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Clocks in the Green Lineage: Comparative Functional Analysis of the Circadian Architecture of the Picoeukaryote *Ostreococcus* [W]

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Biological rhythms that allow organisms to adapt to the solar cycle are generated by endogenous circadian clocks. In higher plants, many clock components have been identified and cellular rhythmicity is thought to be driven by a complex transcriptional feedback circuitry. In the small genome of the green unicellular alga *Ostreococcus tauri*, two of the master clock genes *Timing of Cab expression1* (*TOC1*) and *Circadian Clock-Associated1* (*CCA1*) appear to be conserved, but others like *Gigantea* or *Early-Flowering4* are lacking. Stably transformed luciferase reporter lines and tools for gene functional analysis were therefore developed to characterize clock gene function in this simple eukaryotic system. This approach revealed several features that are comparable to those in higher plants, including the circadian regulation of TOC1, CCA1, and the output gene *Chlorophyll a/b Binding* under constant light, the relative phases of *TOC1*/*CCA1* expression under light/dark cycles, arrhythmic overexpression phenotypes under constant light, the binding of CCA1 to a conserved evening element in the *TOC1* promoter, as well as the requirement of the evening element for circadian regulation of *TOC1* promoter activity. Functional analysis supports TOC1 playing a central role in the clock, but repression of CCA1 had no effect on clock function in constant light, arguing against a simple TOC1 /CCA1 one-loop clock in *Ostreococcus*. The emergence of functional genomics in a simple green cell with a small genome may facilitate increased understanding of how complex cellular processes such as the circadian clock have evolved in plants.

INTRODUCTION

Living organisms are exposed to predictable 24-h cycles in their environment due to the daily rotation of the earth. Organisms have thus evolved endogenous timekeeping mechanisms in order to anticipate the solar cycle and phase essential processes, such as photosynthesis and cell division, at the most appropriate time of the day. These cell-autonomous circadian rhythms persist under constant conditions with a period of ~24 h and can be reset by environmental cues, predominantly light and temperature (Jarrett and Edmunds, 1970; Samuelsson et al., 1983; Harmer et al., 2001). In eukaryotes, this cellular clockwork is thought to comprise multiple interlinked transcriptional/posttranslational feedback loops whereby clock proteins ultimately repress their own expression and also rhythmically regulate output genes (Dunlap, 1999; Lakin-Thomas, 2000). In higher plants, as much as a third of the transcriptome is estimated to be circadian regulated (Michael and McClung, 2003; Covington et al., 2008; Michael et al., 2008).

In plants, the majority of clock genes have been isolated through genetic screens for mutants with altered flowering timing or defects in the circadian expression of reporter genes, such as the *Light-harvesting complex*/*Chlorophyll a/b Binding* (*LHCB*/*CAB*) gene (Millar et al., 1995; Hicks et al., 1996; Wang et al., 1997; Schaffer et al., 1998; Fowler et al., 1999; Park et al., 1999; McWatters et al., 2000; Nelson et al., 2000; Doyle et al., 2002; Mizoguchi et al., 2005; Onai and Ishiura, 2005). For example, the *toc1-1 Arabidopsis thaliana* mutant displays a shorter period in several output rhythms, including *LHCB* (Millar et al., 1995; Somers et al., 1998).

Timing of Cab expression1 (*TOC1*) mRNA is normally expressed around subjective dusk and belongs to the family of pseudo response regulators (PRRs) that all have been shown to play a role in the regulation of circadian rhythms (Matsushika et al., 2000; Strayer et al., 2000; Mizuno, 2004; Mizuno and Nakamichi, 2005). Conversely, the *Late Elongated Hypocotoyl* (*LHY*) and *Circadian Clock-Associated1* (*CCA1*) genes are expressed around subjective dawn and are MYB transcription factors of the REVEILLE family (Wang et al., 1997; Schaffer et al., 1998; Wang and Tobin, 1998; Jin and Martin, 1999; Carre and Kim, 2002). LHY and CCA1 function to repress *TOC1* expression until dusk by binding to a conserved evening element (EE) sequence found in the *TOC1* promoter (Alabadi et al., 2001). Mutants overexpressing either *LHY* or *CCA1* display arrhythmic

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expression of *LHCB* (*CAB2*) and leaf movements, further indicating that both genes are central to the *Arabidopsis* clock (Alabadi et al., 2002; Mizoguchi et al., 2002). The low levels of *CCA1* and *LHY* mRNA observed in the *toc1-2* loss-of-function mutant led to the proposal that *TOC1* is an activator of *CCA1* in a transcriptional feedback loop. The robustness of rhythms in *toc1* mutants, however, as well as the reduction of *CCA1* and *LHY* mRNA levels observed in *TOC1*-overexpressing lines and in *zeitlupe* mutants, which are defective in TOC1 protein turnover, are inconsistent with a single negative feedback loop model (Alabadi et al., 2001; Makino et al., 2002). Many more clock-related genes have subsequently been identified in *Arabidopsis*, and a more complex picture of the circadian clock is now emerging.

Furthermore, mathematical modeling has been used to unravel the clock architecture with great success. For example, a three-loop mathematical model has been iteratively designed to extend the central *TOC1-CCA1/LHY* negative feedback loop. According to this model, two coupled oscillators involving TOC1 and GIGANTEA in the evening and LHY/CCA1 and PRR7/PRR9 in the morning, allow the tracking of dusk and dawn, respectively (Locke et al., 2005a, 2005b, 2006; Gould et al., 2006). Besides *TOC1*, *EARLY FLOWERING3* (*ELF3*) and *ELF4* are also necessary for the activation of *CCA1* and *LHY* under constant light conditions, and loss-of-function mutants in any of these genes leads to arrhythmic phenotypes (Schaffer et al., 1998; Hicks et al., 2001; Doyle et al., 2002; Hazen et al., 2005; Kikis et al., 2005). The putative MYB transcription factor *PCL1/LUX* has also been identified as a master clock gene, since mutants displayed arrhythmia of multiple circadian outputs under free-running conditions. In these *lux/pcl1* mutants, *LHY/CCA1* and *ELF4* expression was constitutively low, while the expression levels of *TOC1* and *GI*, though arrhythmic, were moderately high, suggesting the existence of at least one additional feedback mechanism (Hazen et al., 2005; Onai and Ishiura, 2005).

The multicellular nature of plant circadian models, however, coupled with their inherent genetic redundancy, adds complexity to circadian research in systems such as *Arabidopsis*. By contrast, unicellular, small genome organisms, such as cyanobacteria and miscellaneous fungi, are better suited to molecular approaches toward delineating these multiple interconnected feedback loops and their input and output pathways (Roenneberg and Merrow, 2001). This direction is supported by the results of a large forward genetics screen, recently performed in a *Chlamydomonas reinhardtii* line carrying a chloroplast luciferase reporter gene, which led to the discovery of six plant-like clock genes, among more than a hundred circadian mutants, including several MYB transcription factors related to *CCA1*, *LHY*, and *LUX* as well as a *CONSTANS-like* gene (Matsuo et al., 2008).

Though *Chlamydomonas* is now emerging as a powerful unicellular model system for genetic studies of the circadian clock, it has approximately the same genome size and complexity as *Arabidopsis*. In addition, reverse genetic approaches, including gene silencing and expression of foreign transgenes, remain difficult in the nuclear genome of *Chlamydomonas* (Cerutti et al., 1997; Leon and Fernandez, 2007). Among green microalgae, several genomes of Prasinophyta have been, or are being, sequenced, including the genus *Ostreococcus* (Robbens et al., 2005; Palenik et al., 2007). *Ostreococcus tauri* has a minimal cellular organization and has been described as the smallest living eukaryote (Courties et al., 1994). This marine picoeukaryote has a compact genome (12.56 Mb, i.e., one-tenth the size of *Arabidopsis* or *Chlamydomonas*) that is very gene rich (85% protein coding), with average intergenic regions smaller than 200 bp and very low gene redundancy. As such, it constitutes an appealing circadian model organism, since cell division, regulated by a reduced number of cyclin-dependent kinases, was recently shown to be under circadian regulation (Robbens et al., 2005; Moulager et al., 2007).

In *Ostreococcus*, we identified two putative homologs of higher-plant core clock genes, namely *TOC1*- and *CCA1-like*, referred to as *TOC1* and *CCA1*. To address the function of these genes, we developed stably transformed lines expressing luciferase reporter genes in order to monitor *TOC1* and *CCA1* transcriptional and translational activity under different photoperiods and under constant light. A vector system was designed for gene function analysis, through overexpression or antisense knockdown. Using these techniques, we observed that *TOC1* displayed the hallmarks of a core clock gene, as it was involved in the circadian regulation of its own expression, as well as that of CCA1 and a rhythmically expressed output gene (*CAB/LHCB*). Furthermore, CCA1 was observed to bind to a conserved EE essential to the circadian regulation of *TOC1* promoter activity. Lines that overexpressed CCA1 exhibited an altered rhythmic phenotype and reduced TOC1 levels. Repression of *CCA1*, however, had no effect on the circadian expression of either *TOC1* or *CAB* under free-running conditions, which is inconsistent with a minimal clock architecture based on a simple *CCA1/TOC1* negative feedback loop.

RESULTS

Identification of *TOC1* and *CCA1* Homologs in *Ostreococcus*

Homologs of known *Arabidopsis* clock genes were searched for in the fully sequenced genomes of the green unicellular algae *O. tauri* and *C. reinhardtii* as well as in that of the red alga *Cyanydioschyzon merolae*. Circadian clock genes such as *CCA1* have been shown to be conserved in angiosperms (Murakami et al., 2003; Takata et al., 2008); however, we were not able to identify putative homologs of plant clock genes based on full sequence conservation. Therefore, conserved domains of plant clock genes such as MYB REVEILLE, GARP DNA binding, PAS/LOV, receiver, or CCT were queried in the sequence databases. Two putative homologs of higher-plant master clock genes were found in *O. tauri*. They corresponded to a PRR-like protein (five members in *Arabidopsis*, including *TOC1*) and a member of the REVEILLE family (11 members in *Arabidopsis*, including *CCA1/LHY*) referred to as *Ostreococcus TOC1* and *CCA1*, respectively (Figures 1A and 1B; see Supplemental Table 1 online). An as yet unidentified *TOC1* homolog was also found in the *Chlamydomonas* genome. The phosphate acceptor aspartyl residue, conserved in functional response regulators, was found only in *Ostreococcus* (Mizuno and Nakamichi, 2005). Close homologs of *CCA1/LHY* exhibited the conserved I/LPPPRPKRKPXXPYPQ/

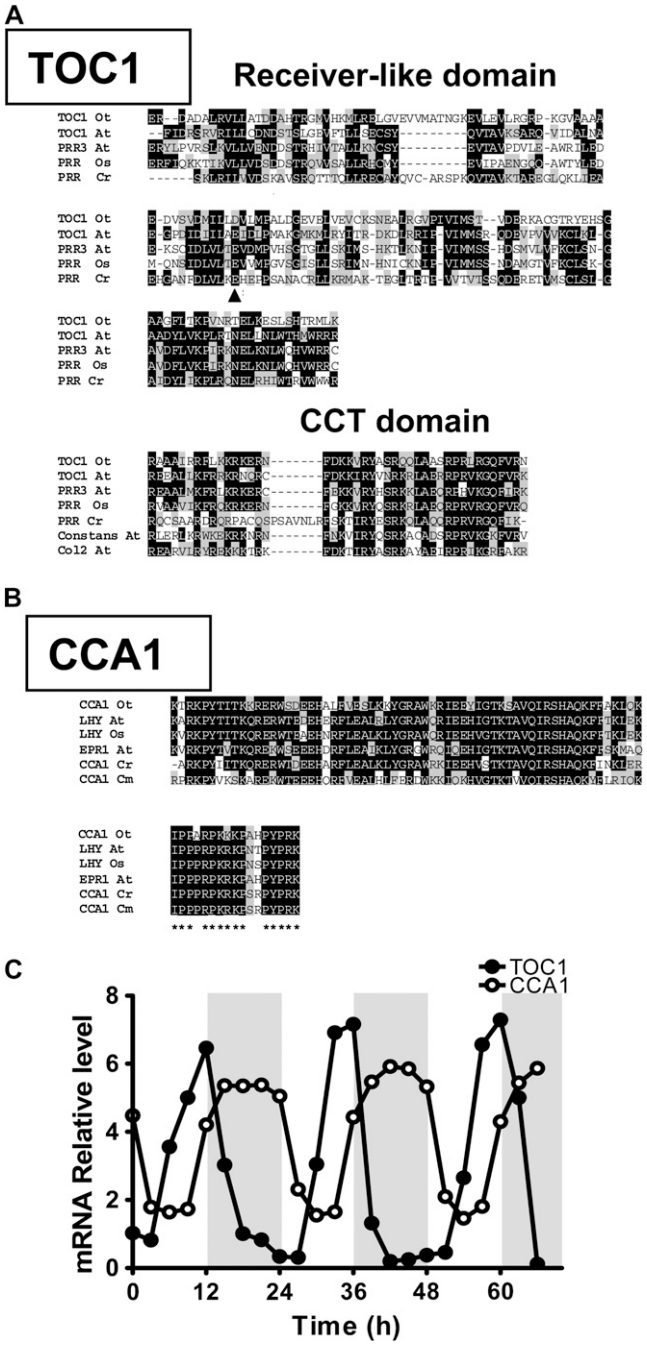

Figure 1. In Silico Identification and Expression Patterns of *TOC1* and *CCA1* Genes.

(A) and **(B)** ClustalW alignment of receiver and CCT domains in TOC1 proteins, PRRs, and CONSTANS-like proteins **(A)** and of MYB domains in the CCA1/LHY REVEILLE family **(B)**. The arrowhead indicates the position of a conserved aspartyl residue (phosphate receiver) that is mutated to glutamyl in all PRRs except *Ostreococcus* TOC1. Asterisks indicate amino acid residues specific to the REVEILLE family. Ot, *O. tauri*; At, *A. thaliana*; Cm, *C. merolae*; Cr, *C. reinhardtii*; Os, *O. sativa*. Accession numbers are given in Methods.

(C) Microarray analysis of *TOC1* (closed circles) and *CCA1* (open circles) mRNA patterns under LD: 12,12 entraining cycles. Gray areas represent nights.

RK signature near the MYB domain in *Cyanidioschyzon*, *Chlamydomonas*, and *Ostreococcus* (Figure 1B). Homologs of other known core clock genes, such as *ELF3*, *ELF4*, *GI*, and *ZTL*, were not identified in *Ostreococcus*, but *CONSTANS* and sequences containing a GARP domain (found in *Arabidopsis LUX*) were identified both in *Ostreococcus* and *Chlamydomonas* (Onai and Ishiura, 2005; Matsuo et al., 2008). In both microalgae, a GARP domain–containing protein displayed an additional receiver domain typical of B-type response regulators and is referred to as *RRB* in *Ostreococcus*.

Since *TOC1* and *CCA1*/*LHY* were the first clock genes to be identified in *Arabidopsis*, and are the best characterized, we hypothesized an evolutionarily conserved role for these genes in the green lineage and therefore chose to focus this first functional study of *Ostreococcus* clock components upon the homologs of *TOC1* and *CCA1*.

Microarray analysis of transcript expression patterns for *TOC1* and *CCA1* from cells cultivated under clock entraining conditions of 12-h-light/12-h-dark cycles (denoted as LD: 12,12) revealed that *TOC1* exhibits a sharp peak of mRNA at the light/dark transition that was coincident with the trough of *CCA1* transcript (Figure 1C). *CCA1* expression closely followed that of *TOC1*, reaching a plateau in the middle of subjective night and decreasing before dawn.

Development of Genetic Transformation and Use of a Luciferase Reporter Strategy to Investigate the Function of TOC1 and CCA1 Genes in *Ostreococcus*

Circadian studies rely on long-term monitoring of biological rhythms. The luciferase reporter gene strategy, initially developed in *Arabidopsis*, is commonly used to follow in vivo promoter activity, and more recently protein synthesis, over long periods of time (Millar et al., 1992; Farre and Kay, 2007). We have implemented this strategy in *Ostreococcus.* The pOtluc vector was designed to introduce transcriptional reporters (promoter of interest driving the expression of luciferase) or translational fusions (gene of interest including coding region upstream of luciferase) into *Ostreococcus* cells (see Supplemental Figure 1 online). Linearized DNA was introduced by electroporation, and efficiencies up to 1000 stable transformants/micrograms of DNA were obtained. DNA gel blot analysis revealed between one and three gene insertion events per line (average of 1.5 copies; see Supplemental Figure 2 online). The luminescence level was not correlated with the number of inserted constructs, indicating that the level of expression may depend mostly on the insertion site as previously reported in plants (van Leeuwen et al., 2001). For all lines, the ratio of hybridization signal between the inserted copy and the endogenous gene was never below 1, suggesting that the genome of *Ostreococcus* is haploid in our culture conditions.

TOC1 and CCA1 reporter lines were entrained under light/dark (LD) cycles of three different photoperiods, LD: 12,12; long days of 18-h-light/6-h-dark cycles (LD: 18,6), and short days of 6-h-light/18-h-dark cycles (LD: 6,18), and transferred to

fresh luciferin-supplemented medium to record luminescence changes under identical conditions (Figure 2; see Supplemental Figure 3 online). Promoters of *TOC1* and *CCA1* (PTOC1:Luc and PCCA1:Luc) displayed rhythmic activities under various photoperiods that were consistent with the patterns of their cognate transcripts (Figures 2A and 2D). TOC1:Luc and CCA1:Luc translational fusions (full gene fused in frame to luciferase) monitored under the same conditions also displayed robust rhythms under all photoperiods (Figures 2B and 2D). In vitro luciferase assays confirmed that TOC1:Luc and CCA1:Luc had rhythmic patterns of expression under LD entrainment (Figure 2C).

Phases of promoter activity and protein synthesis were advanced under short days relative to long days, indicating that both *TOC1* and *CCA1* expressions adjusted to anticipate the time of dusk (see Supplemental Figure 3 online). The amplitude of oscillations increased with the daylength for both translational reporter lines and for PTOC1:Luc (Figure 2D). Our results suggest that TOC1 and CCA1 expression is coregulated by diurnal and circadian factors as they exhibit amplitude and typical circadian patterns of phase adjustment to photoperiod length (Millar and Kay, 1996; Roenneberg et al., 2005; Merrow et al., 2006; Perales and Mas, 2007).

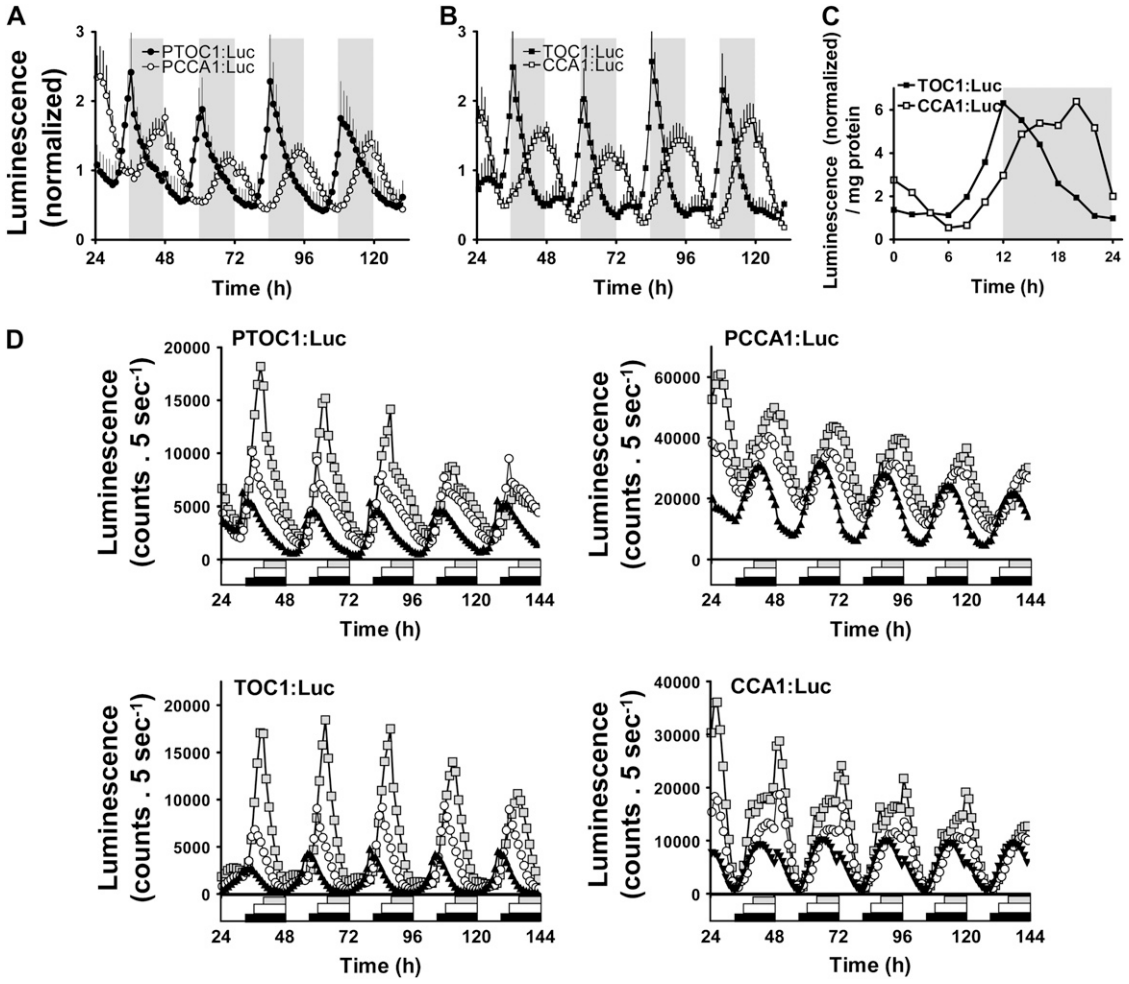

Figure 2. Regulation of *TOC1* and *CCA1* Promoter Activities and Protein Synthesis under 24-h LD Cycles of Different Photoperiods.

Dark periods are represented by gray areas (**[A]** to **[C]**) or scaled on the *x* axis **(D)**. Bioluminescence of lines carrying *TOC1* and *CCA1* transcriptional (promoter fused to the luciferase) or translational (full gene fused to luciferase) fusions.

(A) and **(B)** LD: 12,12 entraining cycles. Means of transcriptional fusions **(A)** (PTOC1:Luc, closed circles, $n = 5$; PCCA1:Luc, open circles, $n = 6$) and of translational fusions **(B)** (TOC1:Luc , closed squares, $n = 4$; CCA1:Luc, open squares, $n = 6$). Error bars represent SE.

(C) In vitro luciferase assay on protein extracts from individual TOC1:Luc (closed squares) and CCA1:luc (open squares) translational fusion lines, representative of three trials.

(D) Phase and amplitude adjustments of *TOC1* and *CCA1* promoter activity and protein synthesis to photoperiod. Transcriptional and translational reporter lines were entrained under LD: 8,16 (gray squares), LD: 12,12 (white circles), and LD: 16,8 (black triangles) for 8 d and transferred at the same cell density for recording under identical conditions. Means of triplicates of representative transcriptional and translational fusion are shown, and the SE is smaller than symbols.

TOC1 and *CCA1* Promoter Activity and Protein Synthesis Are under Circadian Regulation

To monitor circadian rhythms, cell lines were entrained under LD: 12,12 entraining conditions before release into constant light (LL), corresponding to a condition where the circadian clock is free-running. Both TOC1 and CCA1 reporter lines displayed sustained oscillations in LL, indicating that TOC1 and CCA1 transcription and protein synthesis are under circadian regulation (Figure 3). Phases of all reporter lines, except TOC1:Luc, stayed as they were under entrainment conditions, with PTOC1:Luc peaking at the beginning of the subjective night and both PCCA1:Luc and CCA1:Luc peaking at the end of the subjective night (Figures 3A and 3B). Upon transition to constant light, TOC1:Luc luminescence continued to increase until 16 to 18 h after the lights were turned on. This resulted in a higher peak, the phase of which was delayed by about 4 h compared with LD. The free-running period of all reporter lines was close to 24 h, except for TOC1:Luc, which displayed a period ~2 h longer (Figure 3C). Period lengthening resulted, therefore, in a superimposition of TOC1:Luc and PCCA1:Luc (and CCA1:Luc) signals after one cycle in LL. This appears to be globally inconsistent with TOC1 and CCA1 acting in a simple transcriptional feedback loop if TOC1:Luc and CCA1:Luc do not have any effect on the free-running period of the clock. Period lengthening has been documented in *Arabidopsis* when *TOC1* expression was increased upon introduction of additional TOC1 copies through TMG constructs (Mas et al., 2003). Therefore, it is conceivable that the introduction of an extra copy of *TOC1*, in the form of TOC1:Luc, similarly lengthens free-running period in *Ostreococcus* due to gene dosage sensitivity.

Rhythmic robustness in LL of TOC1 and CCA1 clearly indicates that both are under circadian regulation at the transcriptional and posttranscriptional levels.

Misexpression of Either *TOC1* or *CCA1* Disrupts Circadian Entrainment

To characterize the genetic interactions between *TOC1* and *CCA1* and their role within the *Ostreococcus* clock, the pOtox vector was designed to overexpress genes of interest in sense or antisense orientation under the control of the high-affinity phosphate transporter (HAPT) promoter (see Supplemental Figure 4 online). The HAPT gene encodes one of the most abundant transcripts in *Ostreococcus* ESTs. The 100-nucleotide-long HAPT promoter drives luciferase expression at a 10 times higher level than the TOC1 and CCA1 promoters and moreover displays constitutive activity under constant light (see Supplemental Figure 5 online).

Overexpression (-ox) or repression (antisense; -as) constructs of *TOC1* and *CCA1* were introduced into representative TOC1:Luc and CCA1:Luc backgrounds. In addition, a transcriptional reporter line, in which the *CAB/LHCB* promoter drives luciferase expression (PCAB:Luc) was also used to monitor circadian regulation of photosynthetic output pathways, as in plants (Millar et al., 1992). Screening for altered rhythmic phenotypes of transformed lines was achieved in LL after LD: 12,12 entrainment (Figure 4; see Supplemental Figures 6 and 7 online). Each line

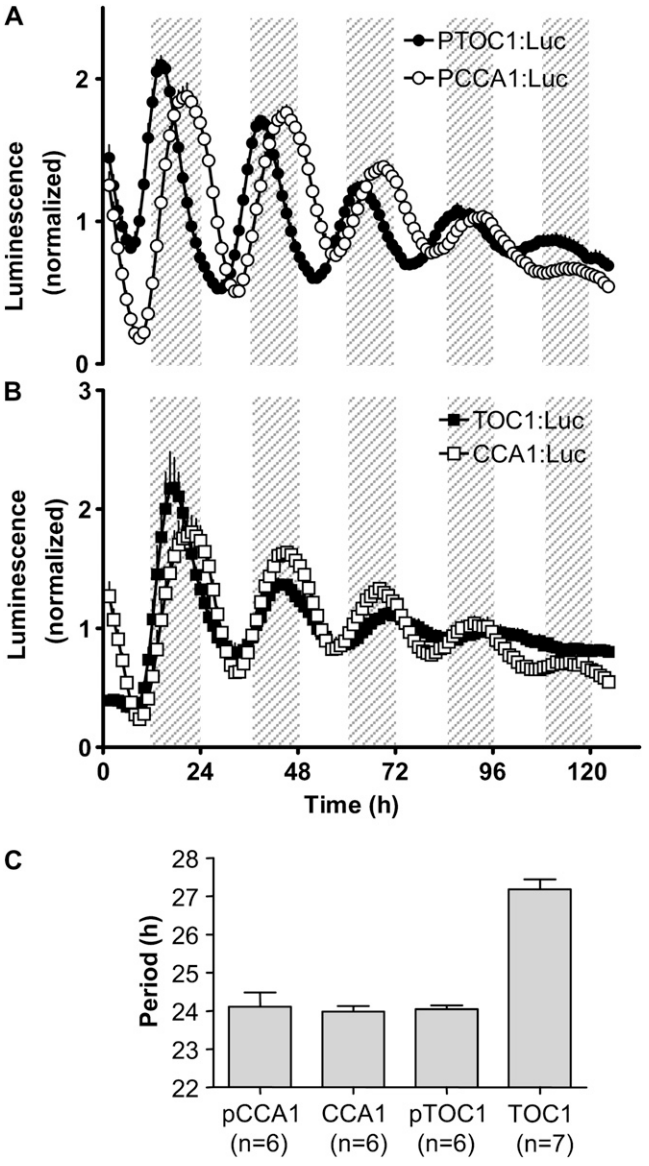

Figure 3. Regulation of *TOC1* and *CCA1* Promoter Activity and Protein Synthesis upon Release from LD: 12,12 into LL.

Subjective night is represented by gray areas.

(A) and **(B)** Bioluminescence of lines carrying TOC1 and CCA1 transcriptional (promoter fused to the luciferase) or translational (full gene fused to luciferase) fusions.

(A) Transcriptional fusions in LL (PTOC1:Luc, $n = 3$; PCCA1:Luc, $n = 3$).

(B) Translational fusions in LL (TOC1:Luc $n = 3$; CCA1:Luc $n = 3$). Means ± SE are represented.

(C) Period length of the different reporter lines in LL. Periods were analyzed from 24 h after release into constant light. Means ± SD are shown. *n* indicates the number of individual reporter lines (plated in duplicate) for monitoring promoter activity or protein synthesis.

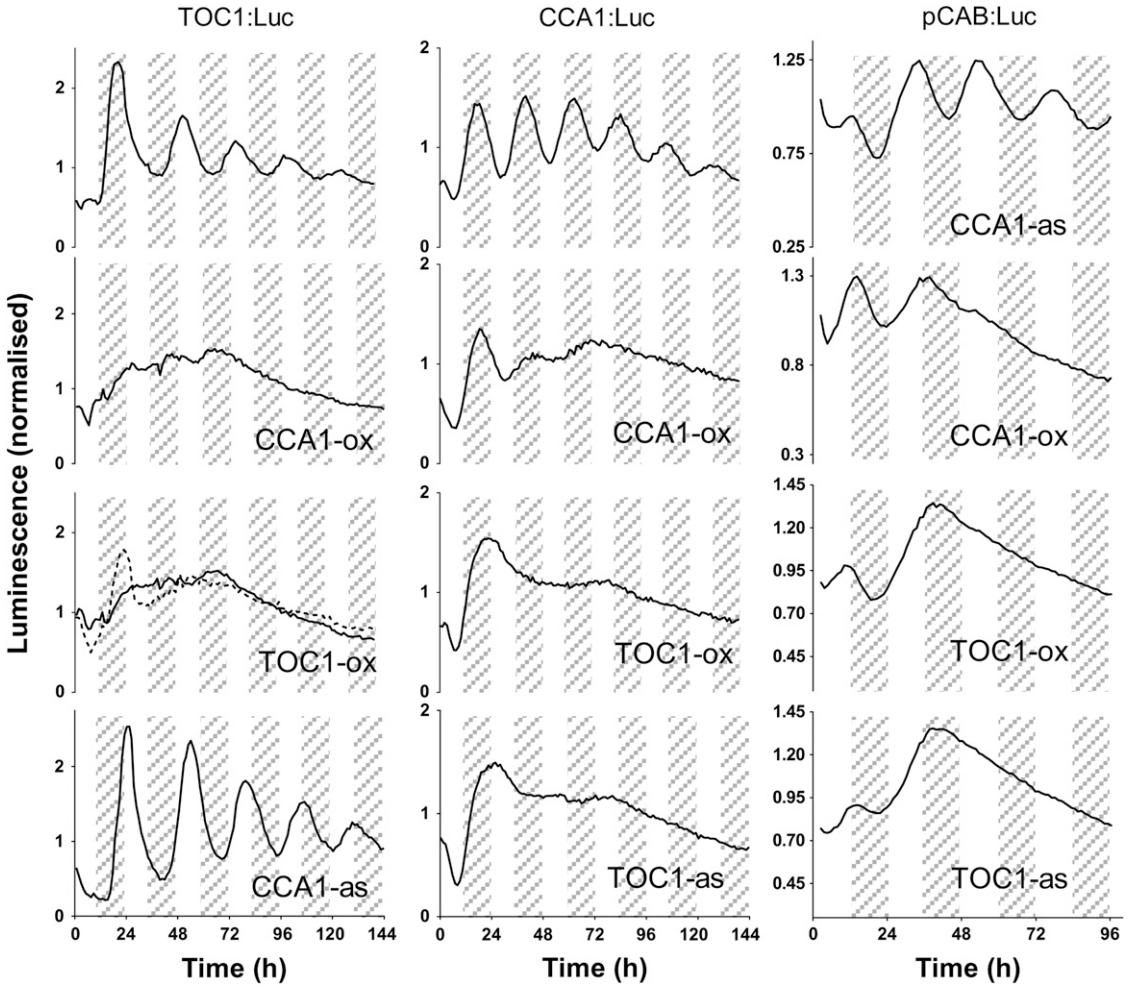

Figure 4. Functional Analysis of *TOC1* and *CCA1* under Free-Running Conditions (LL).

Representative bioluminescence traces of TOC1:Luc, CCA1:Luc, and PCAB:Luc in different backgrounds or the wild type (unlabeled graphs). Data sets are representative of at least of three trials. Subjective night is represented by gray areas. Overexpression of CCA1 (CCA1-ox) altered the rhythmicity of TOC1:Luc (number of arrhythmic lines [ar] = 3; number of lines with dampened rhythmicity [dr] = 10; $n = 23$), CCA1:Luc (ar = 1, dr = 5; $n = 22$), and PCAB:Luc, but CCA1-antisense (CCA1-as) had no effect on either TOC1:Luc (ar = 0, dr = 0; $n = 17$) or PCAB:Luc rhythmicity (ar = 0, dr = 0; $n = 30$). Overexpression of TOC1 (TOC1-ox) altered the rhythmicity of TOC1:Luc (ar = 4, dr = 3; $n = 13$), CCA1:Luc (ar = 17, dr = 12; $n = 42$), and PCAB:Luc (ar = 15; $n = 31$). Downregulation of TOC1 (TOC1-as) had a similar effect on CCA1:Luc (ar = 2, dr = 7; $n = 21$) and PCAB:Luc rhythmicity (ar = 16; $n = 31$). Note that the strongest phenotypes are represented.

with a defective rhythmic phenotype was screened at least three times (see Methods).

CCA1 overexpression greatly dampened or totally abolished the rhythmic expression of TOC1:Luc in most lines, as well as affecting the rhythmicity of CCA1:Luc (Figure 4). By contrast, antisense knockdowns of *CCA1* expression barely affected rhythmicity of TOC1:Luc lines under LL ($n = 17$, eight trials). CCA1 activity might simply be more robust to our antisense strategy for knockdown, as suggested by quantitative RT-PCR experiments (see Supplemental Figure 8 online). Alternatively CCA1 may be only indirectly relevant to the circadian regulation of TOC1 expression under constant conditions.

Overexpression of *TOC1* dramatically disrupted TOC1:Luc and CCA1:Luc rhythmicity under constant light in the majority of lines (Figure 4). Interestingly, downregulation of *TOC1* by antisense also resulted in arrhythmia of the CCA1:Luc signal. Misexpression of *TOC1* or overexpression of *CCA1* led to arrhythmia or dampened rhythmicity in the majority of PCAB:Luc lines, whereas, as in the TOC:Luc background, *CCA1* knockdown (CCA1-as) had no effect ($n = 30$) (Figure 4). Overexpression phenotypes correlated with increased levels of transcripts as confirmed by quantitative RT-PCR, and TOC1 transcript levels in TOC1-as/CCA1:Luc lines were seen to be reduced (see Supplemental Figure 9 online).

Sustained rhythmicity under free-running conditions usually indicates that the clock has readily entrained to LD cycles. Arrhythmia under LL, however, may mask some underlying oscillations or alternatively imply some disruption of entrainment

mechanisms (McWatters et al., 2000; Nozue et al., 2007). Therefore, LD cycles with a photoperiod other than 24 h are a convenient means of investigating the entrainment properties of circadian clocks (Figure 5; see Supplemental Figure 8 online). When the phase of the entraining cue matches the phase of the circadian clock, entrainment can occur and result in the establishment of a stable phase relationship between external and internal time. When the period of entrainment is approximately half of the endogenous free-running period, then circadian rhythms (unlike driven rhythms) skip a cycle every other cycle (Roenneberg et al., 2005). Under LD 6:6, the photoperiod cycle thus occurs twice per circadian cycle entraining the clock with a 24-h periodicity (Merrow et al., 1999). By contrast, expression of genes in clock mutants has been reported to be driven solely by external cues in plants and fungi (Roenneberg et al., 2005; Nozue et al., 2007). Selected arrhythmic lines were therefore transferred from LL to LD: 6,6 cycles. Under these conditions, TOC1 and CCA1 translational reporter lines exhibited a 24-h circadian pattern of protein expression that reflected coincidence between circadian and diurnal regulation at every other cycle, allowing the expression of TOC1 during the light period when CCA1 was low and the converse (Figure 5). By contrast, TOC1-ox/CCA1:Luc, TOC1-as/CCA1:Luc, and CCA1-ox/TOC1:Luc lines that were arrhythmic under LL displayed expression that was directly driven by the LD: 6,6 cycle, implying that the capacity for entrainment was impaired (Figure 5; see Supplemental Figure 8 online). Intriguingly, a similar pattern was observed for four out of six analyzed CCA1-as/TOC1:Luc lines, which responded equally to light every LD: 6,6 cycle, indicating that under these conditions, CCA1 is required for the proper entrainment of TOC1 expression (Figure 5). CCA1 transcript levels in CCA1-as/TOC:Luc lines were reduced at most by 34% (see Supplemental Figure 9 online).

In both TOC1:Luc and CCA1:Luc backgrounds, the majority of CCA1-ox lines with altered rhythmicity (either arrhythmic or with dampened rhythmicity) exhibited a lower luminescence level than did control lines, suggesting that CCA1 overexpression repressed endogenous expression of both CCA1 and TOC1 (Figures 6A and 6B). Conversely, screening of TOC1-ox/CCA1:Luc lines showed that CCA1:Luc luminescence levels were constitutively higher in rhythmically deficient lines, suggesting an activating role of TOC1 on CCA1 expression. While there was also a tendency toward higher luminescence levels for TOC1-ox in a TOC1:Luc background (in four of seven lines), the effect was not significant, as judged from the overlapping error bars (Figure 6D).

The EE Binds CCA1 in Vitro and Is Essential for the Circadian Regulation of the TOC1 Promoter

In *Arabidopsis*, CCA1 was reported to bind the consensus EE in the TOC1 promoter (Alabadi et al., 2001; Michael and McClung,

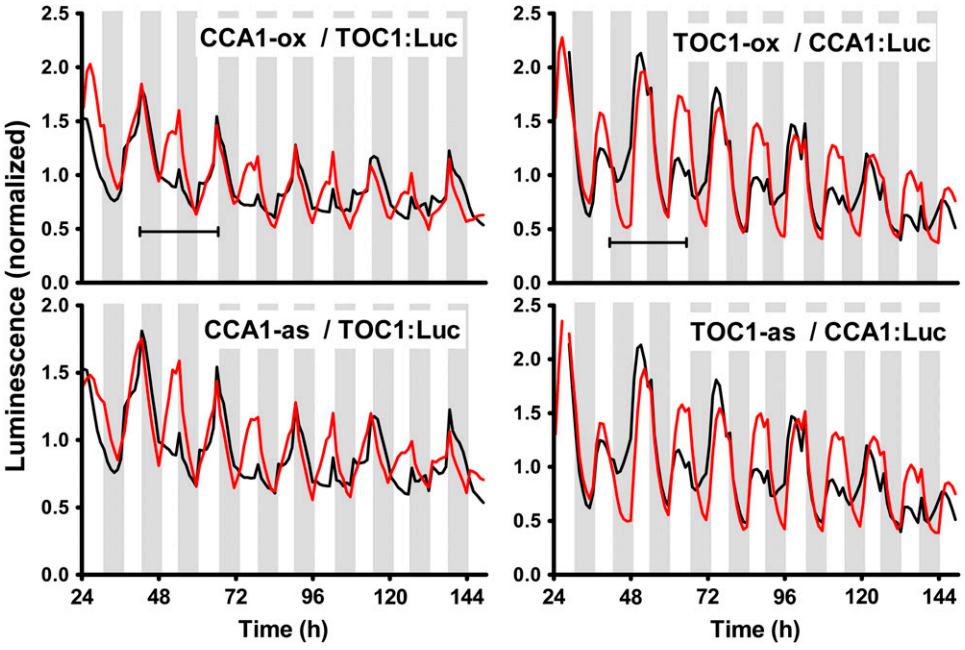

Figure 5. CCA1 Downregulation Disrupts Circadian Entrainment of TOC1:Luc Expression under LD: 6,6.

Bioluminescence traces of representative CCA1- and TOC1-ox and -as lines in the TOC1:Luc and CCA1:Luc backgrounds are shown. Lines grown under constant light were transferred at the same cell density to LD: 6,6. Time zero corresponds to the beginning of the first period of light. TOC1:Luc and CCA1:Luc control lines are represented in black, and ox and as lines are in red. TOC1:Luc and CCA1:Luc display a biphasic 24-h pattern of oscillation (underlined). By contrast, ox/as lines respond directly to each LD: 6,6 cycle. Note that repressing CCA1 in TOC1:Luc (CCA1-as/TOC1:Luc) leads to a loss of the 24-h module in lines found to be rhythmic in LL. Data sets are representative of three trials.

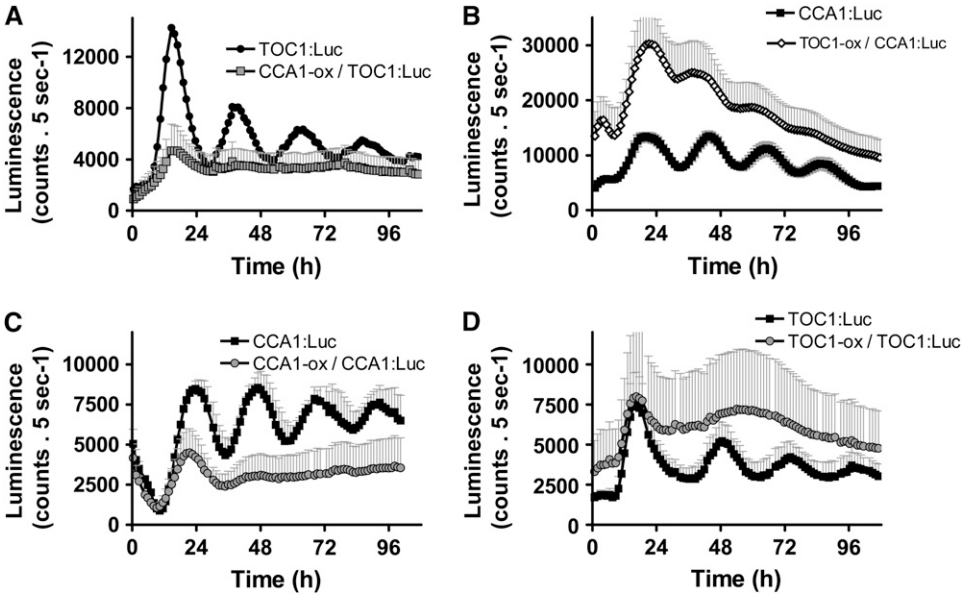

Figure 6. Quantitative Effect of *TOC1* and *CCA1* Overexpression on TOC1:Luc and CCA1:Luc Levels.

Lines with altered rhythmic phenotypes were monitored under constant light. Means ± SE are plotted. Control lines are shown in each panel (n = 3 to 4).
(A) CCA1-ox/TOC1:Luc lines (n = 13).
(B) TOC1-ox/CCA1:Luc lines (n = 19).
(C) CCA1-ox/CCA1:Luc lines (n = 6).
(D) TOC1-ox/TOC1:Luc lines (n = 7).

2002). As a perfectly conserved EE (AAAATATCT) was identified at position −100 in the *TOC1* promoter region, we tested the interaction of recombinant CCA1 protein with EE sequences. Gel electrophoretic mobility shift assays showed a specific interaction between CCA1 and the EE motif in both the *Ostreococcus TOC1* promoter and the *Arabidopis* EE consensus sequence (Harmer and Kay, 2005) (Figure 7A). A change of three nucleotides in the EE to mEE (AAAAgcTtT) was sufficient to abolish this interaction.

A regulatory role for the EE in *TOC1* promoter activity was investigated through analysis of luciferase lines reporting the rhythmicity of the TOC1 promoter when similarly mutated in the EE (mPTOC1:Luc). Under constant light, the rhythmicity of mPTOC1:Luc lines was disrupted (Figure 7B). Ten out of 28 lines were arrhythmic, and 18 displayed dampened rhythmicity. To test whether the change in promoter activity was due to a loss of circadian regulation, and not to a direct light response that might mask circadian regulation, we analyzed arrhythmic lines under LD: 6,6 cycles (Figure 7C). PTOC1:Luc control lines transferred from LL to LD: 6,6 exhibited a 24-h biphasic pattern similar to those observed for the TOC1:Luc reporter. By contrast, arrhythmic mPTOC1:Luc exhibited a direct light response every 12-h cycle. These observations indicate that the EE is an important element for circadian regulation of the *TOC1* promoter activity. These results also suggest a light-dependent activation of the *TOC1* promoter since its activity increases with every light period in mPTOC1:Luc lines but with every other light period in PTOC1:Luc.

DISCUSSION

Conservation of Clock Proteins in Photosynthetic Organisms Supports a Common Evolutionary Origin of Green Clocks

The search for putative master clock genes in the genome of the three algae *Chlamydomonas*, *Ostreococcus*, and *Cyanidioschyzon* revealed that a *CCA1*-like MYB transcription factor is the only known master clock gene found in these distantly related photosynthetic organisms from the green and red lineages. *TOC1*-like genes identified by the receiver domain and the conserved C-terminal region were found only in the two green algae (Figure 1; see Supplemental Table 1 online). Surprisingly, *TOC1* has not been identified by forward genetic approaches in *Chlamydomonas*, although we did identify a putative *TOC1*-like sequence in silico (Matsuo et al., 2008). Interestingly, among TOC1 homologs identified to date, only *Ostreococcus TOC1* may be a true response regulator since it alone displays the conserved aspartyl residue required for phosphotransfer in all response regulators. That conserved clock components are found in evolutionarily distant photosynthetic organisms is presumed to bear witness to a common origin for plant clock genes. Based on functional and in silico analysis, however, it is likely that the specific clock mechanisms have diverged. The *Arabidopsis* clock is currently viewed as three interconnected loops involving CCA1/LHY, at least three PRRs including TOC1, and GI, as well as additional proteins found only in land plants, such as ELF4 and

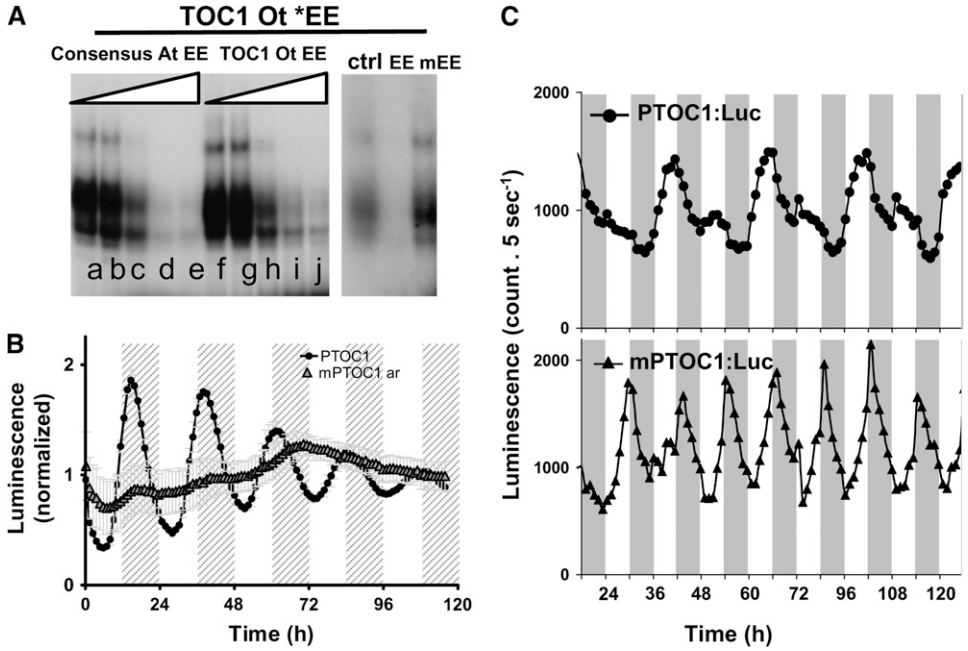

Figure 7. Functional Analysis of the Consensus EE of the *Ostreococcus* TOC1 Promoter.

(A) Gel mobility electrophoretic assay of CCA1 using the EE sequence of the TOC1 promoter as the labeled probe. Competition experiments reveal that the recombinant CCA1 specifically binds to the EE (AAAATATCT) in the 100-bp upstream region of the 250-bp *TOC1* promoter. In the left panel, the labeled EE probe of *Ostreococcus* was incubated with 10 ng of recombinant CCA1 protein. No competitor DNA was added in lanes a and f. The unlabeled competitor DNA corresponded to the *Arabidopsis* consensus sequence (lanes b through e) or the EE motif in the context of the *Ostreococcus* promoter (lanes g through j). White triangles represent increasing amount of competitor, which was present at 5-, 25-, 50-, and 100-fold molar excess to the labeled probe (lanes b to e and g to j). Right panel: No DNA (crtl), the *Ostreococcus* EE, or the mutated mEE (AAAAgcTtT) was added as the unlabeled competitor to the labeled EE incubated with 10 ng of recombinant CCA1 protein. No competition for CCA1 binding was observed at a 100-fold excess of mEE.

(B) Analysis of the activity of the TOC1 promoter with mutations in the EE. Transcriptional TOC1:Luc lines with the mutated EE (mPTOC1:Luc) were generated and analyzed under LL after LD: 12,12 entrainment. Subjective night is represented by hashed gray areas. mPTOC1:Luc lines (n = 28) were either arrhythmic in LL (ar, gray triangle, n = 18) or displayed dampened rhythmicity (n = 10, data not shown) compared with control lines (PTOC1:Luc). Means ± SE are shown.

(C) Analysis of mPTOC1:Luc reporter lines under LD: 6,6 cycles. Representative traces are shown. Top panel: Bioluminescence of control PTOC1:Luc reporter lines. Bottom panel: Bioluminescence of mPTOC1:Luc lines.

ZTL (Locke et al., 2006). In *Ostreococcus*, the simpler cellular organization and reduced number of putative components, compared with higher plants such as *Arabidopsis*, should be of great advantage in understanding the basic mechanisms that sustain circadian systems.

TOC1 and CCA1 Are Differentially Involved in the Circadian Clock of *Ostreococcus*

While *Ostreococcus TOC1* expression is similar to that of its plant homologs, peaking at dusk or at the beginning of the night (Strayer et al., 2000; Nakamichi et al., 2004), *Ostreococcus CCA1* displays a different profile (Wang and Tobin, 1998; Mizoguchi et al., 2002; Nakamichi et al., 2004) (Figure 2; see Supplemental Figure 3 online). In *Arabidopsis*, *CCA1* expression rises late in the night, anticipating dawn and exhibiting a sharp peak after the lights come on. By contrast, *Ostreococcus CCA1* expression begins much earlier, shortly after TOC1 increases, and persists until late in the night. Short days advanced the relative phase of both CCA1 and TOC1 expression, whereas long days slightly delayed them (Figure 2; see Supplemental Figure 3 online).

The *TOC1* gene displays several features expected of a core clock gene as it is able to regulate both its own expression and that of output genes such as *CAB* (Figure 4; see Supplemental Figure 6 online). TOC1 also appears to be involved in sustaining circadian rhythmicity under free-running conditions as *TOC1* overexpression or antisense knockdown resulted in arrhythmia of CCA1:Luc and PCAB:Luc. In addition, TOC1:Luc and CCA1:Luc expressions under LD: 6,6 cycles appear to be driven rather than entrained in TOC1-ox/-as lines (Figure 5; see Supplemental Figure 8 online). *Ostreococcus* TOC1-as lines exhibited an arrhythmic phenotype that contrasts with the short period phenotype of the *Arabidopsis* TOC1 loss-of-function mutant (Alabadi et al., 2001). This difference suggests that in *Ostreococcus*, TOC1 functions as part of a central oscillatory mechanism that, unlike in *Arabidopsis*, cannot be rescued efficiently by other feedback mechanisms (like the PPR7/PRR9-LHY/CCA1 feedback loop) to maintain circadian function (Locke et al., 2006) and

serves to highlight the greater functional redundancy in land plants.

The role of CCA1 is rather puzzling because the CCA1-as/TOC1:Luc and CCA1-as/PCAB:Luc lines remained rhythmic under constant light but exhibited a driven rhythmicity under LD: 6,6, reflecting an overtly altered circadian regulation (Figures 4 and 5). Therefore, CCA1 might not be as critical as TOC1 for clock function in *Ostreococcus*, or it might be more specifically involved in gating cues for resetting, ensuring the proper entrainment of TOC1 expression (Figure 5). It is also possible that CCA1 levels in these lines remained sufficient to sustain rhythmicity in LL but not high enough to block the induction of TOC1 in the light periods of LD 6:6. The development of loss-of-function mutants by knockout should allow this question to be addressed in the future.

Taken together, our results suggest that as in higher plants, TOC1 and CCA1 are involved in the circadian function of *Ostreococcus* but that TOC1 plays a more important role in sustaining rhythmicity under free-running conditions.

The Circadian Clock of *Ostreococcus* Is More Complex Than a Simple TOC1/CCA1 Feedback Loop

Based on the expression patterns of TOC1 and CCA1 and the occurrence of a perfectly conserved EE in the *TOC1* promoter, we first hypothesized that, as proposed in higher plants, CCA1 might repress the expression of *TOC1* through binding to the EE element (Figure 7). We showed that *Ostreococcus* CCA1 binds in vitro to the EE of the *Ostreococcus TOC1* promoter. In addition, the EE appears to be essential for proper regulation of the *TOC1* promoter, since mutation of the EE alters the circadian-regulated activity of the *TOC1* promoter under free-running conditions, as also reported in *Arabidopsis*, as well as under LD: 6,6 (Alabadi et al., 2001, 2002). This illustrates that higher plants and green microalgae share not only common clock components but also similar mechanisms that regulate transcription, which are likely to have evolved before the emergence of land plants. Analysis of luminescence levels indicates that CCA1 likely represses TOC1 because in most of the arrhythmic CCA1-ox/TOC1:Luc lines, expression of TOC1:Luc was constitutively low (Figure 6). Similarly CCA1:Luc levels were low in CCA1-ox/CCA1:Luc as would be expected if the constitutive overexpression of CCA1 inhibited the TOC1-dependent activation of CCA1:Luc. Based on these observations, it is tempting to speculate that CCA1 might repress TOC1 promoter activity, as proposed in higher plants (Alabadi et al., 2001; Perales and Mas, 2007), but this remains to be shown. Further development of tools, such as an inducible expression system, would be required to test this hypothesis in *Ostreococcus*.

TOC1 clearly plays an essential role in the *Ostreococcus* clock since both repression and overexpression of TOC1 leads to arrhythmia of CCA1:Luc under free-running conditions. (Figure 4). In addition, the severity of CCA1:Luc rhythmicity defects was correlated with the level of TOC1 overexpression (see Supplemental Figure 9 online).The higher luminescence levels observed in arrhythmic lines overexpressing TOC1 in CCA1:Luc backgrounds is globally consistent with TOC1 being an activator of CCA1 (Figure 6). Furthermore, CCA1:Luc levels were reduced in TOC1-as lines, consistent with TOC1 being an activator of CCA1. The variability of TOC1:Luc expression levels in arrhythmic TOC1-ox/TOC1:Luc lines is puzzling in the context of a simple TOC1/CCA1 clock model, since overexpression of TOC1 should trigger increased levels of CCA1, which in turn would repress *TOC1* transcription. Together, our results support the notion that the clockwork of *Ostreococcus* is likely to be more complex than a simple TOC1/CCA1 single loop mechanism, as is also the case in higher plants.

In conclusion, we have shown that the only functional core clock genes thus far identified in *Ostreococcus* are differentially implicated in sustaining rhythmicity. The clock is extremely sensitive to changes in TOC1 levels, perhaps because TOC1 is the only PRR-like protein in *Ostreococcus*. In addition, we showed that the CCA1 binding site in the *TOC1* promoter is required for its circadian activity. Functional analysis is globally consistent with TOC1 activating *CCA1* transcription and CCA1 repressing *TOC1* transcription, with the reduced time interval between TOC1 and CCA1 peaks being compatible with such a system. However, several lines of evidences, such as the robustness of the clock to CCA1 repression in LL, suggest the existence of additional unidentified components. Indeed, CCA1 might be more specialized toward gating light entry to the clock, as illustrated in LD: 6,6 cycles. It is therefore unlikely that the *Ostreococcus* clock simply reflects a reduced version of that in *Arabidopsis*. Together, in silico and functional analysis suggest that clock genes, such as *TOC1* and *CCA1*, arose early in evolution, in a common ancestor of Chlorophyta and Streptophyta, whereas other clock genes, such as *GI*, *ELF3*, or *ELF4*, evolved afterwards in terrestrial plants. A future challenge will be to identify other genes involved in the circadian clock of *Ostreococcus* and determine to what extent they are shared with other plant lineages. Though in its infancy, circadian research in simple photosynthetic organisms should aid the understanding of how green clocks have evolved and help in unraveling their architecture. Beyond circadian research, the development of *Ostreococcus* as a new model system opens the way for a better understanding of how complex biological processes are coordinated in simple photosynthetic organisms; this in turn may lend insight to delineating similar systems in higher plants.

METHODS

Cloning Strategy and Vector Construction

PCR amplification of the promoter sequences (entire intergenic region), full genes, or open reading frames for TOC1, CCA1, CAB, Histone H4, and HAPT was achieved with the Triple Master polymerase mix (Eppendorf). Cloning primers are listed in Supplemental Table 2 online. For ease, a subcloning step in the pGEMT vector (Promega) was performed in all cases. The pOtLuc vector (see under accession numbers) was designed to report gene expression by fusion of the promoter or the full gene in frame to the luciferase coding region from pSP-luc+NF (Promega; see Supplemental Figure 1 online). The expression of KanMx, which encodes resistance to G418, is driven by the *Ostreococcus tauri* histone H4 promoter. The pOtox vector (see under accession numbers) uses the *O. tauri* HAPT promoter, which drives the expression of one of the most abundant transcripts in *Ostreococcus* at steady state levels in LL (see Supplemental Figures 4 and 5 online).

Algal Material and Culture Conditions

O. tauri strain 0TTH0595 was grown in aerated flasks (Sarstedt) or white 96-well microplates (Nunc; Perkin-Elmer) under constant illumination or under appropriate LD cycles (at 16 to 32 μmole·quanta··cm^{-2}·s^{-1}). For time course luminescence recordings, cells were refreshed in culture medium with luciferin (20 μM) at a final density of 4 to 10 × 10^6 cell/mL. Entrainment was achieved during 8 d under LD cycles (16 μmole·quanta · cm^{-2}·s^{-1}) during which the final 2 d were performed under recording conditions. Release into LL was performed at the same or decreased intensity for phase shifting experiments (16 μmole·quanta ·cm^{-2}·s^{-1} or at 8 μmole·quanta ·cm^{-2}·s^{-1}). To test entrainment properties, cells grown in constant light were transferred to new medium with luciferin in recording conditions under LD. For mRNA and protein analysis, batch cultures were grown in aerated plastic flasks.

Ostreococcus Genetic Transformation and Line Selection

Fifty milliliters of *O. tauri* (3 × 10^7 cell/mL) grown under LL were harvested by centrifugation (8000*g*, 8 min, 10°C) after addition of pluronic acid F-68 to 0.1% w/v (Sigma-Aldrich). To remove salts, the pellet was gently resuspended in 1 mL 1 M sorbitol and centrifuged (8000*g*, 5 min, 10°C) twice. Cells resuspended in 50 to 80 μL sorbitol (2 to 3 × 10^{10} cells per mL) were incubated on ice for 10 min with 5 μL of linearized DNA (1 μg/μL) and transferred into an electroporation cuvette. Electroporation was performed using a Gene Pulser apparatus (Bio-Rad), field strength 6 kV/cm, resistor 600 Ω, capacitor 25 μF. Cells were carefully transferred into 40 mL of culture medium for 24 h. Stable transformant colonies were selected in semisolid medium at 0.2% w/v agarose (low-melting-point agarose; Invitrogen) in Keller medium supplemented with the appropriate antibiotic (1 mg/mlL G418 or 2 mg/mL nourseothricin). Inclusion was performed by mixing 0.5 to 1 mL of cell culture (1 to 3 × 10^7 cell/mL) with 10 mL of culture medium containing melted agarose in a Petri dish. Plates were placed in a wet chamber under constant light. After 10 to 20 d, individual clones were transferred in liquid medium to 96-well microplates until they reached stationary phase (4 to 6 × 10^7 cell/mL). CloNAT (nourseothricin; WERNER BioAgents) or G418 (Calbiochem) resistant transformants were further checked either by DNA gel blot or by PCR using primers specific for the transgene. DNA was extracted with DNAeasy plant mini kit (Qiagen). For DNA gel blots, 1 μg of DNA was digested with appropriate enzymes, migrated in a 0.8% TAE agarose gel, and transferred onto Hybond N^+ membrane (Amersham) used for DNA gel blot experiments. A 1.4-kb radiolabeled fragment corresponding to the 5′ region of the TOC1 gene, including TOC1 promoter, detects both the endogeneous TOC1 gene in wild-type cells and inserted copies of TOC1 in TOC1:Luc transformed lines.

Screening of Transformants

The pOtLuc vector was used to fuse any promoter or full gene of interest in frame with firefly luciferase (see Supplemental Figure 1 online). Transformants resistant to G418 and selected on the basis of reproducible patterns of luminescence were grown to saturation in microplates under LD conditions. Cells were refreshed at fixed-cell density in luciferin-supplemented culture medium. Two more LD cycles were performed before starting the automated recording of luminescence under constant light. The pOtox vector (see Supplemental Figures 5 and 6 online) was used to overexpress, in sense or antisense orientation, the coding region of interest in selected luciferase reporter backgrounds. Transformants resistant to CloNAt were screened for severe defects in rhythmicity of the luciferase reporter under LL. Experiments were repeated from 3 to 10 times with at least one duplicate per experiment. Bioluminescence traces were analyzed manually and with BRASS (Biological Rhythms Analysis Software System, P.E. Brown, Warwick University). Rhythmicity phenotypes were assessed at various cell densities. Comparison of luminescence levels was achieved using lines at similar cell densities to ensure similar growth between wells.

Data Acquisition and Analysis

Luminescence was acquired for 5 s every hour (Berthold LB Centro automated luminometer). Statistical analyses were performed using BRASS. Normalization of luminescence to the mean level was automatically achieved with the BRASS function. Fast Fourier transform nonlinear least square fit analysis (Plautz et al., 1997) was used to estimate relative amplitude error (RAE; a measure of goodness-fit to a theoretical sine wave) that was taken as an objective measure for rhythmicity of bioluminescence traces (O'Neill and Hastings, 2008). Analysis was performed on at least three independent experiments. Lines displaying RAE above 0.5 in all experiments were considered as arrhythmic (ar), and lines displaying RAE between 0.3 and 0.5, at least once, as dampened rhythmic (dr).

Luciferase in Vitro Assay

Cells were ground in lysis buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, and 10% glycerol). Luciferase assays were performed in a luminometer Centro LB 960 (Berthold Technologies) after injection of luciferase assay reagent (20 mM Tricine, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 270 μM CoA, 500 μM luciferin, and 500 μM ATP). Luminescence was normalized to the total amount of protein.

RNA Extraction, Whole-Genome Expression Analysis, and Quantitative RT-PCR Analysis

RNA was extracted using the RNeasy-Plus mini kit (Qiagen). Quantitative RT-PCR was performed as previously described (Moulager et al., 2007). Real-time PCR was performed on a LightCycler 1.5 (Roche Diagnostic) with LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals). Primers were designed with LightCycler Probe Design2 software (Roche Diagnostic). For quantification of RNA in antisense lines, reverse primers were designed against 3′ untranslated regions so that only the native transcript was quantified. Primers are available in Supplemental Table 2 online. Results were analyzed using the comparative critical threshold (ΔΔ*C*T) method. The *O. tauri* elongation factor 1 α (EF1α) was used as internal reference. The analyses were performed in duplicate. Microarray expression data were obtained from a time courses performed in triplicate, over 27 h, at 3-h intervals over three 12:12 light/dark cycles (Moulager et al., 2007). Pan-genomic *Ostreococcus* slides (24K) manufactured in the Rennes Transcriptome Platform (France) were based on gene-specific 50-mer oligonucleotides. In short, total RNAs (350ng) were amplified and labeled using a two-color labeling protocol. For each hybridization, the reference sample corresponded to a pool of all stages under investigation, so that it represents an average expression of the genome. Detailed methods have been previously described (Moulager et al., 2007)

Gel Shift Mobility Assay

The CCA1 open reading frame was cloned into pGEX bacterial expression vector (Amersham) for glutathione *S*-transferase (GST) recombinant protein production using specific primers (see Supplemental Table 2 online). The GST-CCA1 protein was produced in *Escherichia coli* (BL-21 strain; Stratagene) according to the manufacturer's instructions (induction with 0.4 mM IPTG, 20°C overnight) and loaded on a Histrap GST-sepharose column. Elution was performed after cleavage of the GST tag with the precision enzyme according to the manufacturer's instructions (GE Healthcare). Gel shift mobility assays were performed essentially as previously described (Schwartz et al., 2000). Protein-DNA binding

reactions were performed in a buffer containing 10 ng of recombinant CCA1, 1 ng of 32P-5′-end-labeled double-strand probe (annealed oligonucleotides), 1 μg of poly(dI-dC), 50 ng of sonicated salmon sperm DNA, 10 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 12.5 mM HEPES, pH 7.8, 10% glycerol, and 0.05% Nonidet P-40. Mixtures were incubated for 15 min at 4°C, and protein-DNA complexes were analyzed by electrophoresis on a 6% polyacrylamide gel in 0.25× Tris-buffered EDTA. To check the specifity of the DNA–protein interaction, competing unlabeled probe was added at 5-, 25-, 50-, or 100-fold molar excess to the labeled probe. Sequences of probes are listed in Supplemental Table 2 online

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: TOC1 At (*Arabidopsis*), At5g61380; PRR3 At (*Arabidopsis*), At5g60100; PRR Os (*Oryza sativa*), XP47960; PRR Cr (*Chlamydomonas reinhardtii*), C_530090; TOC1 Ot, AY740079 (*O. tauri*); COL Ot, AY740087 (*O. tauri*); COL2 At (*Arabidopsis*), At3g02380 LHY At (*Arabidopsis*), At1g01060; CCA1/LHY Os1 (*O. sativa*), BAC99516; EPR1 At (*Arabidopsis*), At1g18330; CCA1/LHY Cr (*C. reinhardtii*), C_1060059; CCA1/LHY Cm (*Cyanydioschyzon merolae*), CMK043C; CCA1/LHY Ot (*O. tauri*), AY740077, AY740087; CRY (*O. tauri*), AY740085; CRY DASH (*O. tauri*), AY740084; SRR1 (*O. tauri*), AY740080); pOtLuc vector, FN54878; pOtox vector, FN554877.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. pOtLuc Expression Vector Ddesigned to Generate Promoter or Full Gene Luciferase Fusion in *Ostreococcus*.

Supplemental Figure 2. Analysis of TOC1:Luc Transformants by DNA Gel Blot Analysis.

Supplemental Figure 3. Relative Phase Adjustments of TOC1 and CCA1 Promoter Activity and Protein Synthesis to Photoperiod.

Supplemental Figure 4. Schematic Map of the Overexpression/Antisense pOtox Vector.

Supplemental Figure 5. Analysis of the Promoter Activity of the High Affinity Phosphate Transporter (PHAPT).

Supplemental Figure 6. Screening for Altered Rhythmicity in TOC1 and CCA1 Overexpression/Antisense Lines in the CCA1:Luc and TOC1:Luc Backgrounds.

Supplemental Figure 7. Expression of PCAB:Luc in Constant Light

Supplemental Figure 8. Overexpression of TOC1 or CCA1 Disrupts Circadian-Regulated Expression of CCA1 and TOC1.

Supplemental Figure 9. RT-PCR Analysis of Overexpression and Antisense Lines with Rhythmicity Defects in TOC1:Luc and CCA1:Luc Backgrounds.

Supplemental Table 1. Identification of Putative Homologs of *Arabidopsis thaliana* Clock-Related Genes and Photoreceptors in the Green Alga *Ostreococcus tauri*.

Supplemental Table 2. Primers Used for Cloning, Checking Recombinant Lines, Quantitative RT-PCR, and EMSA.

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REFERENCES

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P., and Kay, S.A. (2001). Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science **293:** 880–883.

Alabadi, D., Yanovsky, M.J., Mas, P., Harmer, S.L., and Kay, S.A. (2002). Critical role for CCA1 and LHY in maintaining circadian rhythmicity in Arabidopsis. Curr. Biol. **12:** 757–761.

Carre, I.A., and Kim, J.Y. (2002). MYB transcription factors in the Arabidopsis circadian clock. J. Exp. Bot. **53:** 1551–1557.

Cerutti, H., Johnson, A.M., Gillham, N.W., and Boynton, J.E. (1997). Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell **9:** 925–945.

Courties, C., Vaquer, A., Troussellier, M., Lautier, J., Chrétiennot-Dinet, M.J., Neveux, J., Machado, C., and Claustre, H. (1994). Smallest eukaryotic organism. Nature **370:** 255.

Covington, M.F., Maloof, J.N., Straume, M., Kay, S.A., and Harmer, S.L. (2008). Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol. **9:** R130.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognar, L., Nagy, F., Millar, A.J., and Amasino, R.M. (2002). The ELF4 gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. Nature **419:** 74–77.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell **96:** 271–290.

Farre, E.M., and Kay, S.A. (2007). PRR7 protein levels are regulated by light and the circadian clock in Arabidopsis. Plant J. **52:** 548–560.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G., and Putterill, J. (1999). GIGANTEA: A circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J. **18:** 4679–4688.

Gould, P.D., Locke, J.C., Larue, C., Southern, M.M., Davis, S.J., Hanano, S., Moyle, R., Milich, R., Putterill, J., Millar, A.J., and Hall, A. (2006). The molecular basis of temperature compensation in the *Arabidopsis* circadian clock. Plant Cell **18:** 1177–1187.

Harmer, S.L., and Kay, S.A. (2005). Positive and negative factors confer phase-specific circadian regulation of transcription in *Arabidopsis*. Plant Cell **17:** 1926–1940.

Harmer, S.L., Panda, S., and Kay, S.A. (2001). Molecular bases of circadian rhythms. Annu. Rev. Cell Dev. Biol. **17:** 215–253.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R., and Kay, S.A. (2005). LUX ARRHYTHMO encodes a Myb

domain protein essential for circadian rhythms. Proc. Natl. Acad. Sci. USA **102:** 10387–10392.

Hicks, K.A., Albertson, T.M., and Wagner, D.R. (2001). EARLY FLOWERING3 encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. Plant Cell **13:** 1281–1292.

Hicks, K.A., Millar, A.J., Carre, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R., and Kay, S.A. (1996). Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. Science **274:** 790–792.

Jarrett, R.M., and Edmunds, L.N., Jr. (1970). Persisting circadian rhythm of cell division in a photosynthetic mutant of Euglena. Science **167:** 1730–1733.

Jin, H., and Martin, C. (1999). Multifunctionality and diversity within the plant MYB-gene family. Plant Mol. Biol. **41:** 577–585.

Kikis, E.A., Khanna, R., and Quail, P.H. (2005). ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. Plant J. **44:** 300–313.

Lakin-Thomas, P.L. (2000). Circadian rhythms: new functions for old clock genes. Trends Genet. **16:** 135–142.

Leon, R., and Fernandez, E. (2007). Nuclear transformation of eukaryotic microalgae: Historical overview, achievements and problems. Adv. Exp. Med. Biol. **616:** 1–11.

Locke, J.C., Kozma-Bognar, L., Gould, P.D., Feher, B., Kevei, E., Nagy, F., Turner, M.S., Hall, A., and Millar, A.J. (2006). Experimental validation of a predicted feedback loop in the multi-oscillator clock of *Arabidopsis thaliana*. Mol. Syst. Biol. **2:** 59.

Locke, J.C., Millar, A.J., and Turner, M.S. (2005a). Modelling genetic networks with noisy and varied experimental data: The circadian clock in *Arabidopsis thaliana*. J. Theor. Biol. **234:** 383–393.

Locke, J.C., Southern, M.M., Kozma-Bognar, L., Hibberd, V., Brown, P.E., Turner, M.S., and Millar, A.J. (2005b). Extension of a genetic network model by iterative experimentation and mathematical analysis. Mol. Syst. Biol. **1:** 0013.

Makino, S., Matsushika, A., Kojima, M., Yamashino, T., and Mizuno, T. (2002). The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: I. Characterization with APRR1-overexpressing plants. Plant Cell Physiol. **43:** 58–69.

Mas, P., Alabadi, D., Yanovsky, M.J., Oyama, T., and Kay, S.A. (2003). Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. Plant Cell **15:** 223–236.

Matsuo, T., Okamoto, K., Onai, K., Niwa, Y., Shimogawara, K., and Ishiura, M. (2008). A systematic forward genetic analysis identified components of the Chlamydomonas circadian system. Genes Dev. **22:** 918–930.

Matsushika, A., Makino, S., Kojima, M., and Mizuno, T. (2000). Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators in *Arabidopsis thaliana*: Insight into the plant circadian clock. Plant Cell Physiol. **41:** 1002–1012.

McWatters, H.G., Bastow, R.M., Hall, A., and Millar, A.J. (2000). The ELF3 zeitnehmer regulates light signalling to the circadian clock. Nature **408:** 716–720.

Merrow, M., Boesl, C., Ricken, J., Messerschmitt, M., Goedel, M., and Roenneberg, T. (2006). Entrainment of the Neurospora circadian clock. Chronobiol. Int. **23:** 71–80.

Merrow, M., Brunner, M., and Roenneberg, T. (1999). Assignment of circadian function for the Neurospora clock gene frequency. Nature **399:** 584–586.

Michael, T.P., and McClung, C.R. (2002). Phase-specific circadian clock regulatory elements in Arabidopsis. Plant Physiol. **130:** 627–638.

Michael, T.P., and McClung, C.R. (2003). Enhancer trapping reveals widespread circadian clock transcriptional control in Arabidopsis. Plant Physiol. **132:** 629–639.

Michael, T.P., et al. (2008). Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. PLoS Genet. **4:** e14.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.H., and Kay, S.A. (1995). Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science **267:** 1161–1163.

Millar, A.J., and Kay, S.A. (1996). Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. Proc. Natl. Acad. Sci. USA **93:** 15491–15496.

Millar, A.J., Short, S.R., Chua, N.H., and Kay, S.A. (1992). A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell **4:** 1075–1087.

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.R., Carre, I.A., and Coupland, G. (2002). LHY and CCA1 are partially redundant genes required to maintain circadian rhythms in Arabidopsis. Dev. Cell **2:** 629–641.

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J., and Coupland, G. (2005). Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in *Arabidopsis*. Plant Cell **17:** 2255–2270.

Mizuno, T. (2004). Plant response regulators implicated in signal transduction and circadian rhythm. Curr. Opin. Plant Biol. **7:** 499–505.

Mizuno, T., and Nakamichi, N. (2005). Pseudo-response regulators (PRRs) or true oscillator components (TOCs). Plant Cell Physiol. **46:** 677–685.

Moulager, M., Monnier, A., Jesson, B., Bouvet, R., Mosser, J., Schwartz, C., Garnier, L., Corellou, F., and Bouget, F.Y. (2007). Light-dependent regulation of cell division in Ostreococcus: Evidence for a major transcriptional input. Plant Physiol. **144:** 1360–1369.

Murakami, M., Ashikari, M., Miura, K., Yamashino, T., and Mizuno, T. (2003). The evolutionarily conserved OsPRR quintet: Eice pseudo-response regulators implicated in circadian rhythm. Plant Cell Physiol. **44:** 1229–1236.

Nakamichi, N., Ito, S., Oyama, T., Yamashino, T., Kondo, T., and Mizuno, T. (2004). Characterization of plant circadian rhythms by employing Arabidopsis cultured cells with bioluminescence reporters. Plant Cell Physiol. **45:** 57–67.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell **101:** 331–340.

Nozue, K., Covington, M.F., Duek, P.D., Lorrain, S., Fankhauser, C., Harmer, S.L., and Maloof, J.N. (2007). Rhythmic growth explained by coincidence between internal and external cues. Nature **448:** 358–361.

Onai, K., and Ishiura, M. (2005). PHYTOCLOCK 1 encoding a novel GARP protein essential for the Arabidopsis circadian clock. Genes Cells **10:** 963–972.

O'Neill, J.S., and Hastings, M.H. (2008). Increased coherence of circadian rhythms in mature fibroblast cultures. J. Biol. Rhythms **23:** 483–488.

Palenik, B., et al. (2007). The tiny eukaryote Ostreococcus provides genomic insights into the paradox of plankton speciation. Proc. Natl. Acad. Sci. USA **104:** 7705–7710.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A., and Nam, H.G. (1999). Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. Science **285:** 1579–1582.

Perales, M., and Mas, P. (2007). A functional link between rhythmic changes in chromatin structure and the *Arabidopsis* biological clock. Plant Cell **19:** 2111–2123.

Plautz, J.D., Straume, M., Stanewsky, R., Jamison, C.F., Brandes, C., Dowse, H.B., Hall, J.C., and Kay, S.A. (1997). Quantitative analysis of Drosophila period gene transcription in living animals. J. Biol. Rhythms **12:** 204–217.

Robbens, S., Khadaroo, B., Camasses, A., Derelle, E., Ferraz, C., Inze, D., Van de Peer, Y., and Moreau, H. (2005). Genome-wide analysis of core cell cycle genes in the unicellular green alga *Ostreococcus tauri*. Mol. Biol. Evol. **22:** 589–597.

Roenneberg, T., Dragovic, Z., and Merrow, M. (2005). Demasking biological oscillators: Properties and principles of entrainment exemplified by the Neurospora circadian clock. Proc. Natl. Acad. Sci. USA **102:** 7742–7747.

Roenneberg, T., and Merrow, M. (2001). Circadian systems: Different levels of complexity. Philos. Trans. R. Soc. Lond. B Biol. Sci. **356:** 1687–1696.

Samuelsson, G., Sweeney, B.M., Matlick, H.A., and Prezelin, B.B. (1983). Changes in photosystem II account for the circadian rhythm in photosynthesis in *Gonyaulax polyedra*. Plant Physiol. **73:** 329–331.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A., and Coupland, G. (1998). The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219–1229.

Schwartz, C., Catez, P., Rohr, O., Lecestre, D., Aunis, D., and Schaeffer, E. (2000). Functional interactions between C/EBP, Sp1, and COUP-TF regulate human immunodeficiency virus type 1 gene transcription in human brain cells. J. Virol. **74:** 65–73.

Somers, D.E., Webb, A.A., Pearson, M., and Kay, S.A. (1998). The short-period mutant, toc1-1, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. Development **125:** 485–494.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A., and Kay, S.A. (2000). Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science **289:** 768–771.

Takata, N., Saito, S., Tanaka Saito, C., Nanjo, T., Shinohara, K., and Uemura, M. (2008). Molecular phylogeny and expression of poplar circadian clock genes, LHY1 and LHY2. New Phytol., in press.

van Leeuwen, W., Ruttink, T., Borst-Vrenssen, A.W., van der Plas, L.H., and van der Krol, A.R. (2001). Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. J. Exp. Bot. **52:** 949–959.

Wang, Z.Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S., and Tobin, E.M. (1997). A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis* Lhcb gene. Plant Cell **9:** 491–507.

Wang, Z.Y., and Tobin, E.M. (1998). Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell **93:** 1207–1217.