the *FT* promoter via its CCT domain [34•]. This raises the hypothesis that these PRR proteins possess intrinsic DNA-binding activity through the CCT domain. Full definition both of this DNA-binding domain in the PRRs and of the DNA element(s) to which it binds requires further experimentation.

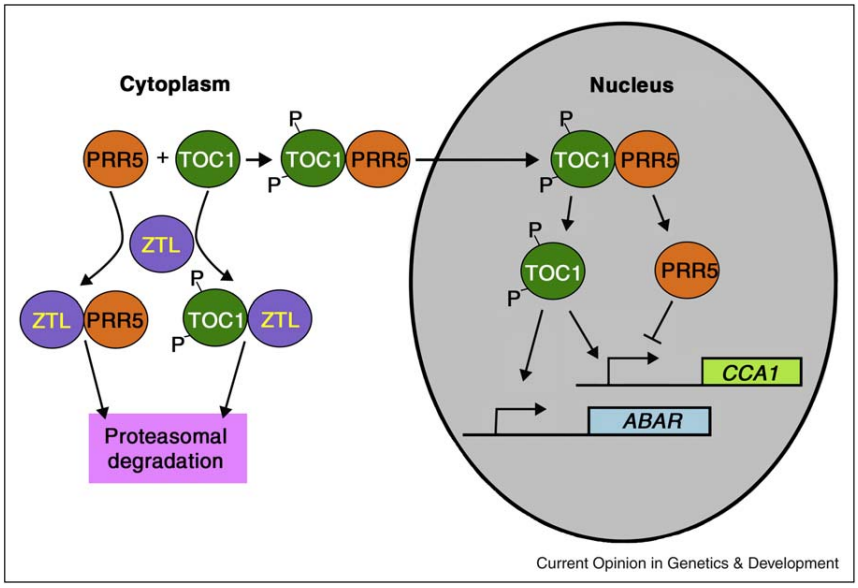
Post-transcriptional regulation

To date, most analyses of plant clock gene regulation have focused at the transcriptional and post-translational levels. However, examples are emerging in which mRNA stability is clock-regulated and, in the case of clock gene *CCA1*, this offers an additional mechanism with which to modulate clock function [35–37]. Alternative splicing is rapidly emerging as an important mechanism in expanding the proteomes of eukaryotes [38], and has been encountered as a mechanism to regulate expression of clock genes, including *CCA1* and *ELF3* [39•,40•], as well as *GLYCINE RICH PROTEIN7* (*GRP7*) [41,42], a component of a circadian slave oscillator implicated in the promotion of flowering [43]. However, the detailed molecular mechanisms by which alternative splicing occurs within circadian networks remain largely obscure.

Post-translational regulation

Post-translational processes, notably phosphorylation and proteolysis, play critical roles in all clock systems [44]. It has been known for some time that casein kinase 2 phosphorylates CCA1 and that this phosphorylation is necessary for CCA1 function [45]. More recently it has been established that all five PRRs are phosphorylated with functionally significant consequences [46]. PRR5 and TOC1 are targeted for degradation through interaction with the F-box protein ZEITLUPE (ZTL) [46–48]. Phosphorylation of TOC1 and PRR3 promotes their interaction and this interaction blocks TOC1 from interaction with ZTL [46], offering mechanistic insight into how PRR3 stabilizes TOC1 [9]. A second PRR, PRR5 also binds to and stabilizes TOC1, although the PRR5–TOC1 interaction itself is independent of the phosphorylation status of both partners [49••] (Figure 2). The PRR5-TOC1 interaction promotes nuclear accumulation of TOC1, which bears striking resemblance to TIMELESS–PERIOD, CYCLE–CLOCK and PERIOD2–CRYPTOCHROME interactions in Drosophila and mammals; in each case, interaction with the former promotes nuclear accumulation of the latter [49••]. Interaction with PRR5 promotes TOC1 phosphorylation, which enhances the interaction of TOC1 with ZTL, but nuclear localization of the PRR5–TOC1 complex sequesters both proteins from cytoplasmic ZTL. This suggests a mechanism by which PRR5 could modulate TOC1 degradation [46]. However, it has not been shown that the PRR5-dependent phosphorylation sites on TOC1 are those that promote the TOC1/ZTL interaction. Phosphorylation of PRR5 promotes its interaction with ZTL, which leads to PRR5 degradation.

Figure 2



A model for post-translational regulation of TOC1 by PRR5. In the cytoplasm, both PRR5 and TOC1 interact with ZTL and are targeted for proteasomal degradation. These interactions with ZTL are promoted by phosphorylation of PRR5 and TOC1. However, PRR5 and TOC1 also interact. This interaction promotes TOC1 phosphorylation and also facilitates the accumulation of TOC1 in the nucleus, where both proteins are protected from interaction with cytoplasmic ZTL [49••]. Within the nucleus, TOC1 and PRR5 accumulate in nuclear foci [49••]. TOC1 directly or indirectly induces transcription of target genes, such as *CCA1* (and *LHY*) [18,19••] and *ABAR* [17•]. PRR5 binds to the promoters of *CCA1* (and *LHY*) to repress transcription [29••]. It is not known if TOC1 and PRR5 remain complexed when recruited to target promoters.

Genetic analysis has made it clear that ZTL is the primary F-box protein responsible for degradation of TOC1 and PRR5. However, ZTL has two close relatives, LOV, KELCH PROTEIN2 (LKP2) and FLAVIN, KELCH, F-BOX1 (FKF1). Single loss of function mutations, *fkf1* or *lkp2*, have at most subtle effects on circadian function. However, compared with the *ztl* single mutant, clock defects are more pronounced in a *ztl fkf1* double mutant and even more pronounced in a *ztl fkf1 lkp2* triple mutant. This establishes that both LKP2 and FKF1 are capable of targeting TOC1 and PRR5 for proteasomal degradation [50•].

The two remaining PRRs, PRR7 and PRR9, function in the 'morning loop' as negative regulators of CCA1 and LHY [29]. Both proteins show progressive phosphorylation as the day progresses [46]. This, in parallel with PRR5 and TOC1, suggests that phosphorylated PRR7 and PRR9 are targeted for proteasomal degradation. However, at this time mechanistic details such as the identity of a putative F-box protein (or other ubiquitin ligase complex) are lacking.

Modeling of the clock

The power of systems approaches for gaining a deeper understanding of plant processes is probably best exemplified by efforts to model the clock machinery. Using published genetic and molecular data, a mathematical model of the original simple feedback loop containing CCA1/LHY and TOC1 was developed several years ago [51]. This model failed to explain reported circadian behaviors such as the pattern of *LHY* mRNA accumulation during the day or the short-period phenotype observed in *lhy* or *cca1* loss of function mutant plants. Adjusting the model structure by introducing interlocked feedback loops including two new components, X and Y, explained these experimental data. Subsequent analysis identified GIGANTEA (GI) as a possible candidate for Y function and later experimental work demonstrated GI as a new essential component of the central clock regulatory network that explains most of the Y functions [52]. Several models, increasingly more sophisticated, followed that added new components and regulatory feedback loops to better explain additional experimental data (reviewed in [53]). Similar approaches are now being utilized to model the integration of light signals, the photoperiod flowering pathway and the circadian clock [54]. Again, adjusting the structure of the original model to fit the experimental data predicts new components or regulatory interactions that can be tested experimentally. Namely, this modeling work predicts a novel positive regulatory interaction between FKF1 and *FLOWERING LOCUS T* (*FT*) controlling photoperiodism [54].

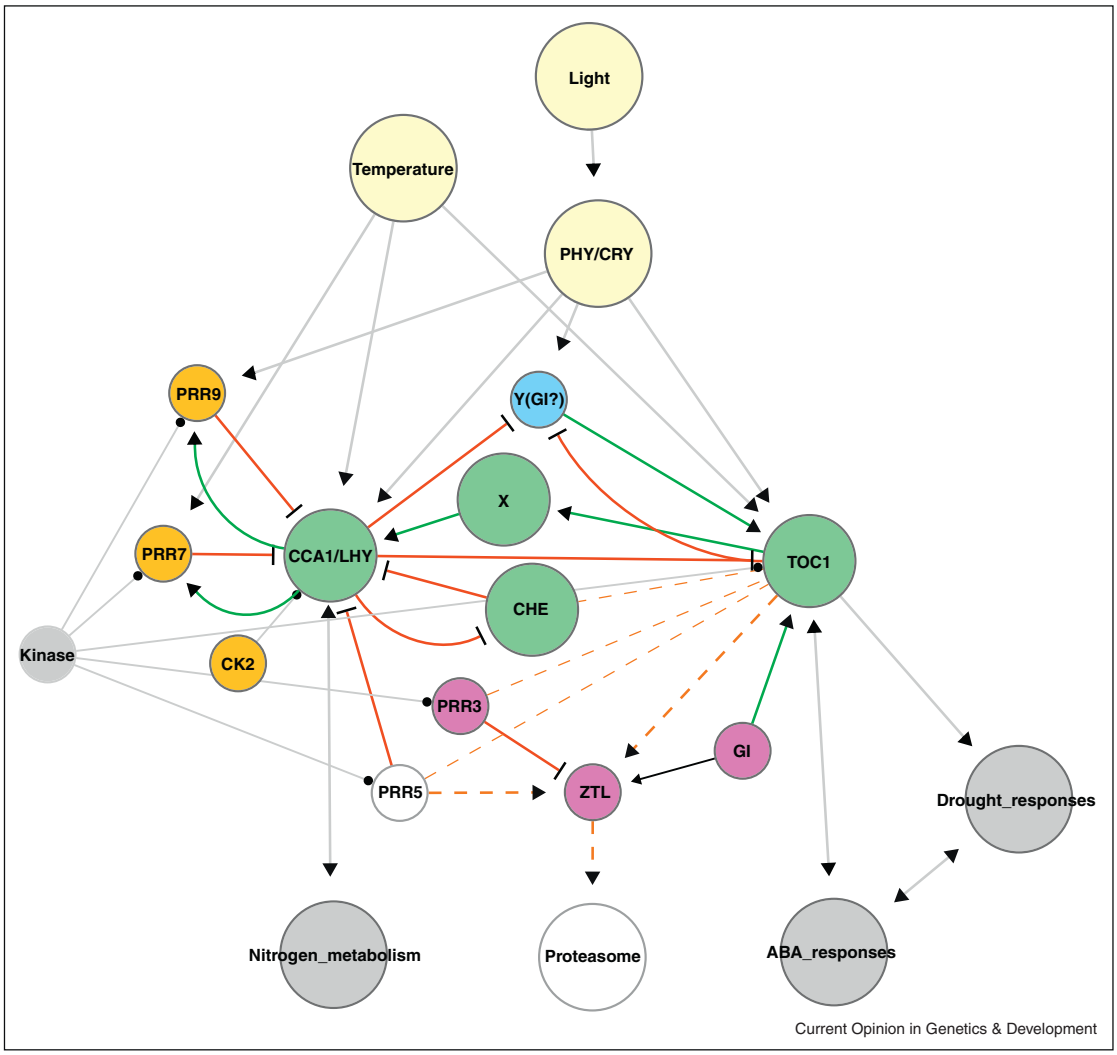
Despite scarce quantitative data, systems biology approaches are also providing novel insight into components and regulatory connections that integrate distinct biological processes such as the central circadian clock and nitrogen metabolism [14]. To identify potential 'master' regulators of the plant response to organic forms of nitrogen (N), transcriptomics data were analyzed in the context of gene networks [55] and several nitrogen-regulated transcription factors were identified on the basis of their regulatory potential [14]. At the top of the list, with 47 connections to targets in the N-regulated gene network, was found the central clock gene *CCA1*. ChIP assays using CCA1 antibodies confirmed binding of CCA1 to the promoter regions of some of its predicted targets, including central nitrogen metabolic genes. These results indicate that the circadian clock regulates N-assimilation by transcriptional regulation of N-assimilatory pathway genes by CCA1. In addition, the finding that *CCA1* mRNA levels are regulated by organic N-sources suggests that N signals act as an input to the circadian clock [14]. The observation that N-treatments resulted in subtle (2 h) but stable phase shifts in *pCCA1:-LUC* expression, indicated that N-status serves as an input to the circadian clock [14]. The recent observation that *CCA1*, *LHY* and *PRR9* genes are differentially expressed under magnesium deficiency [56] suggests that other nutrients may have similar effects on clock function. The emerging view of the circadian clock as a key integrator of metabolic and physiologic processes is that it receives input not only from environmental stimuli but also from metabolic pathways, many of which are subject themselves to circadian regulation (Figure 3).

Genome-wide characterization of circadian-regulated genes and processes

In order to understand the physiological significance of the circadian clock, it is important to identify the genes, pathways and processes that are circadian regulated. Several genome-wide studies have been carried out over the years to address this question using gene microarrays [57–60,61••], tiling microarrays [39•] and deep sequencing technologies [40•]. These studies underscore the importance of clock regulation for the plant as it appears the circadian clock regulates virtually every biological process. Most prominently, hormone and stress response pathways stand out as over-represented among clock-controlled genes [61••] highlighting the relevance of the clock for plant growth and development process and adaptation to changing environmental conditions.

Analysis of the transcriptome in *Arabidopsis thaliana* seedlings revealed temporal integration of hormone pathways as a mechanism to fine-tune phytohormone responses for plant growth regulation. Bioinformatic analysis of genome-wide expression data detected significant enrichment of genes involved in phytohormone metabolism or signaling at the time of day when hypocotyl growth rate is maximal [62•]. The cis-acting element

Figure 3

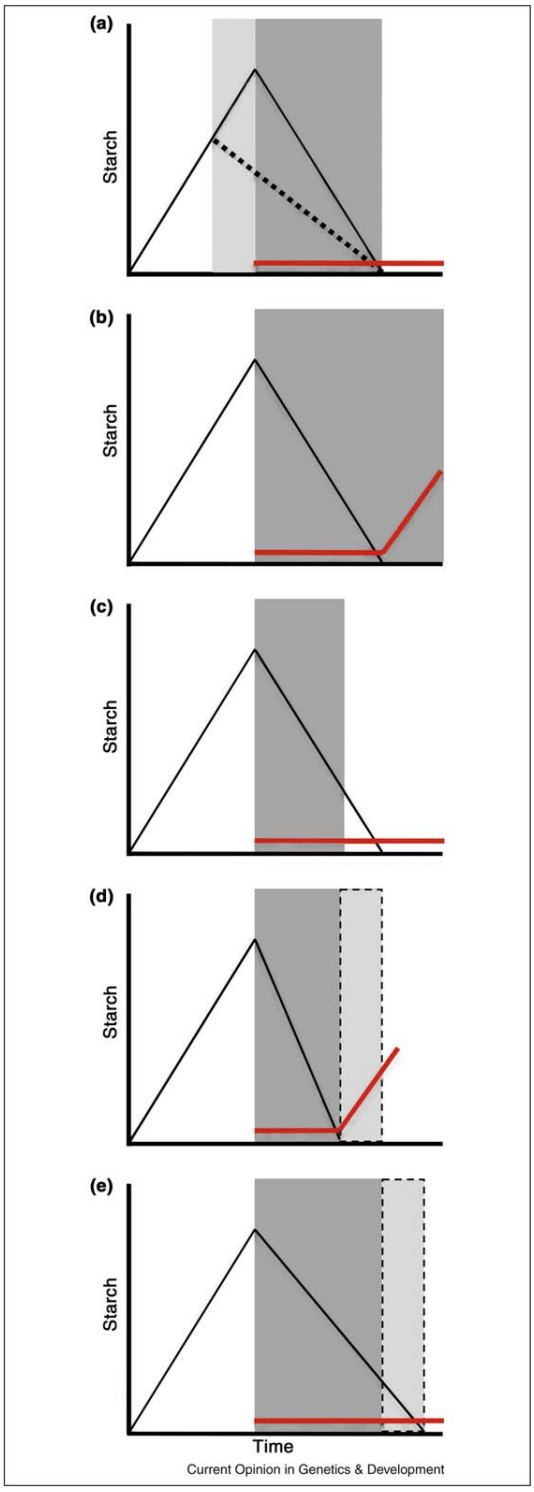


A network model of the circadian clock and its connections to entrainment and output pathways. The central clock regulatory network is composed of multiple interlocked negative feedback loops, each loop involving transcriptional activation and repression. The central loop consists of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which repress expression of *TIMING OF CAB EXPRESSION 1* (*TOC1*). TOC1 activates expression of *CCA1* and *LHY* through an unknown mechanism that may include an unidentified factor X. CCA1 HIKING EXPEDITION (CHE) is a negative regulator of CCA1, which is itself repressed by CCA1. The two pseudo-response regulators, PRR7 and PRR9, repress their activators *CCA1* and *LHY*, to form a second interlocked 'morning loop'. PRR3, PRR5, PRR7, PRR9, and TOC1 are phosphorylated by an unknown kinase(s). PRR5 and TOC1 proteins are degraded through interaction with ZEITLUPE (ZTL) and, in the absence of ZTL, by LOV, KELCH PROTEIN2 (LKP2) and FLAVIN, KELCH, F-BOX1 (FKF1). TOC1 and PRR3 phosphorylation promotes their interaction and this blocks TOC1 from interaction with ZTL. A third 'evening loop' is composed of a hypothetical component 'Y,' that includes GIGANTEA (GI), a positive regulator of TOC1. Additional clock components have been identified but are not included as they cannot be connected with confidence to any node in this network. Green nodes represent components of the central loop; orange nodes represent components of the morning loop; purple nodes represent components of the evening loop; yellow nodes represent entrainment pathways to the central clock; gray nodes represent circadian clock outputs; thick gray edges show known interactions between the entrainment or output pathways and the central clock; green edges with an arrow head represent positive regulatory interactions (e.g., induction); red edges with a terminal perpendicular line represent negative regulatory interactions (e.g., repression); gray edges with a black circle at the end represent phosphorylation; thin dashed orange edges represent physical interactions between proteins; thick dashed orange edges with an arrow head show physical interactions for protein degradation.

(CACATG) was identified as sufficient to confer the predicted diurnal and circadian expression patterns *in vivo* of phytohormone genes [62•]. Examination of the behavior of the phytohormone genes in circadian and light signaling mutants with defective hypocotyls growth showed that the circadian clock indirectly controls growth by gating light-responsive phytohormone transcript levels [62•].

The circadian clock modulates (gates) the ability of a plant to respond to environmental cues such as low

Figure 4



Starch accumulation and utilization in wild type and circadian clock mutant plants during light/dark cycles. **(a)** In wild type, starch accumulates throughout the day and is degraded during the night (dark gray) at a constant rate such that starch is depleted at dawn, as anticipated by the circadian clock. In the event of an early dusk (light gray), starch accumulation ceases and starch is degraded at a reduced rate (dotted line) such that that starch is depleted at dawn. In both cases, carbon starvation gene expression (red line) is not induced. **(b)** In wild type subjected to an unexpected extension of the night, starch is depleted at predicted dawn, which precedes the real dawn, and carbon starvation gene expression (red line) is induced. **(c)** In wild type subjected to a short night, starch is not fully depleted at dawn. Carbon starvation gene expression (red line) is not induced. Nonetheless, growth is not maximal because of the failure to fully use accumulated starch (although it is unlikely that a single such short night would have a large effect). **(d)** In a short-period *cca1 lhy* mutant in 12/12 light/dark cycles, the circadian clock incorrectly predicts an early dawn and starch degradation rates are adjusted accordingly. Thus, starch is depleted before real dawn, with the consequent induction of carbon starvation genes. **(e)** In a long-period mutant in 12/12 light/dark cycles, the circadian clock would be expected to incorrectly predict a late dawn, with concomitant decrease of starch degradation rates. As a consequence, starch would not be fully depleted by real dawn. Carbon starvation genes would not be induced, but growth would not be maximal because of the failure to fully use accumulated starch.

temperatures [63]. A comparison of the transcriptome of wild-type and *prr9/prr7/prr5* triple mutant plants using Affymetrix technology showed that there was a significant overlap between cold-responsive genes and clock-controlled genes with a peak expression between subjective dawn and midday. These results suggested PRR9, PRR7 and PRR5 are important for anticipating diurnal stress by low temperature [64]. Cold acclimation responses require transcription factors of the CBF/DREB family and PRR5, PRR7 and PRR9 would gate the induction of DREB1 by low temperature [64]. Although the CBF/DREB transcription factors have a central role in the cold response, the regulators of the majority of the cold-responsive genes are unknown. The integration of cold-regulated and clock-regulated gene expression occurs through the interaction of regulatory proteins that bind to evening element (EE) and EE-like (EEL) elements with transcription factors acting at nearby ABA response element (ABRE)-like (ABREL) sequences; these two classes of elements are highly enriched in cold-induced genes and play a significant role in configuring the low-temperature transcriptome [65]. Microarray experiments in poplar [66•] and in Arabidopsis [67•] have established that the circadian clock gates the transcriptome-level response to drought; while a core set of genes responded to drought throughout the day, the magnitude of the response was strongly time-dependent. *TOC1* is a critical player linking the clock to ABA-mediated drought responses, and not only contributes to output from the clock to the ABA network but also is implicated in input to the clock from ABA; *TOC1* expression is acutely induced by ABA in a clock-gated manner [17•].

Global studies have focused on plants found in temperate and sub-tropical climates. However, little is known about the circadian gene networks of plants that grow under constant day lengths and temperatures over the years. Recent genomic and computational analysis of the circa-

dian transcriptome of *Carica papaya* indicated that despite its current tropical habits, this plant exhibits conserved transcriptional networks with circadian clock genes cycling with the same phase as Arabidopsis [68]. These results suggest that circadian timing has played an important role in the evolution of plant genomes.

Fitness

An underlying premise to the study of circadian rhythms has been that the circadian clock allows coordination with the temporal environment, which enhances fitness. Data have accumulated showing that a functioning circadian clock enhances survival and biomass accumulation [69,70]. Intriguingly, altered clock function contributes to the increased growth, called 'hybrid vigour,' observed in hybrids and allopolyploids [71••]. Intuitively, given the plethora of processes regulated by the clock, one might expect that the mechanisms by which the circadian clock confers a growth advantage may be both many and complex. In Arabidopsis, net photosynthesis is greatest when the endogenous circadian period matches the environmental period [70]. This is satisfying, but may not be the whole story.

In Arabidopsis, starch synthesis and starch utilization are among those processes regulated by the clock [72••]. During the day some photosynthate accumulates as starch to support metabolism and growth at night (Figure 4). Starch degradation commencing at dusk proceeds at an essentially linear rate such that almost all of the starch is used by dawn, with the timing of dawn predicted by the circadian clock to be ~24 h after the last dawn (Figure 4). Thus, in wild type plants grown in long (28 h) days starch is depleted before dawn resulting in the induction of carbon starvation stress. Similarly, in the short-period *cca1lhy* mutant grown in 24 h days, the clock predicts an early dawn with the result that starch is depleted before dawn, again with carbon starvation stress. Both these conditions result in reduced growth relative to growth when the endogenous circadian period matches the environmental period. Conversely, wild type plants grown in short (17 h) days fail to fully utilize starch at night and greet the dawn with residual starch. This suboptimal allocation of carbon to storage and growth extracts a penalty of reduced growth. Although it is tempting to conclude that it is the match of endogenous and environmental period length that is critical, data with the short-period *toc1-2* and long period *ztl-3* mutants argue that this is too simple. Both mutants grow better in 24 h days than in days matching their periods (20 or 28 h, respectively). Graf *et al.* [72••] suggest that perhaps these mutations fail to perturb normal control of starch degradation, noting that not all clock outputs are equally affected by these mutations. The undeniable importance of the influence of the circadian clock on growth and biomass in Arabidopsis and, by extension, on yield in agricultural systems, impels further investigation.

Conclusions

Interlocked molecular feedback loops at the heart of the clock are responsible for the rhythmic behaviors observed in plants and other systems. Determining how the central oscillators control their targets to explain biochemical, physiological, and behavioral rhythms will allow a deeper understanding of the roles of biological rhythms in enhancing growth and fitness. Genome-wide approaches to measure molecule levels such as microarray, next generation sequencing technologies, proteomics and metabolomics will continue to play important roles in the characterization of the output pathways of the circadian clocks. However, detailed information on physical and regulatory interactions is needed for a better understanding of the molecular networks underlying circadian rhythms. Moreover, integrating these data coherently in systems level models is essential for understanding biological rhythms in plants and other systems.

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- • of special interest
- •• of outstanding interest

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