Title:

Global Mapping at the Core of the Arabidopsis Circadian Clock Defines the Network Structure of the Oscillator

Authors: W. Huang[1], P. Pérez-García[1], A. Pokhilko[2], A.J. Millar[2,3], I. Antoshechkin[4], J.L. Riechmann[1,4,5] and P. Mas[1]*

Affiliations:

[1]Center for Research in Agricultural Genomics (CRAG), Barcelona 08193, Spain.

[2] School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom.

[3]Centre for Systems Biology at Edinburgh, C.H. Edinburgh EH9 3JD, United Kingdom.

[4]California Institute of Technology, Division of Biology, Pasadena, CA 91125, USA.

[5]Institució Catalana de Recerca i Estudis Avançats, Barcelona 08010, Spain.

*Correspondence to: paloma.mas@cragenomica.es

Abstract:

In many organisms, the circadian clock is composed of functionally coupled morning and evening oscillators. In Arabidopsis, oscillator coupling relies on a core loop in which the evening oscillator component TOC1 (TIMING OF CAB EXPRESSION1) was proposed to activate a subset of morning-expressed oscillator genes. Here we show that TOC1 does not function as an activator but as a general repressor of oscillator gene expression. Repression occurs through TOC1 rhythmic association to the promoters of the oscillator genes. Hormone-dependent induction of *TOC1* and analysis of RNAi plants show that TOC1 prevents the activation of morning-expressed genes at night. Our study overturns the prevailing model of the Arabidopsis circadian clock showing that the morning and evening oscillator loops are connected through the repressing activity of TOC1.

One Sentence Summary: The morning and evening feedback loops at the core of the Arabidopsis oscillator are connected through the repressing activity of the key clock component TOC1.

Main Text:

The molecular circuitry underlying circadian rhythms relies on the reciprocal regulation of clock components forming negative feedback loops at the core of the oscillator (*1*). However, deciphering the oscillator transcriptional regulatory code is a major challenge due to the interplay among clock activators and repressors, which are responsible for the generation of the loops. In Arabidopsis, two MYB transcription factors, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (*2, 3*) together with the pseudo-response regulators PRR5, 7 and 9 (*4*) function as key components of a so-called morning loop, which interlocks with an evening loop (*5, 6*) composed of TOC1 (or PRR1) (*4, 7*), GIGANTEA (GI) (*8, 9*) and a hypothetical component Y (*10*). The evening loop also includes the Evening Complex (EC) composed of EARLY FLOWERING 3 (ELF3), 4 (ELF4) and LUX ARRHYTHMO (LUX) (*11, 12*).

Regulation of TOC1 rhythmic expression is essential for proper functioning of the clock (*13, 14*). Mechanisms that contribute to this regulation include changes in chromatin structure (*15*), transcriptional regulation (*16*) and protein degradation (*17*). Alterations of this regulation affect clock function and result in disrupted circadian gene expression and changes in time to flower (*7, 18, 19*). TOC1 also connects environmental signals with clock-controlled photo-morphogenic and hormonal outputs (*18, 20*). Gene expression analysis using *toc1* mutant plants led to the hypothesis that TOC1 inhibits its expression by activating the repressors CCA1 and LHY (*16, 21*). The direct interaction of TOC1 with CCA1 HIKING EXPEDITION (CHE) was proposed as a regulatory link connecting TOC1 with the activation of *CCA1* (*21*). Our study changes the sign of TOC1 function at the core of the clock and shows that the morning and evening feedback loops are connected through the repressing activity of TOC1.

We used chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) to obtain a high-resolution map of TOC1 chromatin occupancy on a genome-wide scale (*22*). The ChIP experiments were performed with TOC1 Minigene (TMG) seedlings expressing the genomic fragment of *TOC1* fused to the Yellow Fluorescent Protein (*18*) in the *toc1-2* (hypomorphic mutant) background (*7*) (fig. S1). ChIP-Seq data analysis in search for genes that contained one or more TOC1 binding regions identified 867 peak-to-gene associations (ChIP-Seq peak p-value≤0.001) corresponding to 772 potential TOC1 target genes (Supporting Online Material, table S1 and fig. S2). Consistent with previous studies (*20, 21*), we found that *CCA1*

and *ABAR/CHLH/GUN5*, binding targets of TOC1, were present in our gene list. The *ABAR* circadian expression is repressed by TOC1 (*20*).

As TOC1 is a key component of the Arabidopsis circadian system, we analyzed the proportion of TOC1-binding genes that were under the control of the circadian clock. Using different circadian gene expression datasets (*23, 24*), we found that 40% of TOC1 targets were regulated by the circadian clock (table S1, S2, S6 and fig. S3, S4). Analysis of peak phases of expression revealed a phase enrichment around dawn (Fig. 1A-C, table S3), which suggests that TOC1 modulates clock genes expressed during the morning phase. We also found that the promoters of the oscillator components were occupied by TOC1. The targets included genes from both the morning (*CCA1, LHY, PRR9, PRR7*) and the evening loops (*GI, TOC1, ELF4* and *LUX*) (Fig. 1D-G and table S4). We also identified other components associated to the oscillator such as *PRR5* (*13*) whereas other clock-related genes more indirectly linked to the transcriptional regulation of the oscillator such as *ZTL, FKF1* or *LKP2* (*13*) or clock genes expressed only in specific tissues (e.g. *PRR3*) (*13*) were not found as putative TOC1 targets. Independent ChIP-Q-PCR analyses showed specific amplification of the regions identified by ChIP-Seq, consistent with the binding of TOC1 to the promoters of the oscillator genes (fig. S5). We further validated our results by using TOC1 over-expressing plants (TOC1-ox), in which TOC1 is constitutively expressed. In these plants, TOC1 occupancy at the oscillator loci was significantly higher than occupancy at the promoters of genes not present in our ChIP-Seq list (fig. S5). We therefore report the association of TOC1 to the promoters of the morning and evening oscillator genes. Binding might be indirect and may require other co-factors and/or the formation of higher order protein complexes.

Analysis of TOC1-bound oscillator sequences identified as significantly enriched a motif closely related to the GBox (named GBox-expanded) (Fig. 1H, fig. S6 and table S5). We also found that a motif very similar to the circadian motif, Evening Element (EE), was present in both morning and evening-expressed oscillator genes (named EE-like-expanded) (Fig. 1I, fig. S6). The EE motif was proposed to be associated with evening-expressed genes (*16, 25*). The motifs were positioned very close to the center of the TOC1-bound ChIP-Seq genomic regions (fig. S6). A similar positional enrichment at the peak maximum was observed when sequences of the circadian genes bound by TOC1 were analyzed (fig. S6). The results were consistent with a

ChIP-Q-PCR screening of the *CCA1* promoter, showing that amplicon enrichment decreased as the distance from the GBox and EE-like motifs increased (fig. S6).

We next analyzed the role of the circadian clock in modulating TOC1 interaction with its target genes by performing ChIP assays with TMG plants synchronized under 12h light:12h dark cycles (LD). The results revealed a rhythmic oscillation of TOC1 binding with a peak at Zeitgeber Time 15 (ZT15 - 3 hours after lights off; ZT0: lights on), the proposed time of TOC1 function (Fig. 2A-D). Similar oscillatory binding was observed in plants grown under constant light (LL) conditions (Fig. 2E-F) indicating a role for the circadian clock. The rhythmicity was abolished in TOC1-ox plants (Fig. 2G-H), suggesting that TOC1 binding is dictated by its protein abundance, which is under circadian regulation. These results together with the arrhythmic phenotypes of TOC1-ox (*18*) support the notion that the circadian pattern of TOC1 binding is relevant for TOC1 function in the clock.

We next examined oscillator gene expression in WT and TOC1-ox samples under LD and LL cycles. The peak expression of transcripts from the morning loop genes (*PRR9, PRR7, CCA1* and *LHY*) was significantly reduced in TOC1-ox (Fig. 2I-J, fig. S7, table S7). This is noteworthy as TOC1 was previously assumed to be an activator of *CCA1* and *LHY* expression (*16*). The reduced expression cannot be attributed to the activation of their known repressors PRR7 and PRR9, as their expression is also decreased in TOC1-ox plants. Analysis of the evening loop also revealed gene repression. For instance, *GI* and *ELF4* were reduced around dusk (Fig. 2K-L) and plants expressing the *LUX* promoter fused to the *LUCIFERASE* (*LUX::LUC*) also displayed a reduced promoter activity particularly during the night (fig. S8). In the morning loop, oscillator components that are expressed at a similar phase repress each other expression (e.g. CCA1 and LHY) (*2, 3*). Our results suggest that similar regulation occurs in the evening loop, where TOC1 might also repress genes expressed during the night. The observed function of TOC1 as a repressor is not likely due to constitutive over-expression of TOC1 as we also observed decreased oscillator expression in TMG plants (expressing *TOC1* under its own promoter) (Fig. 2I-L).

To further confirm the transcriptional repressing function of TOC1, we generated transgenic plants expressing TOC1 fused to the GR domain of the glucocorticoid receptor (*26*). In the absence of a steroid ligand, the GR domain retains a nuclear factor in the cytoplasm but

nuclear localization is restored in the presence of the synthetic glucocorticoid Dexamethasone (Dex). Treatment of TOC1-GR plants with Dex resulted in a reduction of *CCA1* and *GI* expression relative to the mock-treated controls (Fig. 3A-B and fig. S9). Our analysis revealed statistically significant differences between TOC1-GR+Dex and the other genotypes (+/-Dex) (Fig. 3D-E) while no significant variations were found in WT plants in the presence or in the absence of Dex. We also analyzed the effects of TOC1-GR induction by Dex on *CCA1::LUC* activity. The in vivo studies were consistent with the RT-Q-PCR data and showed a clear reduction of *CCA1::LUC* luminescence in Dex treated plants (Fig. 3C). To further address the repressing function of TOC1, we examined *CCA1* expression in TOC1-GR plants treated with Dex in the presence or in the absence of the protein synthesis inhibitor Cycloheximide (CHX). Our results showed that *CCA1* expression was reduced after Dex treatment in the presence of CHX (Fig. 3F) indicating that *CCA1* repression does not require de novo protein synthesis. We also utilized the previously described induction of *TOC1* by the plant hormone abscisic acid (ABA) (*20*). We analyzed *TOC1* induction by ABA and in parallel we examined *CCA1* and *PRR9* expression. The results showed that the induction of *TOC1* was accompanied by a concomitant reduction of *CCA1* and *PRR9* expression (Fig. 3G-I). Our studies, together with the results of *toc1* mutant plants (see Fig. 4) indicate that in contrast to its previously assigned activating role, TOC1 functions as a repressor of oscillator gene expression.

The repressive function of TOC1 provides an explanation for recently published experimental data that cannot be reconciled with an activating role for TOC1. For instance, analysis of *ztl* mutant plants, with increased TOC1 accumulation (*17*), revealed a reduced expression of *LHY* and *CCA1* (*27*), which is not consistent with TOC1 activating function. The data also showed low abundance of *PRR9* and *PRR7* expression (*27*). Thus, the reduced expression of *LHY* and *CCA1* cannot be attributed to increased abundance of their inhibitors. To clarify TOC1 function in the clock, we examined oscillator gene expression in TOC1 RNAi and *toc1-2* mutant plants grown under LD cycles. Our results showed an advanced phase for *LHY* expression at the onset of transcription (Fig. 4A and fig. S10) suggesting that the absence of TOC1 alleviates the repression normally observed in WT plants around dusk. The advanced phase of *LHY* expression correlated with a slight increase of *PRR7* (Fig. 4B and fig. S10). These results suggest that TOC1 temporally extends the repressing function of the other sequentially expressed PRRs inhibitors (*28*) (Fig. 4E). The absence of TOC1 in *toc1* mutant plants shortens

the duration of this repression and as a consequence, the onset of *LHY* transcription is advanced (Fig. 4F). The effects on the trough of *LHY* are subtle, most likely because the expression of other *CCA1/LHY* repressors is also increased (Fig. 4C-D). In TOC1-ox, the abnormally high expression of TOC1 during the day reveals its repressing function at times when TOC1 is not normally expressed (Fig. 4G). Altogether, our studies support the repressing function of TOC1 on oscillator gene expression, which changes the regulatory circuit at the core of the Arabidopsis circadian clock (fig. S11).

Conclusions about the circadian transcriptional networks are complicated by the existence of multiple feedback loops and gene redundancies. Our study shows the complex and dynamic function of TOC1 as a regulator of oscillator gene expression, with a repressing activity of the morning and evening loops. Binding of TOC1 to the promoters of the oscillator genes may antagonize transcriptional activation around the time of peak expression. The mechanisms of oscillator gene activation are largely unknown but chromatin remodelling and light-dependent regulation may play key roles at defined times of day. The challenge remains now to identify the mechanisms and components responsible for the activation of oscillator gene expression.

References and Notes

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Acknowledgments:

Fig. 1. Analysis of TOC1 ChIP-Seq circadian targets. Global trend of expression phases within TOC1 circadian targets (**A**, **B**) and for a random list of circadian genes (**C**). Phase enrichments were graphically portrayed within a range of ChIP-Seq p-values (legends) at different Zeitgeber Times (radial axis: 0-dawn; 12-dusk). The white and grey areas represent day and night, respectively. Peak traces of *LHY* (**D**), *PRR7* (**E**) *PRR9* (**F**) and *GI* (**G**) from TOC1 ChIP-Seq data. The exon-intron-UTR structure of the different gene models above and below each panel indicates forward and reverse orientation, respectively. Position weight matrix representation of consensus motifs present within the TOC1 target oscillator sequences. The Gbox-expanded (**H**) and EE-like-expanded (**I**) motifs were identified using the *SCOPE* motif finder. Both strands were used to compute the significance value. ChIP-Seq, peak visualization, circadian phase analysis and motif discovery were performed as described in Supporting Online Material.

Fig. 2. TOC1 binds to the promoters of the oscillator genes and inhibits their expression. ChIP-Q-PCR assays of TMG plants sampled every four hours over a 24-hour LD cycle. Primers encompassing target sequences obtained in the ChIP-Seq analysis were used to amplify *CCA1* and *LHY* (**A**); *PRR7* and *PRR9* (**B**), *GI* and *PRR5* (**C**) or *LUX/PCL1* and *ELF4* (**D**). Binding was also examined under LL conditions following entrainment in TMG (**E-F**) and in TOC1-ox plants (**G-H**). Values are represented as means ± SEM. Expression profiling of *PRR9* (**I**), *PRR7* (**J**), *GI* (**K**) and *ELF4* (**L**) in WT, TMG and TOC1-ox plants. Gene expression was analyzed in plants grown under LD cycles. mRNA abundance was normalized to *IPP2* expression. Values represent means ± SEM. White boxes: day; grey boxes: night.

Fig. 3. Effects of TOC1 transient induction on oscillator gene expression. Analysis of *CCA1* (**A**) and *GI* (**B**) in WT and TOC1-GR plants mock-treated or treated with 5 µM dexamethasone (+Dex). Seedlings were treated at ZT10 and samples were analyzed at the indicated ZT. (**C**) Analysis of *CCA1::LUC* luminescence in TOC1-GR seedlings under 8h light:16h dark cycles. Luminescence was recorded 24 hours after the seedlings were transferred to medium containing 5 µM of Dex. Data are presented as means ± SEM of luminescence signals from 6-7 independent plants. (**D**, **E** and **F**) Comparisons of *CCA1* expression in TOC1-GR plants mock-treated or treated with 5 µM of Dex at ZT10 and analyzed at ZT21 and ZT23 in the absence (-CHX) or in the presence (+CHX) of 50 µM of cycloheximide. The statistical relevance of the differences is presented (*p-value<0.05; **p-value<0.01; ***p-value<0.001). (**G**, **H** and **I**) Time course analysis of *TOC1*, *CCA1* and *PRR9* expression over the diurnal cycle in TMG plants in the presence or in the absence of the hormone Abscisic Acid (ABA). Seedlings were sprayed with 100 µM (±) ABA at ZT5. The Y axes of the *CCA1* and *PRR9* graphs were divided into segments so that repression at trough can be clearly observed. White boxes: day; grey boxes: night.

Fig. 4. Gene expression analysis in TOC1 RNAi plants. Analysis of *LHY* (**A**) and *PRR7* (**B**) expression under 12L:12D conditions. Gene expression analysis of *PRR9* (**C**) and *GI* (**D**) during the night in TOC1 RNAi under 12L:12D conditions. Values are represented as means ± SEM. Analysis was performed as described in Supporting Online Material. Schematic diagrams depicting the waveform of *CCA1/LHY* expression and the different waves of PRRs repression. Schemes depict the regulations in WT (black line) (**E**), *toc1* mutant (blue line) (**F**) and TOC1-ox (red line) (**G**). The small lines ending in perpendicular dashes represent repression. White boxes: day; black boxes: night.

Supporting Online Material

Materials and Methods

SOM Text

References (*29-45*)