Circadian Rhythm of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 in the Dark

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The cyanobacterium *Synechocystis* sp. strain PCC 6803 exhibited circadian rhythms in complete darkness. To monitor a circadian rhythm of the *Synechocystis* cells in darkness, we introduced a P_{dnaK1}::*luxAB* gene fusion (S. Aoki, T. Kondo, and M. Ishiura, J. Bacteriol. 177:5606–5611, 1995), which was composed of a promoter region of the *Synechocystis dnaK1* gene and a promoterless bacterial luciferase *luxAB* gene set, as a reporter into the chromosome of a dark-adapted *Synechocystis* strain. The resulting *dnaK1*-reporting strain showed bioluminescence rhythms with a period of 25 h (on agar medium supplemented with 5 mM glucose) for at least 7 days in darkness. The rhythms were reset by 12-h-light–12-h-dark cycles, and the period of the rhythms was temperature compensated for between 24 and 31°C. These results indicate that light is not necessary for the oscillation of the circadian clock in *Synechocystis*.

Circadian rhythms are biological oscillations with a period of about 1 day and are found ubiquitously in organisms from cyanobacteria to humans. Since circadian rhythms in diverse organisms persist under constant conditions (i.e., constant light or darkness at a constant temperature), an endogenous mechanism called "circadian clock" that generates the rhythms is postulated.

Light resets the phases of circadian rhythms in all organisms examined so far. In green plants, a phase-resetting light signal or signals are supposed to be mediated by phytochrome (8, 23) or a blue-light receptor (21). Light is also required for sustaining circadian rhythms in plants and algae (7, 10, 11, 15, 20, 22, 24), because circadian rhythms are dampened in complete darkness (DD), even in the presence of exogenously added energy sources. In *Lemna gibba* (12), *Samanea saman* (23), and *Albizzia julibrissin* (20), daily exposure to brief red-light pulses sustains the circadian rhythms in darkness. In wheat, a red-light pulse prevents the damping of the circadian rhythms of *CAB-1* gene expression in DD, and far red reverses the red-light effect (17). In tobacco, overexpression of rice phytochrome makes the circadian rhythms of *CAB* gene expression persistent in DD (10). Therefore, a light signal mediated by phytochrome has an important role in sustaining the expression of the circadian rhythms in these plants. However, whether such a light signal is necessary (i) for the oscillation of the circadian clock itself, (ii) for the expression of a specific gene (for example, the *CAB* gene family), or (iii) for the ability to utilize exogenously added energy sources which are necessary for sustaining the expression of circadian rhythms remains to be resolved.

Previously, by using a bacterial luciferase *luxAB* gene set as a reporter, we demonstrated that a circadian clock controls the expression of the *dnaK1* gene, which encodes a heat shock protein, DnaK, in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (2). The *dnaK1*-reporting strain CFC2 cultured under continuous illumination (LL) exhibited circadian rhythms of bioluminescence, which reflect the rhythmic activation of the *dnaK1* promoter (2). However, the bioluminescence declined completely within 1 or 2 days when the cells were maintained in DD. In cyanobacteria, it remains to be determined whether a light signal is necessary for the oscillation of the circadian clock.

We took advantage of the fact that *Synechocystis* can grow heterotrophically on glucose in darkness when the cells are exposed to a daily, brief light pulse (light-activated heterotrophic growth [LAHG] [1]). The *Synechocystis* cells grown under LAHG conditions (dark-adapted cells) can continue to grow for 6 to 8 days even in DD (1). We constructed a dark-adapted *dnaK1*-reporting strain and found that this strain showed a persistent circadian rhythm of bioluminescence even in DD, indicating that the oscillation of the clock does not need any light signal in *Synechocystis*.

MATERIALS AND METHODS

Bacterial strains, media, and cultures. Wild-type cells of *Synechocystis* sp. strain PCC 6803 were maintained in BG-11 liquid medium (19) or on BG-11 agar that contained 1 mM sodium thiosulfate, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES [pH 8.2]), and 1.5% (wt/vol) Bacto-Agar (Difco Laboratories, Detroit, Mich.) under LL (intensity of about 35 μmol m^{-2} s^{-1} from white fluorescent lamps), as described previously (2). A bioluminescent *dnaK1*-reporting strain of *Synechocystis*, CFC2 (2), was maintained in BG-11 medium (or agar) supplemented with 40 μg of spectinomycin sulfate per ml. Dark-adapted cells of *Synechocystis* were maintained under LAHG conditions, in which cells were grown in the presence of 5 mM glucose in darkness except for a 15-min light pulse of 40 μmol m^{-2} s^{-1} every 24 h (1). (Although Anderson et al. [1] used daily 5-min light pulses, we used 15-min light pulses for technical convenience.) A *dnaK1*-reporting strain of the dark-adapted *Synechocystis* constructed in this study, CFC4, was maintained under the LAHG conditions in the presence of 40 μg of spectinomycin sulfate per ml. Unless otherwise stated, cells were cultured at 27°C.

Construction of a *dnaK1*-reporting strain of dark-adapted *Synechocystis*. We first adapted wild-type cells of *Synechocystis* and CFC2 cells gradually to LAHG conditions as follows (23a). Cells were inoculated into 100 ml of BG-11 liquid medium containing 5 mM glucose and cultured with vigorous shaking (rotation speed of about 180 rpm) under constant illumination (LL) of 35 μmol m^{-2} s^{-1} on the first day. The light period was then decreased stepwise by 2 h a day. On the 13th day, the cells were transferred to and then maintained under LAHG conditions and used as dark-adapted cells. The dark-adapted cells grew continuously under LAHG conditions, both in liquid medium and on agar medium.

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Next, we transformed the dark-adapted *Synechocystis* cells with a *dnaK1* reporter plasmid pCF5 to introduce a P_{dnaK1}::*luxAB* gene fusion into a specific targeting site of the genome (2), as described previously (25) with some modifications. pCF5 carries a P_{dnaK1}::*luxAB* gene fusion, namely, the promoter region of the *Synechocystis dnaK1* gene (P_{dnaK1}) (5) connected to the coding region of the *Vibrio harveyi* luciferase gene set (*luxAB*) (4), and the Ω fragment (18) that contains the spectinomycin-streptomycin resistance gene (2). The dark-adapted cells were grown in BG-11 liquid medium under LAHG conditions with vigorous rotation (speed of about 180 rpm) until the cell density reached 3.5×10^8 cells per ml. The cells were then harvested by centrifugation and resuspended in BG-11 medium at a density of 10^9 cells per ml. pCF5 DNA was added to the cell suspension at a final concentration of 100 μg/ml, and the DNA-cell mixture was incubated for 4 h in darkness. A 100-μl aliquot of the mixture per plate was then plated onto BG-11 agar plates supplemented with 5 mM glucose (BG-11–glucose agar) and maintained for 20 h at 27°C in darkness, and then 200 μl of 6 mg of spectinomycin sulfate per ml was added to the bottom of the agar at a final concentration of 40 μg/ml to select spectinomycin-resistant transformants. The agar plates were maintained under LAHG conditions until transformant colonies developed. About 20 days after plating, we obtained 10 to 20 transformant colonies per plate. We confirmed by Southern blotting analysis that the resulting transformants carried P_{dnaK1}::*luxAB* and the Ω fragment at a specific targeting site of the chromosome as predicted (data not shown). We selected a transformant clone named CFC4 for further experiments.

Assay of bioluminescence rhythms and data analysis. CFC4 cells which had been maintained on agar medium under LAHG conditions were inoculated onto new BG-11–glucose agar plates and grown under LAHG conditions for 3 days. During the LAHG light pulse administered on the 3rd day after inoculation, an agar block (15 by 15 by 7 mm) containing a patch of cells was cut out and transferred into a 20-ml vial, and then an open, sterile microcentrifuge tube which contained *n*-decanal dissolved in vacuum pump oil at a concentration of 1.5% (vol/vol) was placed upright in the vial. Then the vial was maintained in DD to monitor bioluminescence. We monitored the bioluminescence from CFC4 cells on an agar block with a photomultiplier tube apparatus, as described previously (2). CFC2 cells which had been cultured under LL were also inoculated onto BG-11–glucose agar plates and grown under LL for 3 days, and then we also monitored the bioluminescence from CFC2 cells similarly. Measurements were repeated every 30 min for 7 days in DD, and the bioluminescence signal was collected for 100 s for each measurement with a low-noise photomultiplier tube. The periods of bioluminescence rhythms were computed by regression procedures, as described previously (13).

RESULTS AND DISCUSSION

Bioluminescence rhythms of the dark-adapted *Synechocystis* strains. In a previous study, we constructed a *dnaK1* reporter plasmid, pCF5, which carries a P_{dnaK1}::*luxAB* gene fusion composed of the promoter region of the *dnaK1* gene (P_{dnaK1}) and the promoterless *luxAB* gene set derived from *V. harveyi* and introduced the gene fusion into a specific targeting site of the chromosome of wild-type cells of *Synechocystis* via homologous recombination (2). We found that CFC2 cells exhibit circadian rhythms of bioluminescence under LL (2).

We first examined the bioluminescence from CFC2 cells maintained in DD. CFC2 cells were cultured for 3 days under LL in the presence of 5 mM glucose and then transferred to DD for monitoring of bioluminescence. The bioluminescence of CFC2 cells declined completely within 2 days in DD (Fig. 1), and most cells lost viability rapidly in DD.

We found that dark-adapted cells of *Synechocystis* could develop colonies under LAHG conditions on agar plates which were prepared with Bacto-Agar without purification. (Anderson et al. reported that unpurified Difco Bacto-Agar failed to support light-activated heterotrophic growth of the *Synechocystis* cells [1].) The dark-adapted cells could be transformed with the pCF5 plasmid, although the efficiency of transformation was very low: 10 to 20 transformant colonies per 10^8 cells per 10 μg of plasmid DNA. Thus, we addressed the question of whether *Synechocystis* cells which grow heterotrophically on glucose exhibit circadian rhythms of bioluminescence when the cells are maintained in DD. We transformed the dark-adapted cells with the pCF5 plasmid to introduce a P_{dnaK1}::*luxAB* gene fusion into the chromosome and obtained a *dnaK1*-reporting strain, CFC4. The cells were cultured under LAHG conditions
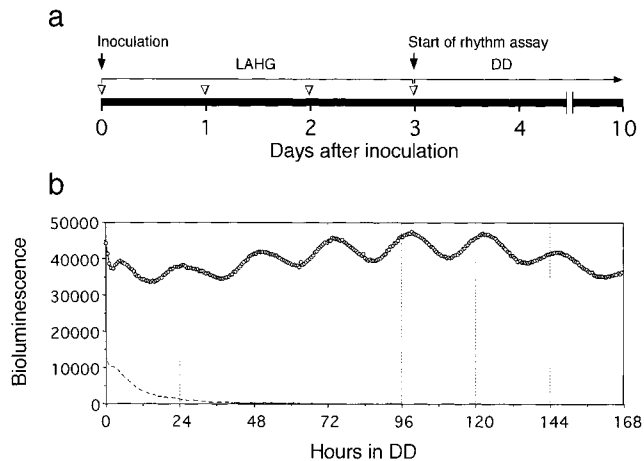

FIG. 1. Bioluminescence rhythm of CFC4 cells in DD. CFC4 cells were grown on BG-11 agar under LAHG conditions. The cells were inoculated onto fresh BG-11-glucose agar and cultured under LAHG conditions for 3 days, and after the last LAHG pulse, the cells were transferred to DD for monitoring of bioluminescence (open circles). The bioluminescence of CFC2 cells not been adapted to LAHG conditions is also shown for comparison (dashed line). CFC2 cells were grown on BG-11-glucose agar at 27°C under LL (46 μmol m^{-2} s^{-1}). The cells were inoculated onto fresh BG-11-glucose agar and cultured under LL for 2 days, and then the cells were grown in DD for monitoring of bioluminescence. The vertical axis indicates bioluminescence intensity as photon counts per second. The first peak of the bioluminescence, which occurred about 4 h after the onset of DD, was not used for data analysis because the peak was not observed in some cases. (a) Time course of the experiment. The times of the LAHG light pulses are shown by open inverted triangles, and the times of inoculation of cells and the start of bioluminescence monitoring are shown by arrows. (b) Bioluminescence rhythm.

for 3 days and then maintained in DD for monitoring of bioluminescence.

Figure 1 shows the time course of bioluminescence measured from CFC4 cells maintained in DD. The bioluminescence oscillated with a period of 25 ± 1 h at 27°C ($n = 4$). The first peak of the bioluminescence rhythm occurred at about 25.3 h after the onset of DD. The rhythm persisted for at least 7 days without dampening, and the peak/trough ratio of the rhythms was about 1.1 to 1.2 during measurement. Thus, the bioluminescence rhythm of CFC4 cells in DD satisfied one of the three criteria for circadian rhythms, namely, persistence under constant conditions with a period of about 1 day.

Entrainment of the bioluminescence rhythms to daily light-dark cycles and the phase shift of the rhythms by illumination. We examined further whether light-dark cycles entrain the bioluminescence rhythms of CFC4 cells. We exposed two cultures to three 12-h-light–12-h-dark (LD) cycles (light intensity of about 1.2 μmol m^{-2} s^{-1} from white fluorescent lamps) that were 180° out of phase and then maintained them in DD to monitor their bioluminescence (Fig. 2).

We found that the bioluminescence from these two cultures oscillated with opposite phases which depended on the timing of the preceding LD cycles, although the level of bioluminescence declined. The peak of bioluminescence rhythms occurred about 18.7 h after the onset of DD.

We also examined the phase-response curve (PRC) by light (Fig. 3). After the last LAHG pulse, we maintained cultures of CFC4 cells in DD and then exposed them to a 3-h illumination (1.2 μmol m^{-2} s^{-1}) at various times. We then monitored the bioluminescence rhythms in DD and calculated the magnitude of phase shift as the difference between the times at which the fourth peaks occurred in the rhythms of the control (without the light illumination) and experimental cultures. Phase ad-
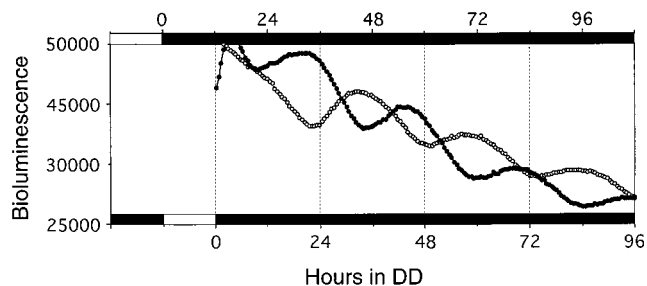
FIG. 2. Entrainment of the bioluminescence rhythms of CFC4 cells to LD cycles. To synchronize the circadian clock, two CFC4 cultures were subjected to LD cycles (light intensity was about 1.2 μmol m^{-2} s^{-1} from white fluorescent lamps) which were 180° out of phase, and then the cells were maintained in DD for monitoring of bioluminescence (photon counts per second). The last 12-h light periods in the LD cycles (open bars) and the DD (filled bars) are shown on the upper abscissa for the open circles and lower abscissa for the solid circles. The lower portions of the vertical axes are omitted for precise comparison of the phases of the two rhythms. Other conditions were the same as described in the legend to Fig. 1.

FIG. 4. Temperature compensation during the period of the bioluminescence rhythms. After culture of CFC4 cells under LAHG conditions for 3 days, cells were maintained in DD at three different temperatures (24, 27, and 31°C) to have their bioluminescence (photon counts per second) monitored. Bioluminescence rhythms observed at the three different temperatures are shown. Other conditions were the same as those described in the legend to Fig. 1.

vance was induced when cells were exposed to the illumination 0 or 20 h after the last LAHG pulse, whereas phase delay was induced by the illumination given 8 or 12 h after the last LAHG pulse. Essentially, no phase shift was observed 4 or 16 h after the last LAHG pulse. We found that the maximal phase advance (4.8 ± 0.9 h [$n = 4$]) and delay (1.8 ± 0.7 h [$n = 4$]) were induced by the illumination given 20 and 8 h after the last LAHG pulse, respectively.

Temperature compensation of the bioluminescence rhythms. We examined the effects of temperature on the period length of the bioluminescence rhythms. CFC4 cells were cultured under LAHG conditions for 3 days, and then the cells were maintained at 24, 27, or 31°C in DD to monitor their bioluminescence (Fig. 4).

We found that the period lengths of the rhythms were 25.7, 25.0, and 27.2 h at 24, 27, and 31°C, respectively. The Q_{10} values (temperature coefficients) for 24 to 27°C and for 27 and 31°C were calculated separately to be 1.1 and 0.8, respectively. The phases of the rhythms observed at 24 or 31°C significantly
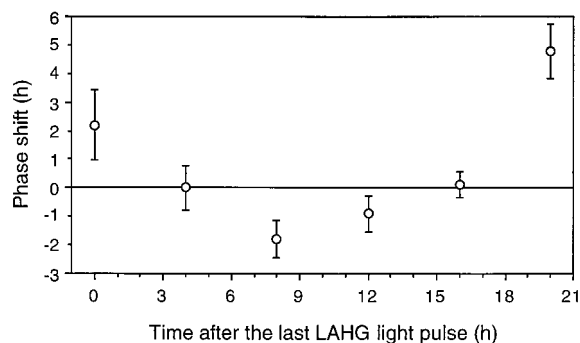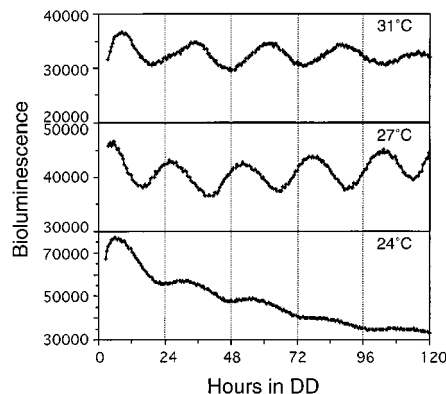

FIG. 3. Phase response induced by light. A 3-h illumination (1.2 μmol m^{-2} s^{-1}) was given to CFC4 cells at various times during the first day after the last LAHG pulse. Other conditions were the same as those described in the legend to Fig. 1. The magnitude of phase shifts was calculated as the difference between the times when the fourth peak occurred in control (without illumination) and experimental cultures and is shown as a function of the time of onset of illumination after the last LAHG pulse. Positive and negative values in the ordinate indicate phase advances and delays, respectively. Data points are means for four replicate experiments, and the bars on each point indicate the standard deviation. We did not draw lines between the data points because it is not clear whether the *Synechocystis* PRC is continuous.

differed from those observed at 27°C. The rhythms were unstable at temperatures higher than 31°C or lower than 24°C. These data indicate that the period length of the rhythms was compensated against changes in ambient temperature.

The data described above indicate that the bioluminescence rhythms of CFC4 cells maintained in DD satisfied the three criteria for circadian rhythms, as did those of CFC2 cells cultured under LL (2), namely, persistence under constant conditions (Fig. 1), entrainment to daily LD cycles (Fig. 2), and temperature compensation of the period (Fig. 4). Thus, we can conclude that the bioluminescence rhythms of CFC4 cells maintained in DD are circadian rhythms. Furthermore, the magnitude and direction of the phase shifts induced by an illumination depended on the time of day at which the light illumination was administered (Fig. 3). This observation represents the basic character of light responsiveness of circadian rhythms.

We demonstrated here that no light signal was necessary for the oscillation of the circadian clock or for the expression of bioluminescence rhythms in the dark-adapted *Synechocystis* strain CFC4. Strain CFC2 could not grow heterotrophically on glucose and therefore lost viability in DD (Fig. 1).

In contrast to cyanobacteria, several higher plants require a light signal which is supposed to be mediated by a phytochrome to sustain the expression of circadian rhythms (10, 12, 17, 20, 23). It is uncertain whether such a light signal is necessary to sustain the oscillation of the circadian clock itself in these plants, because downstream events which are used for monitoring the clock in these plants may depend on light. In the case of the *CAB* gene reporter, we cannot monitor the expression of the *CAB* gene in DD because a light signal is required for its expression (10, 17). Thus, it is possible that no light signal is required for the oscillation of the clock in these higher plants as well as in cyanobacteria. We can examine this possibility by monitoring the expression of a clock-controlled gene whose expression occurs even in DD. With *Synechocystis*, we used the *dnaK1* gene as an efficient clock reporter gene to monitor the oscillation of the clock in DD because the expression of the *dnaK1* gene occurs in DD.

There are several differences between the bioluminescence rhythms of the CFC2 strain cultured under LL (2) and the CFC4 strain maintained in DD. First, the free-running period of CFC4 cells maintained in DD (25 h) is longer than that of

CFC2 cells cultured under LL (22.6 ± 0.1 h; *n* = 4 [2a]) by about 2.4 h at 27°C. The free-running period of circadian rhythms in many organisms observed in DD is often different from that observed under LL (3). An extreme example of this phenomenon is seen in *Arabidopsis* seedlings, in which the period in DD is greater than 30 h, while the period under LL is about 25 h (15). Mutations of *Arabidopsis* seedlings that activate phototransduction pathways shorten the period length of circadian rhythms in *CAB2* gene expression, suggesting that activation of phototransduction pathways is supposed to affect the period of circadian rhythms (15).

Second, the rhythms of strain CFC4 maintained in DD persisted stably at least for a week, whereas those of strain CFC2 cultured under LL persisted only for 5 days with significant dampening (the peak/trough ratios of the bioluminescence rhythms observed in strain CFC2 were about 3 and 1.3 on the first day and the fifth day of LL, respectively [2]). At present, we do not know why the rhythms of strain CFC2 cultured under LL were dampened more rapidly. In the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, the bioluminescence rhythms persist very stably for more than 2 weeks under LL (14).

Third, the average level of bioluminescence from the CFC4 strain maintained in DD (Fig. 1) was about 10 times higher than that from strain CFC2 cultured under LL (2). This increase in bioluminescence observed in DD was mainly due to the increase in a nonoscillating component of bioluminescence. Previously, we demonstrated by Northern blotting analyses that the level of *luxAB* mRNA in CFC2 cells immediately after a dark-to-light transition was significantly higher than that in the cells at later times under LL (2). This suggests that the level of *luxAB* mRNA may be down-regulated by light. Furthermore, the bioluminescence reaction catalyzed by bacterial luciferase is also affected by changes in the levels of its substrates, *n*-decanal, reduced flavin mononucleotide ($FMNH_2$), and O_2. The levels of the latter two substrates may be different, because the metabolic states of cells should be different between CFC2 cells cultured under LL and CFC4 cells maintained in DD.

We have demonstrated that single light pulses induce a significant phase shift in the bioluminescence rhythm of strain CFC4 (Fig. 3). We entrained the *Synechocystis* circadian clock by LAHG light pulses to obtain a PRC (Fig. 3). Thus, it is uncertain which parts of DD after the last LAHG light pulse correspond to subjective day and night, respectively. We also tried to entrain the clock by LD cycles (light intensity of about 1.2 μmol m^{-2} s^{-1} from white fluorescent lamps) but failed to obtain a PRC, because CFC4 cells which had been entrained by the light-dark cycles rapidly lost bioluminescence after a 3-h illumination for the phase shift. There are two types of PRC: type 0 and type 1 (26). Type 1 displays relatively small phase shifts and has a continuous transition between delays and advances, whereas type 0 shows large phase shifts and a discontinuity at the transition between delays and advances. Our *Synechocystis* PRC seems to be type 1, but detailed analysis is necessary to determine whether the *Synechocystis* PRC is type 0 or type 1.

Our success in monitoring the circadian rhythms of *Synechocystis* cells cultured in DD is the first step toward elucidating the phototransduction pathway involved in the light-mediated phase-resetting mechanism for the circadian clock system in cyanobacteria. The dark-adapted *Synechocystis* strain CFC4 may be a useful experimental system, because molecular genetic techniques, such as efficient transformation and gene replacement, which enable us to disrupt or modify a specific gene and then overexpress it, have been established for *Syn-echocystis* (25). We can also easily isolate various mutants in *Synechocystis* by chemical mutagenesis and clone a specific gene that complements each mutant by gene transfer of a library for wild-type genomic DNA (6). The entire genome of *Synechocystis* has been sequenced (9), and it reveals that *Synechocystis* has various candidate molecules for photosignal transduction for the circadian clock system, such as photoreceptors, including a phytochrome, two-component systems (16), and adenylate cyclases, among others. Thus, we can directly determine whether these molecules are involved in the photosignal transduction for the clock system by modifying specific genes (through disruption and/or overexpression).

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