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Review

Chlamydomonas reinhardtii as a new model system for studying the molecular basis of the circadian clock

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ABSTRACT

The genome of the unicellular green alga *Chlamydomonas reinhardtii* has both plant-like and animal-like genes. It is of interest to know which types of clock genes this alga has. Recent forward and reverse genetic studies have revealed that its clock has both plant-like and algal clock components. In addition, since *C. reinhardtii* is a useful model organism also called "green yeast", the identification of clock genes will make *C. reinhardtii* a powerful model for studying the molecular basis of the eukaryotic circadian clock. In this review, we describe our forward genetic approach in *C. reinhardtii* and discuss some recent findings about its circadian clock.

1. Introduction

Chlamydomonas reinhardtii is a unicellular green alga that belongs to the Chlorophytes division, which diverged from the Streptophytes division (including land plants) more than one billion years ago [1]. It has a pair of anterior flagella, a single cup-shaped chloroplast, a nucleus, and an eyespot [2], and this alga shows robust circadian rhythms in many cellular processes [3,4]. *C. reinhardtii* is often called the "green yeast", because it has many properties that are advantageous for molecular genetic studies, such as rapid growth, colony formation on agar plates, a haploid genome, the ease of genetic manipulation, and high-frequency transformation of nuclear, chloroplast, and mitochondrial genomes [2,5]. In addition, many mutant strains of *C. reinhardtii* are publicly available from the *Chlamydomonas* Center (http://www.chlamy.org/). Previously, it was difficult to express foreign genes in *C. reinhardtii*, such as antibiotic resistance and reporter genes due to codon usage bias, because its nuclear and chloroplast genomes are highly GC and AT rich, respectively. However, the development and widespread use of artificial gene synthesis has solved this problem; several codon-adapted reporter genes, including luciferases and green fluorescence proteins, can now be expressed in the nucleus and chloroplasts of *C. reinhardtii* [6–12]. In addition to the chloroplast and mitochondrial genome sequences [13–15], the sequencing of entire nuclear genome (~120 Mbp) was completed in 2007 [16]. As a result, *C. reinhardtii* became one of the models for post-genomic studies. In the following sections, we discuss the development of forward genetic methods for studying circadian rhythm and the identification, regulation, and evolution of circadian genes in *C. reinhardtii*.

2. Studies on circadian rhythms in *C. reinhardtii*

Research on the circadian rhythm of *C. reinhardtii* began about 40 years ago. At the time, *C. reinhardtii* was already a model organism for investigating the genetic basis of circadian rhythms. In 1970, Victor Bruce discovered that the photoaccumulation (positive phototaxis) of *C. reinhardtii* is regulated by a circadian clock. Specifically, more *C. reinhardtii* cells accumulate near a light source during the day/subjective day than at night/subjective night [17]. During the next few years, he isolated several mutants of this process [18,19], which about the same time that circadian rhythm mutants in *Drosophila melanogaster* and *Neurospora crassa* were isolated [20,21]. Although *C. reinhardtii* mutants share the same name "*per*" as clock genes in insects and mammals, they are probably unrelated because

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the genome of *C. reinhardtii* does not contain any homologs of insect or mammalian *per* [22]. Four *per* mutants in *C. reinhardtii* (*per-1*, *per-2*, *per-3*, and *per-4*) have long-period circadian rhythms (27–28 h) [19]. The underlying mutations in these mutants are in different genes, and their period-lengthening effects are additive [19]. Analyses of vegetative diploids of *per-1*, *per-2*, and *per-4* mutants revealed that *per-1* is dominant, *per-2* is recessive, and *per-4* is probably incompletely dominant to their respective wild-type alleles [23]. Furthermore, the *per-4* mutation is genetically linked to arginine-requiring markers on linkage group I [23]. In addition to these long-period mutants, Dieter Mergenhagen isolated a short-period mutant and demonstrated that the periodicity of the mutant and wild-type strains are maintained even under the zero-gravity condition in space [24,25]. However, the corresponding genes for Bruce's and Mergenhagen's mutants have not been identified yet.

Although *C. reinhardtii* is an simple unicellular organism, it shows circadian rhythms in many biological processes, such as phototaxis, chemotaxis, cell division, cell adhesion, starch content, sensitivity to ultraviolet (UV) irradiation, and nitrogen metabolism [17,26–31]. The circadian regulation of these processes confers adaptive advantages to *C. reinhardtii*. For example, positive phototaxis during the day allows cells to accumulate in light-rich environments and achieve efficient photosynthesis. Similarly, chemotaxis during the night enables them to find nitrogen-rich environments before their nitrogen uptake and metabolism peaks in the morning [4,26].

The circadian rhythms in these processes suggest that the underlying gene expression also is regulated by a circadian clock. Indeed, many studies have described circadian rhythms at the mRNA level [32–42]. For instance, a genome-wide DNA microarray analysis revealed that the expression of ~2.6% of the nuclear genes in *C. reinhardtii* is regulated by the circadian clock [32]. In some photosynthesis genes, the transcriptional regulation is thought to be a major target of the circadian control. For example, the transcriptional rate of *LHCA1* is stronger during the day, the peak of *LHCA1* mRNA level, than during the subjective night, whereas its mRNA stability rhythm is relatively weak compared to the transcriptional rhythm [34]. The expression of chloroplast genes also follows a circadian rhythm (see Section 7).

The post-transcriptional regulation of nuclear genes also have important roles in the circadian system of *C. reinhardtii*. For example, the RNA-binding protein CHLAMY1 from *C. reinhardtii* is an analog of the circadian-controlled translational regulator (CCTR) of the dinoflagellate *Lingulodinium polyedrum* [43]. CHLAMY1 binds to 3′ untranslated region (3′-UTR) of various mRNAs, especially whose products are involved in nitrogen and carbon metabolism [44]. Since the activity of NII, which is a key enzyme for nitrogen metabolism, is negatively correlated with the binding activity of CHLAMY1, CHALMY1 seems to act as a translational repressor of the circadian expression of *nii1* [43,44].

Interestingly, CHLAMY1 is involved in the circadian oscillator itself [31]. CHLAMY1 is a multimeric protein complex that is composed of C1 and C3 subunits [45]. The C1 subunit contains three lysine homology (KH) domains and a WW protein–protein interaction domain, while the C3 subunit is a CUG-BP-ETR-3-like factor (CELF) family protein that has three RNA recognition motifs (RRMs) (see Fig. 2) [45]. Both overexpression and RNA interference (RNAi)-mediated knockdown of the C3 subunit advanced the circadian phase in phototactic activity by about 6 h, whereas similar changes of the C1 subunit resulted in arrhythmicity [31]. Since these effects also were observed in the circadian rhythms in other processes, such as nitrate reductase activity, which is considered independent of phototaxis, CHLAMY1 is involved in the core mechanism of the circadian oscillation in *C. reinhardtii* [31]. Furthermore, since overexpression and knockdown of C1 also increased and decreased the level of C3 expression, respectively, it is likely that the regulation of these clock protein expressions are interconnected [31].

In addition to CHLAMY1, another nuclear gene, casein kinase, which is a Ser/Thr protein kinase, is involved in the mechanism of circadian oscillation in *C. reinhardtii* as well as other models of the circadian clock (except cyanobacteria) [46,47]. In *C. reinhardtii*, the casein kinase gene (*CK1*) affects various cellular processes, including the circadian rhythm [46]. The periodicity of a *CK1* knockdown strain of *C. reinhardtii* was 1.5 h shorter than that of the wild-type strain and tended to have arrhythmic photoaccumulation [46].

3. Development of bioluminescence reporters for *C. reinhardtii*

Luciferase-based bioluminescent reporters of circadian gene expression are powerful tools to monitor circadian rhythms and have been used in a wide range of model organisms for studying the molecular basis of the circadian clock [48–55]. A significant advantage of this tool is that it is amenable to high-throughput screening of circadian rhythm mutants. For example, several studies have used it to identify circadian rhythm mutants in *Arabidopsis thaliana* and cyanobacteria [56–58]. To develop similar reporters in *C. reinhardtii*, we generated reporter strains [10] by introducing a luciferase reporter gene into its chloroplast genome since this genome is very easy to transform [59]. We used a synthetic codon-optimized firefly luciferase gene that was driven by circadian-regulated chloroplast promoters (*tufA* and *psbD*). The bioluminescence levels of the transformants exhibited several characteristics of the circadian rhythm, namely self-sustained oscillation, phase resetting by external cues, and temperature compensation of the length of the period [10]. Importantly, when *per* mutants were used as the host strain, these reporter strains exhibited the period lengthening effects of *per* mutations [10]. These results indicated that the bioluminescence rhythm of the chloroplast is under the control of a nuclear-encoded circadian clock that is based on *per* [10], and that this reporting system can be used to isolate circadian rhythm mutants that are due to mutations in nuclear genes.

Since the bioluminescence rhythms of these reporter strains can be monitored in a 96-well format, we were able to monitor the circadian rhythm of *C. reinhardtii* by using an automated high-throughput bioluminescence detection and analysis system [60,61]. However, we did not detect any circadian rhythm in about half of our samples due to low amplitudes of the bioluminescence rhythms [62]. Since we knew that the genetic background of the reporter strain strongly affects the amplitudes of the bioluminescence rhythms, we crossed the original reporter strain and wild-type strains that had different genetic backgrounds to obtain a progeny clone called CBR34 that showed extremely robust rhythm in bioluminescence [62]. In addition, CBR34 has other characteristics that facilitate genetic studies, such as a high transformation rate and uniform growth and mating in 96-well microtiter plates without shaking.

It should be noted that the choice of the wild-type strain is critical for the efficiency of mutant screening and has important ramifications for future studies. For example, many advances in circadian research in *N. crassa* can be attributed to the selection and use of the band (*bd*) mutant, which exhibits a robust circadian rhythmicity in conidia formation in growth-tube culture, as a wild-type strain in early genetic studies [63,64]. Similarly, the isolation of the CBR34 strain has been pivotal for systematic forward genetic studies that have identified the components of the circadian clock in *C. reinhardtii*.

4. Identification of circadian clock genes in *C. reinhardtii*

In *C. reinhardtii*, gene tagging (insertional mutagenesis) has been used in forward genetic studies to identify genes of interest [65–67]. In this method, marker genes, such as antibiotic resistance genes, are introduced into random loci in the nuclear genome of *C. reinhardtii*, where they disrupt the endogenous gene (Fig. 1). After screening for mutants that show the desired phenotypes, the disrupted gene is easily identified by using the inserted marker gene as a "tag". Then, the flanking sequences of the inserted marker gene can be cloned or sequenced by using polymerase chain reaction (PCR)-based methods (e.g., thermal asymmetric interlaced (TAIL)-PCR, inverse PCR) (Fig. 1). Before the complete genome sequence of *C. reinhardtii* was determined, identifying the disrupted gene required screening and sequencing of bacterial artificial chromosome (BAC) or fosmid clones that contained the flanking sequence. Now, in the post-genomic era, the disrupted gene can be identified simply by searching for the flanking sequences in the genome sequence. Thus, it enable a large scale identification of mutated genes. Several studies have demonstrated the usefulness of this strategy for identifying mutated genes in the photosynthesis and nitrate assimilation pathways of *C. reinhardtii* [68,69].

We have used this strategy to perform large-scale identification of circadian genes in *C. reinhardtii* (Fig. 1) [62]. Specifically, we obtained about 16,000 transformants by insertion of the hygromycin-resistant marker gene into the nuclear genome of *C. reinhardtii*, screened them by using bioluminescence, and then isolated 105 mutants that exhibited abnormal circadian rhythms. Among these mutants, there were five kinds of phenotypes: short (10%) and long (7%) periods, advanced (2%) and delayed (3%) phases, and low amplitude rhythm (78%) [62].

We also investigated whether these mutant phenotypes were caused by insertion of the marker gene. If this were the case, then genetic cosegregation between the abnormal rhythm and marker gene in the progeny clone would be expected. As a result, if insertion of the marker gene disrupted a critical circadian gene, then all of the drug-resistant progenies should exhibit abnormal rhythms, whereas all of drug-sensitive progenies should have normal rhythms. Out of the 105 circadian rhythm mutants that we isolated, 51 showed cosegregation [62]. Since it is possible that additional copies of marker gene, which were not expressed, could have been inserted into the genome, we performed Southern blot analysis to determine the number of insertion sites of the marker gene in the mutants. As a result of this analysis, we excluded one mutant. The remaining 50 mutants only had single insertions in their genomes [62]. Therefore, we concluded that the marker gene had indeed "tagged" the genes responsible for the circadian rhythm defects in these mutants.

To identify the unknown flanking sequences of the known marker sequence, we used TAIL-PCR, which is effective for high-throughput analysis [70,71], to analyze the genomic DNA of the 50 tagged mutants. As a result, we identified the flanking sequences of the marker gene in 37 of these mutants [62]. Since there were some allelic mutants, we finally identified the 30 genes (or gene loci) as putative circadian genes in *C. reinhardtii* [62]. Some of these genes were unknown and, as a result of their mutant circadian rhythmicity of chloroplast bioluminescence, they were named "*RHYTHM OF CHLOROPLAST*" (*ROC*).

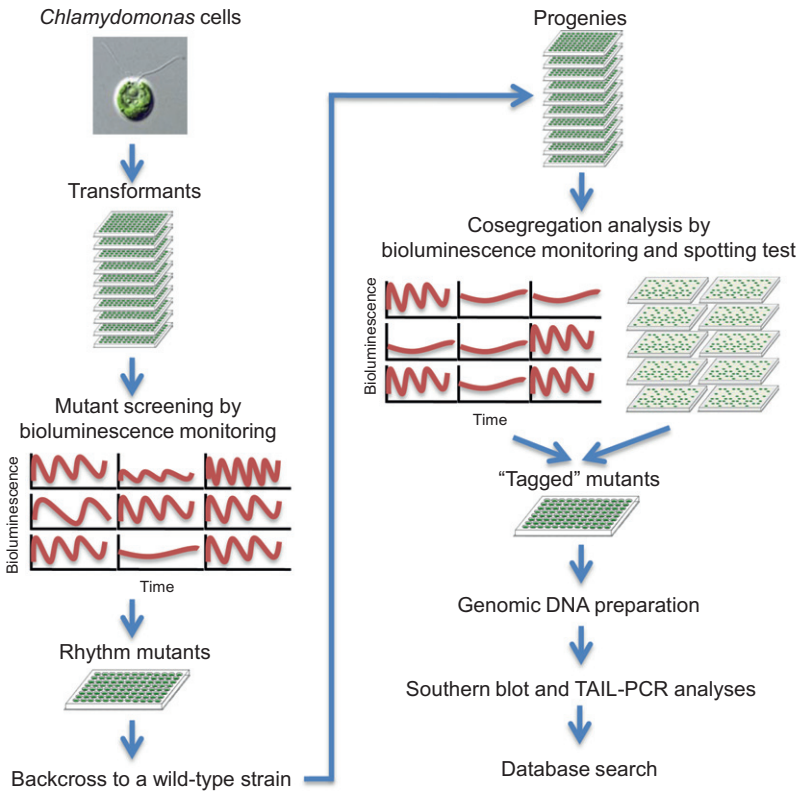

Fig. 1. Flow chart of the systematic forward genetic study that was used to identify novel circadian clock components in *Chlamydomonas reinhardtii*. First, *C. reinhardtii* cells (about 10 μm in diameter) were transformed with an antibiotic resistance gene. Subsequently, the transformants were screened with a bioluminescence assay to identify circadian rhythm mutants. These mutants were backcrossed with a wild-type strain, and then the progenies were further screened with a bioluminescence assay. In addition, a spotting test was performed to confirm the cosegregation of circadian rhythm defects and antibiotic resistance. Finally, the novel circadian genes were identified by TAIL-PCR analysis of the genomic DNA of the mutants.

To rule out the possibility that these mutant phenotypes were caused by other genes that are closely linked to the integration site of the marker gene, we performed genetic complementation experiments. Specifically, we tested six mutants that were thought to have insertions in critical clock genes (see below) by transforming them the mutant strains with genomic fragments that contained the corresponding wild-type allele. As a result, the phenotypes of these mutants were complemented [62].

The proteins that were encoded by the putative circadian genes that we identified can be classified into several groups according to their known or predicted functions. These functional groups included diverse biological processes, such as flagella function, ubiquitin–proteasome protein degradation, transcription and transcript metabolism, gene silencing, membrane trafficking and transport, signal transduction, DNA damage response, and apoptosis [62]. These processes are thought to be involved in the maintenance of the circadian rhythmicity of chloroplast bioluminescence. In particular, the mutation of six genes, namely, *ROC15*, *ROC40*, *ROC55*, *ROC66*, *ROC75*, and *ROC114*, caused severe circadian rhythm defects [62]. For example, *roc15* and *roc55* mutants showed short-period circadian rhythms (21 and 18 h, respectively), while *roc75* and *roc114* mutants exhibited arrhythmicity. Interestingly, *roc40* and *roc66* were conditional mutants; they only had a long period (30 h) circadian rhythm in continuous light (LL) or constant darkness (DD) conditions, respectively. In addition to their mutant phenotypes in the circadian rhythmicity of chloroplast bioluminescence, these six mutants exhibited altered circadian rhythms in their growth rate [62]. Collectively, these results demonstrated that these 6 genes are involved in the central mechanism of the circadian clock of *C. reinhardtii*.

5. Circadian clock proteins in *C. reinhardtii*

Four *ROC* genes (*ROC15*, *ROC40*, *ROC66*, and *ROC75*) encode putative transcription factors, and *ROC114* encodes an F-box protein (Fig. 2). In addition, casein kinase, which is an important regulator of phosphorylation, is involved in the circadian clock of *C. reinhardtii* (Fig. 2) [46]. These indicate that the transcription, phosphorylation, and ubiquitination are involved in the *C. reinhardtii* clock as well as in the other eukaryotic models for circadian clock (*Mus musculus*, *D. melanogaster*, *N. crassa*, and *A. thaliana*) [47,72,73]. Since the mRNA levels of *ROC15*, *ROC40*, *ROC66*, and *ROC75* oscillate in circadian manner with different phases [62], they are predicted to interact genetically with each other and form transcriptional feedback loops. Furthermore, the involvement of CHLAMY1 in the circadian clock of *C. reinhardtii* suggests that post-transcriptional regulation are critical regulatory mechanism of the *C. reinhardtii* clock [31]. As described above, CHLAMY1 binds to UG-repeat sequences in the 3′-UTR of mRNAs [44]. Interestingly, the 3′-UTR of *ROC40* mRNA contains an UG-repeat sequence that fits the consensus binding sequence of CHLAMY1 [44,62]. Thus, CHLAMY1 may regulate ROC40 translation and the regulation might be one of the critical parts in the circadian oscillatory mechanisms in *C. reinhardtii*.

6. Evolution of plant circadian clocks

Interestingly, the amino acid sequences of the putative transcription factors (ROC15, ROC40, ROC66, and ROC75) share some similarity with *A. thaliana* proteins that are involved in its circadian clock and photoperiodic flowering pathway. For example, the GARP DNA-binding domains of *C. reinhardtii* ROC15 and ROC75 are similar to that of *A. thaliana* PHYTOCLOCK1 (PCL1; also known as LUX ARRHYTHMO (LUX)). In addition, the single MYB repeat of *C. reinhardtii* ROC40 is similar to those of *A. thaliana* Late Elongated Hypocotyl (LHY) and Circadian Clock Associated 1 (CCA1) (Fig. 2) [62,74–76]. Recently, it also was shown that CCA1 and Timing Of Cab Expression 1 (TOC1) homologs are core components of the circadian clock in the marine green alga *Ostreococcus tauri* [55]. Likewise, CCA1/LHY homologs have a circadian clock function in the moss *Physcomitrella patens* [77] and *Lemna* plants [78,79]. These findings suggest that plant circadian clocks evolved from a

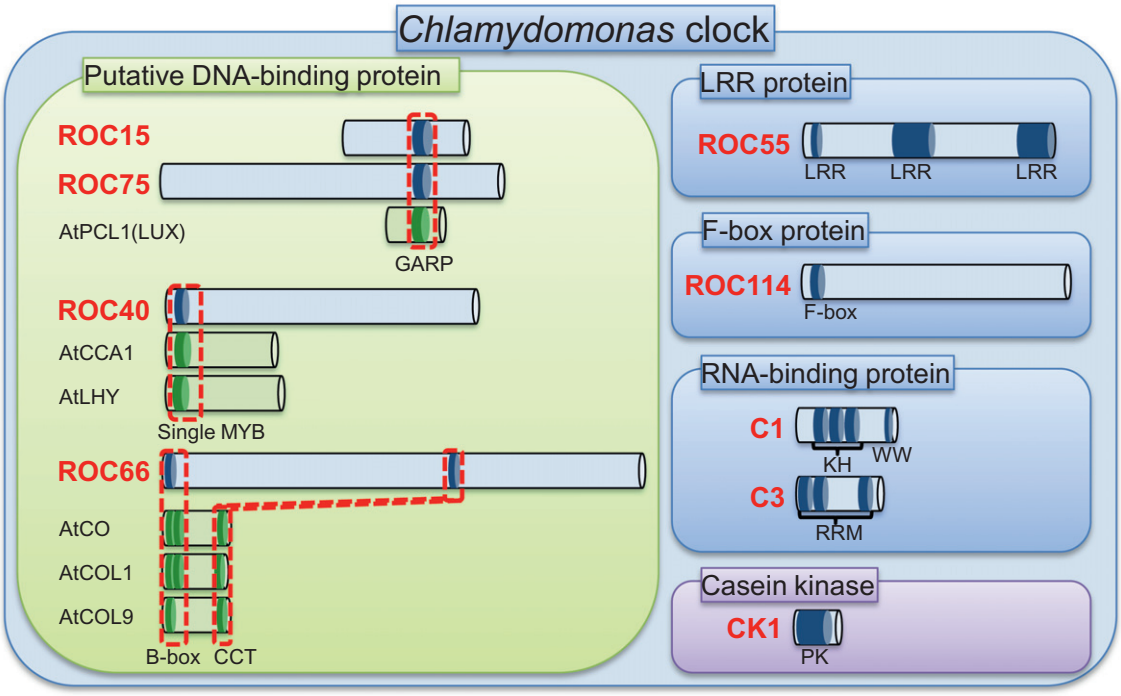

Fig. 2. Schematic representation of circadian clock proteins in *C. reinhardtii*. The predicted functional domains are shown with shaded boxes. The DNA-binding proteins are aligned with homologous *A. thaliana* proteins and regions with significant sequence similarity are outlined by dashed red lines.

common ancestral clock. However, the sequences of *C. reinhardtii* and *A. thaliana* clock proteins do not appear to have any significant similarities except for their DNA binding domains (Fig. 2). Moreover, *C. reinhardtii* protein sequences are much longer than *A. thaliana* proteins (Fig. 2). Furthermore, unlike *A. thaliana PCL1* (*LUX*) and *CCA1*/*LHY*, which are expressed in antiphase and form reciprocal negative feedback loops [62,74,75,80,81], *C. reinhardtii ROC15* and *ROC40* are expressed in the similar phase [62]. Thus, the extent to which molecular mechanisms of the circadian clock are conserved in plant evolution is not yet clear.

The B-box zinc finger and the CONSTANS, CONSTANS-like, TOC1 (CCT) domains of *C. reinhardtii* ROC66 are similar to those of *A. thaliana* CONSTANS (CO), which is a key regulator of the photoperiodic flowering pathway [82], and CO-like (COL) proteins (Fig. 2) [62]. Although the functions of many COLs are not known, COL1 is known to be involved in the circadian clock. For example, overexpression of COL1 shortens the circadian period of *A. thaliana* [83]. Recently, it was shown that the knockdown and overexpression of another CO homolog, CrCO, in *C. reinhardtii* affects the growth, diurnal rhythmicity of starch content, and synchrony of cell cycle-related gene expression [84]. In addition, CrCO can complement the *A. thaliana co* mutation and affect in a similar manner the timing of flowering and the expression of *FLOWERING LOCUS T* (*FT*) when it is expressed under different promoters in *A. thaliana* [84]. As a result, CrCO is thought to be a true ortholog of *A. thaliana* CO [84]. Taken together, these findings suggest that CO/COL family proteins may be evolutionary conserved coordinators of the photoperiodic pathway and the circadian clock in plants.

In contrast, ROC55 and ROC114 do not have any strong similarities to clock proteins in *A. thaliana* or other model organisms [62]. Instead, they are thought to be specific protein components of the circadian clock of *C. reinhardtii* among the clock model organisms. Similarly, the C1 and C3 subunits of CHLAMY1 do not have any strong similarity to known clock proteins. Interestingly though, the C3 subunit shares significant homology with the rat CUG-binding protein 2, and an anti-C3 antibody can recognize the rat homolog in various parts of the brain, including the suprachiasmatic nuclei, which is the location of the mammalian circadian pacemaker [45]. However, the role of CUG-binding protein 2 in the mammalian circadian clock is unknown.

Although chloroplasts are believed to have evolved from an endosymbiosis of an ancestral cyanobacterium [1,85], there are no obvious similarities between the circadian clock genes of *C. reinhardtii* and cyanobacteria, which consists of three genes (*kaiA*, *kaiB*, and *kaiC*) [86]. It is estimated that this endosymbiotic event occurred about 1600 million years ago (MYA) [1]. At the time, the ancestral cyanobacterium is thought to have had *kaiBC* cluster which appeared before 2320 MYA [87]. Subsequently, cyanobacteria acquired the *kaiA* gene about 1000 MYA ago [87] and evolved *kai*-based circadian clocks. However, the *kaiBC* genes derived from the endosymbiotic cyanobacterium seems not to have become an origin of the circadian clock in eukaryotic plants.

The considerable similarity between the circadian clocks of *C. reinhardtii* and *A. thaliana* suggests that green algae and land plants share a common ancestral clock, but each clock are thought to have evolved some specific features. The common mechanisms conserved between the *C. reinhardtii* and *A. thaliana* clocks might be essential parts for the plant clocks.

7. Circadian regulation of chloroplasts

The six aforementioned *ROC* genes that are closely related to the core mechanisms of the circadian clock of *C. reinhardtii*. How are the other *ROC* genes involved in the circadian rhythmicity of chloroplast bioluminescence? There are two possibilities: (1) they are involved in the same clock oscillation mechanisms as the six core *ROC* genes or (2) they are involved in a specific output pathway for the circadian rhythmicity of chloroplast bioluminescence. Although most of these analyses are still being performed, a mutant of the *ROC81* gene has suggested to be the latter. The *roc81* mutant showed normal circadian rhythmicity in growth rate, in spite of its low amplitude phenotype in the chloroplast bioluminescence [62]. This suggests that its core circadian mechanisms are intact but an output pathway for the bioluminescence rhythm may be impaired. Further functional analyses of ROC81 and other ROC proteins are needed to elucidate the molecular mechanisms connecting the circadian clock and chloroplast in *C. reinhardtii*.

The chloroplast is believed to have evolved through the primary endosymbiotic association between a eukaryotic host and a photosynthetic prokaryote. As a relic of this evolutionary past, its genetic system is more similar to that of prokaryotes than that of the nucleus [88]. *C. reinhardtii*, which was the first organism in which circadian regulation of chloroplast gene expression was discovered, is a good model organism to study how the circadian clock exerts its control on heterogeneous genetic systems in a cell. For example, Herrin and co-workers demonstrated that the transcription rate and mRNA accumulation level of the chloroplast *tufA* gene cycle with a robust circadian rhythmicity [33]. In addition, inhibitor experiments for cytoplasmic translation have shown that nuclear-encoded factors having relatively short half-life are involved in regulating the circadian rhythm of *tufA* transcription [89].

One candidate circadian transcriptional regulator is the sigma factor, which is a transcription initiation factor that is responsible for circadian transcriptional regulation of a subset of genes in cyanobacteria [90,91]. In addition, in eukaryotic plant models, such as wheat (*Triticum aestivum*) and moss (*P. patens*), the expression of nuclear-encoded sigma factor genes is regulated by the circadian clock [92,93]. Consequently, targeted disruption of a sigma factor gene in moss decreases the amplitude of the diurnal rhythmicity of the expression of the chloroplast-encoded *psbD* gene [94]. The nuclear genome of *C. reinhardtii* has a single copy sigma-like gene, *RPOD*, which is expressed in a circadian-regulated manner and targeted to the chloroplast [42,95]. The peak phase of *RPOD* mRNA expression is the subjective mid-to-late night [42], which is consistent with the finding that chloroplast transcription peaks in the subjective early morning [33].

It also has been known that the supercoiling of chloroplast DNA is regulated by the circadian clock in *C. reinhardtii* [96]. Specifically, its chloroplast DNA is supercoiled during the subjective day and relaxed during the subjective night, and the timing of the peak superhelicity was consistent with that of its peak transcription rate [96]. Since the degree of DNA supercoiling is an important factor in transcription initiation in chloroplasts [97,98], it is not surprising that the regulation of superhelicity may be a target of circadian transcriptional regulation. A candidate regulator of DNA topology in the chloroplast genome is DNA gyrase. In higher plants, two nuclear-encoded DNA gyrase subunits, GyrA and GyrB, are transported to chloroplasts and mitochondria [99,100]. The nuclear genome of *C. reinhardtii* also contains genes that encode GyrA-like and GyrB-like proteins (Protein ID: 115934 [GyrA-like] and 114600 [GyrB-like]; Joint Genome Institute (JGI) *C. reinhardtii* v4.0), which are homologous to GyrA and GyrB in higher plants; however, these genes have not been characterized yet.

No *roc* mutants having mutations in the sigma factor or DNA gyrase-like genes have been found. However, it should be noted that since these genes would be essential for cell viability, we might not have been able to isolate any mutants by disrupting genes with insertional mutagenesis.

8. Perspectives

By the recent identifications of clock components, *C. reinhardtii* has joined to the important model organisms for molecular dissection of the circadian clock. Several properties of *C. reinhardtii*, such as its haploid genome and ease of culturing, have facilitated these discoveries by shortening the time and increasing the throughput for genetic analyses and bioluminescence assays. At present, the rate-limiting step of forward genetic studies is not gene identification but the throughput of the monitoring system. By developing a high-throughput bioluminescence monitoring system that can measure tens of thousands of samples simultaneously, a comprehensive forward genetic analysis of *C. reinhardtii* will be completed within 2 months. Such a systematic forward genetic analysis of *C. reinhardtii* could potentially identify many novel genes that are involved in not only circadian oscillation but also output pathways to various cellular processes, such as taxis, cell cycle, cellular metabolism, and chloroplast gene expression. Especially, *C. reinhardtii* may be advantageous for studying the output pathway to the chloroplast compared to other clock models because of its simple cellular organization. The *C. reinhardtii* cell contains one nucleus and one chloroplast, and both of them are transformable, thus enabling the development of a dual reporter system for real-time monitoring of the nuclear and chloroplast gene expressions in the same cells.

In addition to forward genetic studies of *C. reinhardtii*, much work remains to elucidate the genetic and biochemical interactions of its circadian genes and proteins. For example, it would be very interesting to know whether ROCs interact with CHLAMY1. Since *C. reinhardtii* can be grown in large quantities, it is a good candidate for proteomic analyses [101,102]. Such large-scale studies of proteins in *C. reinhardtii* will not only reveal the structural and functional relationships of clock proteins but also improve our understanding of and stimulate further research about the molecular basis of the circadian rhythm, which regulates numerous processes in eukaryotic cells.

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