Genome Biology

RESEARCH HIGHLIGHT

The *Arabidopsis* clock: time for an about-face?

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Abstract

Three recent studies have altered thinking on the role of TIMING OF CAB EXPRESSION 1 (TOC1), a core element in the plant circadian oscillator.

The circadian clock controls rhythmic changes in processes and development as wide ranging as gene expression and flowering time. This 24-hour time-keeper coordinates many molecular, physiological and metabolic processes to optimize the plant's health and survival in an ever-changing environment. It is the regular cycle of light and dark, warm and cool that serves to keep the oscillator in register with the natural world.

The search for the molecular components of the *Arabidopsis* central oscillator and for an understanding of how they interrelate has occupied plant clock biologists for at least 20 years. In that time genetic and molecular approaches have been successful in identifying more than 30 genes that either participate directly in the oscillator mechanism or contribute to its maintenance. Two central classes of plant clock genes are: the transcription factors CCA1 and LHY, which have strong morning-phased oscillations in transcript and protein levels (that is, with a peak in the morning); and the five closely related pseudo-response regulator (PRR) proteins, whose defining member is the evening-phased TOC1 (TIMING OF CAB EXPRESSION 1, also called PRR1). Loss of either class causes the clock to run significantly faster, and a molecular connection between the two has been characterized whereby CCA1/LHY bind to the *TOC1* promoter, repressing its expression, and *TOC1* mutant alleles result in strongly diminished *CCA1* and *LHY* expression levels [1]. This latter finding implied that *TOC1* normally activates *CCA1*/*LHY* expression, neatly forming an autoregulatory negative feedback loop consistent with models of clock regulation in animals and fungi.

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The role of TOC1 as an activating element remained dogma for many years, despite evidence that three other PRR family members work together in a temporal series to sequentially repress *CCA1/LHY* expression throughout the afternoon and night [2]. However, three new reports [3-5] present compelling experimental evidence and revised computational modeling to show that TOC1 acts extensively as a circadian transcriptional repressor.

TOC1 as a DNA-binding transcription factor and repressor

Approaching the role of TOC1 comprehensively, Huang and co-workers [3] first performed genome-wide chromatin immunoprecipitation sequencing (ChIP-Seq) to obtain a high-resolution map of TOC1 chromatin occupancy. They identified 772 potential TOC1 target genes, 40% of which were regulated by the circadian clock. Interestingly, the great majority displayed an early morning phase, antiphase to the early evening peak of TOC1 expression [6]. Many known oscillator components were among this group, both morning-phased (*CCA1, LHY, PRR9* and *PRR7*) and evening-phased (*GIGANTEA* (*GI*), *EARLY FLOWERING 4* (*ELF4*) and *LUX ARRHYTHMO* (*LUX*)) genes, with the latter group including *TOC1* itself. Analysis of TOC1-bound sequences identified a G-box (CACGTG)-related motif and a region similar to the evening element previously associated with evening-expressed genes. ChIP validation of specific clock genes showed oscillations in TOC1 chromatin occupancy that coincided with the evening peak of TOC1 protein rhythms.

As chromatin residency does not define function, these findings were extended with experimental manipulations of TOC1 expression. Overexpression of *TOC1* was able to suppress all morning (including *CCA1*) and evening genes tested in a dose-dependent manner, as was transient expression of a dexamethasone-inducible TOC1 construct, suggesting that TOC1 acts as a repressor at all times of the day. Conversely, constitutive reduction in *TOC1* expression by RNA interference or the *toc1-2* mutant raises the levels of *LHY, PRR7, PRR9* and *GI* expression at times generally correlating with TOC1 protein accumulation. Taken together, Huang *et al.* [3] concluded that TOC1 represses a wide range of clock-correlated genes throughout the day, altering the basic

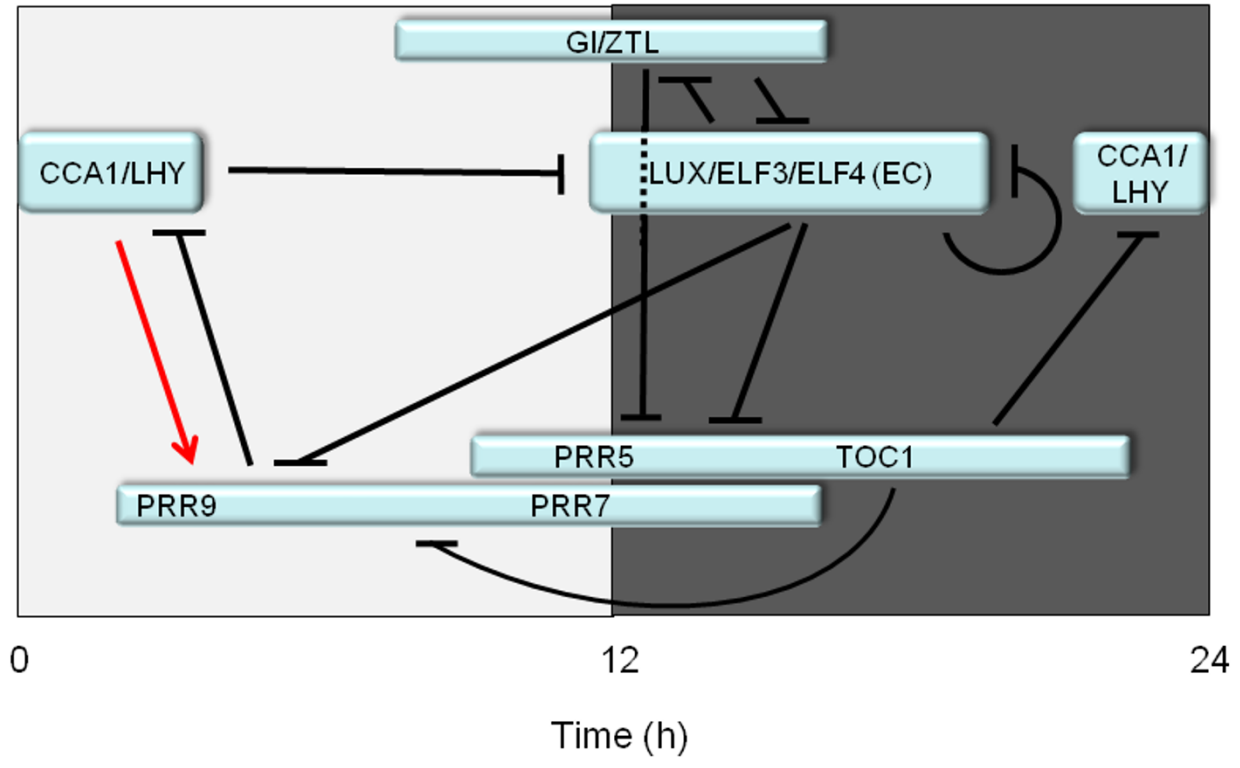

Figure 1. Simplified scheme of regulatory interactions of some core *Arabidopsis* clock components. The morning proteins CCA1/LHY are the sole directly activating elements (red line) in this overview, acting to promote the transcription of *PRR9* and *PRR7*. Repressive relationships (black lines) are based on the papers discussed [3-5] and previously published work. Positioning along the 12-hour:12-hour light:dark time axis approximates the period of maximal protein expression, as does the placement of the PRR/TOC1 names within the respective bars. PRR9 and PRR7 have been grouped separately from PRR5 and TOC1 on the basis of similarities between the partners within each group with respect to single mutant phenotypes, known dimerizations, effects on expression of certain genes and phase of expression. The LUX-ELF3-ELF4 evening complex (EC) consists of a DNA-binding transcription factor (LUX) that requires ELF3 and ELF4 as co-factors for full activity. The co-stabilized GI-ZTL (ZEITLUPE) complex functions to degrade PRR5 and TOC1 proteins, but GI probably has a separate role in its effects on the EC. The dotted line indicates passage of the relational indicator behind the EC bar.

model of reciprocal regulation of *CCA1/LHY* and *TOC1* (note these are genes being regulated not proteins) that first founded the molecular basis of the plant circadian oscillator.

Similar conclusions have come through different approaches by Gendron and colleagues [4]. Their work is the first to establish that TOC1 can bind DNA directly, by performing high-resolution ChIP at the *CCA1* promoter followed by *in vitro* electrophoretic mobility shift assays. This new result has resolved the fundamental question of how TOC1, and the closely related PRRs, function molecularly - as DNA-binding proteins. They further identified a TOC1-binding T1ME element within the *CCA1* promoter, which contains a motif shared with the morning element and Hormone Up at Dawn element [7] but differs from the sequences reported by Huang and co-workers [3].

The T1ME element further differs from a second set of motifs that Gendron *et al.* [4] identified through an extensive microarray analysis using an ethanol-inducible *TOC1* transgenic line. Here, *TOC1* was transiently induced and global gene expression changes in either 12:12 light:dark or constant light cycles were observed over a 24-hour period. In both conditions, almost equal numbers of genes were upregulated and downregulated, potentially positioning TOC1 as both an activator and repressor. Differentially expressed genes were enriched for both the dawn and dusk phases of light transition, corresponding to a potential direct regulation of dawn-phased genes and indirect regulation of dusk genes through effects on dawn targets. When examining *cis* elements shared among the up- and downregulated genes, three sequence elements were found to be enriched, with each motif associated with a specific change in direction of expression. Although the T1ME element was not among this group, a variant of the G-box, remarkably similar to the sequences reported by Huang *et al.* [3], was found among the upregulated genes.

Using transient bombardments and the ethanol-inducible system, Gendron *et al.* [4] confirmed the same *CCA1* suppression phenotype described by Huang *et al.* [3] and showed that this required the DNA-binding capability of TOC1. Using a Gal4-LexA/UAS system, the repressive activity of TOC1 was again confirmed and narrowed down to the amino-terminal PRR domain. This is the same region responsible for TOC1/PRR protein heterodimerization, and it also mediates TOC1 degradation [6,8], pointing to a complex role for this domain *in vivo*. The PRR domain alone was insufficient for *CCA1* repression, indicating that the carboxy-terminal CCT domain is additionally necessary for this function *in vivo*.

A dearth of activators

How do these findings affect our view of the plant clock? Based on the new experimental work [3,4] and on recent modeling efforts from Pokhilko and co-workers [5], the currently annotated plant circadian system is one replete with transcriptional repressors and remarkably low in transcriptional activators (Figure 1). Indeed, all five PRR/TOC1 proteins are now designated repressors, as are the core morning proteins, CCA1 and LHY, in their functional interaction with evening genes. The new models lack any activating elements at all as part of the evening oscillator and retain only *CCA1/LHY* (note this is a genetic interaction) as positively acting on *PRR9* and *PRR7* transcription in the morning loop [3,5]. However, a double negative of serial repression can activate transcription: repression by A of repressor B of gene C will cause derepression (activation) of gene C. Indeed, Pokhilko *et al.* [5] point out similarities between aspects of their model and a 'repressilator', which creates oscillations through successive repressions of one gene by the previous one, in a loop of three or more genes [9]. Although the three-gene repressilator embedded in the Pokhilko model [5] oversimplifies the plant oscillator by bundling all PRR functions into one repressor, its inclusion does emphasize the reality of the current preponderance of repressors. It is possible that such a repressilator mechanism underlies much of the plant clock system, but it is more likely that many other components and relationships have yet to be fitted to these newest schemes.

Most lacking in current models is an adequate incorporation of the role of post-transcriptional processes. TOC1 and all the PRR proteins undergo phase-dependent changes in phosphorylation state [6], and for the most part the significance of these modifications is unknown. In addition, among the PRR family a number of heterodimeric interactions are known. TOC1 can homodimerize, as well as heterodimerize, with PRR3, PRR5 and PRR9 [6,8]. These interactions have the potential of not only altering DNA-binding affinity, but changing the recruitment of co-factors that could turn repressors into activators, and *vice versa*. The Gendron *et al.* [4] work supports this notion of a more varied and nuanced transcriptional role for TOC1 in the finding of both up- and downregulated gene expression associated with its expression.

Current models also poorly explain findings reported in triple and higher-order mutant backgrounds. Most notably, the nearly complete removal of the PRR repressor family - the *prr9 prr7 prr5 toc1* mutant - might be expected to cause a strong and consistent upregulation of *CCA1* and *LHY*. Although overall clock gene expression becomes arrhythmic and *CCA1* levels do rise significantly during the normal trough times, the normal strong peak of *CCA1* expression around dawn is entirely eliminated, with expression at this time four- to five-fold lower in the quadruple (or *prr9 prr7 prr5* triple) mutant than in wild type [10]. Not all clock-controlled transcripts descend to trough levels in these mutants - some flatten towards peak expression levels - further emphasizing the likely incomplete nature of current clock mechanism models.

Finally, a difficult but real complication in dissecting the clock mechanism is the strong intersection between light signaling and the circadian system. Huang *et al.* [3] performed the majority of their work under light:dark cycles of either 12:12 or 8:16, overlaying photic oscillations onto the circadian mechanism. Their evidence is strong that TOC1 represses many genes under these conditions, but in extended constant light *CCA1* and *LHY* levels in *toc1* mutants do drop markedly. Experiments using thermal cycles (warm:cold) to maintain entrainment in constant light or darkness may be necessary to unravel these closely connected processes.

Taken together, the genomic and molecular approaches used in these recent studies both clarify and complicate our understanding of the basis of the plant circadian system. TOC1 has been cast into a much wider role than previously expected, and there is now a fuller appreciation of how pervasive its effect is both within the circadian oscillator itself and in connection to physiological, metabolic and developmental pathways.

Competing interests
The author declares that he has no competing interests.

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