Wheels within wheels: new transcriptional feedback loops in the Arabidopsis circadian clock

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Abstract

The circadian clock allows organisms to temporally coordinate their biology with the diurnal oscillation of the environment, which enhances plant performance. Accordingly, a fuller understanding of the circadian clock mechanism may contribute to efforts to optimize plant performance. One recurring theme in clock mechanism is coupled transcription-translation feedback loops. To date, the majority of plant transcription factors constituting these loops, including the central oscillator components CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and TIMING OF CAB2 EXPRESSION 1 (TOC1), and the related PSEUDO-RESPONSE REGULATORS (PRRs), are transcriptional repressors, leading to a model of the clock emphasizing repressive interactions. Recent work, however, has revealed that a subset of the REVEILLE (RVE) family of Myb transcription factors closely related to CCA1 and LHY are transcriptional activators in novel feedback transcription-translation feedback loops. Other recently identified transcriptional activators that contribute to clock function include LIGHT-REGULATED WD 1 (LWD1) and LWD2 and night light-inducible and clock-regulated transcription factors NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED1 (LNK1) and LNK2. Collectively, these advances permit a substantial reconfiguration of the clock model.

Introduction

The alternation of day and night, a consequence of the rotation of the earth on its axis, means that organisms experience dramatic yet rhythmic and hence predictable environmental change. The circadian clock is an endogenous timekeeping mechanism that enables organisms not simply to respond as they experience environmental change but, importantly, to anticipate and prepare for coming change. This ability to coordinate with the environment enhances fitness in bacteria, plants, and animals [1]. Any fitness advantage accruing from circadian clock function has multiple potential bases because the circadian clock regulates many aspects of biology, including, in plants, basic metabolism, hormone signaling, and responses to biotic and abiotic stresses [2-4].

The molecular mechanisms of the circadian clock in eukaryotes studied to date, including fungi, flies, mammals, and plants, are rooted in coupled transcription-translation feedback loops (TTFLs) [5,6]. Initially these were thought to be relatively simple loops, but it has become clear that most eukaryotic circadian oscillators are based on multiple interlocked TTFLs [6]. Although eukaryotic clocks share this common architecture of multiple interlocked TTFLs, the transcription factors constituting TTFLs are largely distinct among plants and animals and fungi. Thus, efforts to manipulate the circadian clock in order to improve plant performance will require enhanced understanding of the oscillatory mechanism in plants. Excitingly, the last several years have witnessed tremendous advances in our understanding of the mechanism of the plant circadian

clock. This report will describe some recent advances that pertain directly to the roles of transcriptional regulation in the oscillator mechanism.

Evolution in the model of the plant circadian clock

The initial model of the plant circadian clock was a negative feedback loop in which the *PRR* gene, *TOC1*, was posited to encode a positive regulator of two genes encoding MYB transcription factors, *CCA1* and *LHY*, which themselves encode repressors of *TOC1* expression [7].

Over the next several years, a number of new interlocking loops were characterized and integrated into the clock model, primarily through analysis of mutants and of gene expression data. CCA1 HIKING EXPEDITION (CHE) is a TCP transcription factor that binds specifically to the *CCA1* promoter as a transcriptional repressor [8]. CHE was identified through a yeast-one-hybrid screen of a library of Arabidopsis transcription factors. A variety of *in vitro* and *in vivo* studies confirmed that CHE binds to the *CCA1* promoter as a repressor. This relationship is reciprocal, as CCA1 binds to the *CHE* promoter to repress transcription [8]. This study also established via chromatin immunoprecipitation (ChIP) studies that TOC1 binds to the *CCA1* promoter [8].

Experimental and modeling studies supported a morning loop in which CCA1/LHY positively regulates *PRR7* and *PRR9*, which encode repressors of *CCA1* and *LHY* [9,10]. Modeling also predicted an evening loop in which a hypothetical component, Y, possibly including GIGAN-TEA (GI), activated TOC1, which repressed Y [9,11].

Biochemical analyses have dramatically enriched our understanding of the evening loop. A set of three evening-peaking clock components, including EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX, also called PHYTOCLOCK 1 (PCL1) [12-16], were shown to assemble into a so-called "evening complex" (EC) [17,18]. LUX is a DNA-binding protein and recruits the EC, which functions as a transcriptional repressor, to targets that include *PRR9* and *LUX* itself [16-20]. This negative autoregulation of the EC allowed a refinement of the model of the Arabidopsis clock, replacing hypothetical component Y with the EC [21].

A second set of studies revisited the long-standing interpretation of TOC1 as an activator of *CCA1* and *LHY* expression, initially proposed because in a loss-of-function *toc1* mutant background mRNA accumulation of *CCA1* and *LHY* was diminished [7,22]. However, later studies showed that TOC1 overexpression reduced expression of *CCA1* and *LHY* [23]. Moreover, the three

TOC1-related PRRs, PRR9, PRR7, and PRR5, were established as transcriptional repressors whose overlapping expression patterns served to provide sequential and extended repression of *TOC1* transcription throughout the day [24]. Thus, the role of TOC1 as a transcriptional activator was called into question.

Recent studies have established unambiguously that TOC1, like its PRR relatives, is a transcriptional repressor [25,26]. Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) identified TOC1 targets, which included morning-phased (*CCA1*, *LHY*, *PRR9*, and *PRR7*) and evening-phased (*GI*, *ELF4*, and *LUX*) clock genes. TOC1 binding peaked antiphase to target gene expression, and experimental manipulation of (either elevated or reduced) *TOC1* expression affected target gene expression consistent with repression by TOC1 [26]. A second study showed that TOC1 bound the *CCA1* promoter *in vitro* and *in vivo*. Chemical induction of *TOC1* repressed *CCA1* and *LHY* expression [25]. Transient overexpression of TOC1 allowed the identification of both upregulated and downregulated genes [25], leaving open the possibility that TOC1 may also function, directly or indirectly, as a transcriptional activator. Although both TOC1 and its three PRR relatives share repressor function, there are intriguing differences among them; the repressor function of PRR9, PRR7, and PRR5 requires a co-repressor, encoded by members of the *TOPLESS/TOPLESS-RELATED* (*TPL/TPR*) gene family [27], whereas the repressor function of TOC1 seems to be intrinsic [25]. Another recent demonstration of co-repressor function in the clock mechanism is that CCA1 and LHY recruit the COP10-DET1-DDB1 (CDD) complex to the *TOC1* and *GI* promoters and that DET1 (DE-ETIOLATED1) serves as a transcriptional co-repressor necessary for CCA1- and LHY-mediated inhibition of *TOC1* and *GI* transcription [28].

The characterization of TOC1, PRR9/PRR7/PRR5, and the EC as transcriptional repressors was integrated into a new model of the clock as a three-component repressilator, a ring oscillator consisting of three repressors, CCA1/LHY, the EC, and PRR9/PRR7/PRR5/TOC1 [21]. Although this model is quite attractive and does a very good job in matching a variety of experimental data, it is clearly an oversimplification. For example, TOC1, PRR9, PRR7, and PRR5 are pooled into a single repressor, and CCA1 and LHY are pooled as a second repressor. However, these components are not simply redundant. For example, CCA1 and LHY can be distinguished by their temperature response, with LHY more important than CCA1 for clock function at higher temperatures and CCA1 more important than LHY at lower temperatures [29]. Similarly, among EC components, ELF3 and ELF4

show dampened transcript cycling in the cold, whereas LUX cycling maintains a robust cycling amplitude [30]. TOC1 and the PRRs are all transcriptional repressors, but they exhibit temporally distinct expression patterns, and mutants defective in PRR function display different phenotypes—most obviously *toc1* mutants have a short period [31] whereas *prr7* and *prr9* mutants have a long period and the double *prr7 prr9* mutant has an extremely long period at high temperatures but has a wildtype period at low temperatures [32-34]. A second over-simplification is that the model fails to incorporate post-transcriptional control, which is becoming increasingly prominent in the plant clock. Among the PRRs, PRR5 has been shown to interact with TOC1 and this interaction regulates TOC1 phosphorylation and nucleocytoplasmic partitioning [35]. Considerable recent work emphasizes a role for alternative splicing in plant clock function [36-42]. Phase-dependent phosphorylation of CCA1 alters its DNA-binding affinity [43]. TOC1 and all the PRRs undergo clock-dependent changes in the phosphorylation state [44]. TOC1 and PRR5 show clock-regulated proteasomal degradation mediated by interaction with the F-Box protein ZEITLUPE (ZTL) [44-46].

A second prominent feature of the repressilator model is, as noted by David Somers, "a dearth of activators" [47]. Of course, a repressor of a repressor is formally an activator. In this sense, CCA1 and LHY could be activators of PRR7 and PRR9 by virtue of repression of the EC, itself a repressor of PRR7 and PRR9. Nonetheless, it seems unlikely that the plant clock TTFL would function without transcriptional activators. Indeed, several recent studies have defined roles for several transcriptional activators in the plant oscillator mechanism. Although CCA1 is a repressor, it can also function as a transcriptional activator when transiently expressed in protoplasts [41]. Indeed, in the *cca1-11 lhy-21* double-mutant background, cold induction of the three *C-REPEAT BINDING FACTOR* (*CBF*) genes is diminished, implicating CCA1 and LHY as transcriptional activators of these targets.

A second example of activation of gene expression is by LIGHT-REGULATED WD1 (LWD1) and LWD2. The *lwd1 lwd2* double mutant has a significantly shortened period, and the expression of multiple clock genes is greatly reduced [48,49]. ChIP experiments established that LWD1 binds directly to the *PRR9*, *PRR5*, and *TOC1* promoters, implicating it as a transcriptional activator of these genes [49]. *LWD1* and *LWD2* expression is greatly attenuated in a *prr9* mutant, suggesting that PRR9 may be an activator of their expression. However, direct interaction of PRR9 with the *LWD1* and *LWD2* promoters has not been demonstrated and, given the determination that the PRRs are repressors [24], it may be that this positive regulation of *LWD1* and *LWD2* by PRR9 is indirect, via the repression of a repressor.

CCA1 and *LHY* are members of a larger clade of Myb transcription factor genes, including eight *REVEILLE* (*RVE*) genes, and most of these *RVEs* show circadian-regulated expression [50,51]. Three of these, *RVE1*, *RVE2* (also called *CIRCADIAN 1/CIR1*), and *RVE7* (also called *EARLY-PHYTOCHROME-RESPONSIVE1/EPR1*), chiefly play roles in clock output pathways [52-54]. However, *RVE8* (also called *LHY-CCA1-LIKE5/LCL5*) seems to function more centrally in the clock oscillator. RVE8 binds to the *TOC1* promoter, where it is associated with increased acetylation of histone H3, which is associated with increased transcription [55]. RVE8 also binds to the evening element (EE) in the *PRR5* promoter to activate *PRR5* transcription [51,56]. The four remaining RVEs (RVE3, 4, 5, and 6) also bind to the EE [51,57], raising the possibility of functional redundancy among them. Indeed, mutants defective in RVE4 or RVE6 function have little effect on period length but, when combined in either double- or triple-mutant combinations, enhance the long-period phenotype of *rve8-1* [56]. Earlier work had identified an EE-binding activity present in wildtype plant extracts in the afternoon, which had been suggested as a potential activator of evening-phased clock genes, such as *PRR5* and *TOC1* [58]. This recent work strongly suggests that RVE4, RVE6, and RVE8 constitute this afternoon-phased activator of *PRR5* and *TOC1* as well as of other evening-phased clock genes, including *GI*, *ELF4*, and *LUX*, and apparently of the morning-phased *PRR9* [56]. Several pieces of evidence indicate that the PRRs feed back to repress *RVE8*. *RVE8* expression increases in the *prr5 prr7 prr9* triple mutant [51], and PRR5 binds directly to the *RVE8* promoter [59]. Thus, RVE8 (and presumably RVE4 and RVE6) and PRR5 (and possibly PRR7 and PRR9) constitute a negative feedback TTFL [56].

Very recently, two LNK transcription factors, LNK1 and LNK2, have been shown to activate transcription of afternoon-peaking clock-regulated genes, including the critical clock genes *PRR5* and the EC component *ELF4*, as well as the clock regulated F-BOX protein gene *FKF1* that plays a critical role in flowering time [60]. TOC1 and PRR9/7/5 each bind to the *LNK1* and *LNK2* promoters, and *LNK* mRNAs accumulate to increased levels in *toc1* and in *prr7 prr9* mutants. Thus, the LNKs and the PRRs form negative feedback loops in which the LNKs activate *PRR* transcription, and the PRRs feed back to repress *LNK* transcription.

Future directions

Although Somers's "dearth of activators" has been at least partially redressed by the identification of LWDs as

transcriptional activators of *PRR9*, of RVE8 (and RVE4 and RVE6) as transcriptional activators of *TOC1*, *PRR5*, and other EE-regulated genes, and of LNKs as transcriptional activators of *PRR5* and *ELF4*, a number of key questions remain. The regulation of *CCA1* and *LHY* remains incompletely resolved. Are there transcriptional activators of these two critical clock genes? Moreover, although these two genes are typically pooled in our consideration and in our models, they can be distinguished on a number of grounds. As mentioned above, the expression of *CCA1* and *LHY* differs in response to temperature, contributing to temperature compensation of the clock, but it remains unclear how this is effected. More broadly, chromatin modifications have only been mentioned in passing.

This report has focused on transcriptional regulation but included a partial enumeration of some of the levels of post-transcriptional regulation employed by the plant circadian clock. One emerging area is the role of nucleocytoplasmic partitioning in the regulation of clock protein function. As mentioned above, PRR5 regulates not only the phosphorylation of TOC1 but also the nuclear import and subnuclear localization of TOC1 [35], with an obvious implication for TOC1 as a transcriptional repressor. Two recent papers extend this mode of regulation, via subnuclear and nuclear-cytoplasmic partitioning, to a second clock component, GI [61,62].

The circadian clock is not unusual in employing regulatory mechanisms that include transcriptional and post-transcriptional aspects, but the implications for complexity and the challenges of assembling a complete and nuanced model of the clock mechanism are quite obviously significant. While the challenges are great, the rewards will be commensurate. Plant biologists face the daunting challenge of providing increased agricultural production in the face of a declining agricultural landmass that is being altered by resource depletion, pollution, and a changing climate. Impaired circadian function reduces plant growth and fitness, offering the hypothesis that optimizing circadian function will enhance crop productivity, particularly in crops grown over broad latitudinal ranges. Greater refinement of our understanding of the circadian clock mechanism is necessary to inform manipulation of the circadian clock towards the goal of enhancing agricultural productivity.

Abbreviations

CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CHE, CCA1 HIKING EXPEDITION; ChiP, chromatin immunoprecipitation; EC, evening complex; EE, evening element; ELF, EARLY FLOWERING; GI, GIGANTEA; LHY, LATE ELONGATED HYPOCOTYL; LNK, NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED, LNK; LUX, LUX ARRHYTHMO; LWD, LIGHT-REGULATED WD; PRR, PSEUDO-RESPONSE REGULATOR; RVE, REVEILLE; TOC1, TIMING OF CAB2 EXPRESSION 1; TTFL, transcription-translation feedback loop.

Disclosures

The author declares that he has no disclosures.

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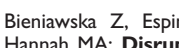
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