

Title: Characterization of circadian rhythms of various duckweeds

Running head: Circadian rhythms of duckweeds

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Abbreviations

CCA1, *CIRCADIAN CLOCK ASSOCIATED1*; *LHY*, *LATE ELONGATED HYPOCOTYL*; *PRR*, *PSEUDO RESPONSE REGULATOR*; *TOC1*, *TIMING OF CAB EXPRESSION1*; *GI*, *GIGANTIA*; *ELF3*, *EARLY FLOWERING 3*; *UBQ1*, *UBIQUITIN1*; *CaMV35S*, *cauliflower mosaic virus 35S*; FFT-NLLS, fast Fourier transform–nonlinear least squares; RAE, relative amplitude error; SEM, standard error of the mean

ABSTRACT

The plant circadian clock controls various physiological phenomena which are important for adaptation to natural day-night cycles. Many components of the circadian clock have been identified in *Arabidopsis thaliana* (Arabidopsis), the model plant for molecular genetic studies. Recent studies revealed evolutionary conservation of clock components in green plants. Homologues of clock related genes have been isolated from *Lemna gibba* and *Lemna aequinoctialis*, and it has been demonstrated that these homologues function in the clock system in a manner similar to their functioning in Arabidopsis. While clock components are widely conserved, circadian phenomena display diversity even within the *Lemna* genus. In order to survey the full extent of diversity in circadian rhythms among duckweed plants, we characterized the circadian rhythms of duckweed plants by employing a semi-transient bioluminescent reporter system. Using a particle bombardment method, circadian bioluminescent reporters were introduced into nine strains representing five duckweed species: *Spirodela polyrhiza*, *Landoltia punctata*, *Lemna gibba*, *Lemna aequinoctialis*, *Wolffia columbiana*. We then monitored luciferase (luc+) reporter activities driven by *AtCCA1*-, *ZmUBQ1*-, *CaMV35S* promoters under entrainment- and free-running conditions. Under entrainment conditions, *AtCCA1::luc+* showed similar diurnal rhythms in all strains. This suggests that the mechanism of biological timing under day-night cycles is conserved throughout the evolution of duckweed plants. Under free-running conditions, we observed circadian rhythms of *AtCCA1::luc+*, *ZmUBQ1::luc+* and *CaMV35S::luc+*. These circadian rhythms showed diversity in period length and sustainability, suggesting that circadian clock mechanisms are somewhat diversified among duckweed plants.

INTORODUCTION

The circadian clock, a self-sustaining oscillator with a period of approximately 24 h, is ubiquitous across life, including cyanobacteria, plants, insects, and mammals. It is synchronized to day–night cycles and controls various biological phenomena to adapt organisms to diurnal environmental changes (Dunlap *et al.* 2005). The plant circadian clock controls various physiological behaviors such as leaf movements, stomatal movements, metabolism, and photoperiodic flowering (Barak *et al.* 2000). The circadian clockwork is important for optimizing productivity, starch utilization, seed yield and seed viability (Green *et al.* 2002; Dodd *et al.* 2005; Graf *et al.* 2010). Understanding the mechanism of circadian regulation in plants is therefore important for improving plant performance in agriculture, as well as for applications such as bioremediation.

In plants, the circadian clock system consists of interconnected transcription–translation feedback loops (Pokhilko *et al.* 2013). Components of these feedback loops have been identified in *Arabidopsis*, the model plant for molecular genetic studies (Nakamichi 2011). Recent studies revealed evolutionary conservation of clock components within plants. In particular, homologues to myb domain transcription factors *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and the *PSEUDO RESPONSE REGULATOR* (*PRR*) family, including *TIMING OF CAB EXPRESSION1* (*TOC1*) were identified in species as diverse as green algae (*Ostreococcus tauri*), moss (*Physcomitrella patens*), and many higher plants (McClung 2013). Characterization of these homologues in many species provided additional evidence of conserved clock architecture (Song *et al.* 2010). Circadian clock systems of duckweed plants have been well analyzed for a long time. Physiological rhythms, such as CO₂ output and potassium uptake, were monitored in duckweed plants, and their circadian traits were characterized (Miyata and Yamamoto 1969; Hillman 1970; Kondo and Tsudzuki 1978). As clock-related genes, *LHY*, *PRRs*, *GI* and *ELF3* have been identified in *Lemna gibba* and *Lemna aequinoctialis* (*Lemna paucicostata*), and a semi-transient bioluminescent reporter system introduced by particle bombardment has been successfully applied to analyze *Lemna* circadian systems (Miwa *et al.* 2006). Functional assessment using a reporter-effector assay (knockdown and overexpression) of these homologues revealed functional conservation of clock components between *L. gibba* and *Arabidopsis* (Serikawa *et al.* 2008). While clock components are widely conserved in plants, circadian phenomena show diversity even within the *Lemna* genus (Kondo and Tsudzuki 1978; Miwa *et al.* 2006). Under constant light

conditions, *L. gibba* demonstrate clear circadian rhythms but *L. aequinoctialis* does not.

In this study we aimed to capture the full extent of diversity in circadian rhythms among duckweed plants. Duckweed plants are suitable for comparative analysis of circadian systems because, as described above, the clock systems of *Lemna* plants have been well characterized and duckweed circadian rhythms are easily monitored by employing a semi-transient bioluminescent reporter system (Miwa *et al.* 2006; Serikawa *et al.* 2008). When the semi-transient bioluminescent reporter system is applied for monitoring circadian rhythms of various plants, short handling time for gene transfection by particle bombardment and its versatility are remarkable advantages. Furthermore, tiny and flat duckweed plants grow well in a small dish for a long period. These advantages allow us to employ an automated bioluminescent monitoring system for high throughput characterization of circadian rhythms in duckweed plants. Many duckweed strains of various species are capable of being cultured as intact mature plants in the same, strictly controlled conditions in the laboratory (Landolt 1986). This feature is necessary for an accurate comparison of circadian clock systems since circadian rhythms in higher plants are affected by culture conditions (Chen *et al.* 2013; Kusakina *et al.* 2014). Thus, using a particle bombardment method for the circadian bioluminescent reporter system, duckweed plants provide a convenient platform for an analysis of natural variations in circadian clock systems.

We characterized circadian rhythms in nine strains representing five duckweed species and four genera; *Lemna gibba* G3 and p8L, *Lemna aequinoctialis* 6746 and Nd, *Landoltia punctata* NS and IS, *Spirodela polyrhiza* 7003 and biwa1, *Wolffia columbiana* 7972 (Fig. S1 and S2). The circadian rhythms of *L. gibba* G3 and *L. aequinoctialis* 6746 were previously analyzed (Miyata and Yamamoto 1969, Hillman 1970, Kondo and Tsudzuki 1978; Miwa *et al.* 2006; Serikawa *et al.* 2008; Muranaka *et al.* 2013). Bioluminescent reporter genes were introduced into the nine strains by a particle bombardment method and reporter activities were monitored under entrainment and free-running conditions. Under entrainment conditions, reporter activities showed similar diurnal rhythms in all strains. Under free-running conditions, circadian rhythms showed diversity in period length and sustainability, suggesting that circadian clock systems are somewhat diversified among duckweed plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Lemna gibba G3 strain and *Lemna aequinoctialis* 6746 strain (previously called *Lemna paucicostata* 6746 or *Lemna perpusilla* 6746) were used as previously described (Miwa *et al.* 2006). *L. gibba* p8L strain is a pure line (eight generations of selfing) produced from the G3 strain. *Spirodela polyrhiza* 7003 and *Wolffia columbiana* 7972 strains originated from the Landolt collections and were the kind gifts of Dr. Todd Michael. *L. aequinoctialis* Nd (original strain), *Landoltia punctata* NS and IS and *S. polyrhiza* biwa1 strains were isolated in our laboratory. The *S. polyrhiza* biwa1 strain was collected at Shiga, Japan and the others were collected at Kyoto, Japan. *L. aequinoctialis* Nd strain is a pure line (six generations of selfing) of the original strain. *L. gibba* plants were kept in E medium with 1% sucrose; other plants were kept in 0.5 × H medium with 1% sucrose. Plants were maintained in the same growth medium as that used in the bioluminescence monitoring experiments for at least three weeks before starting the monitoring. E medium was made by adding 30 μM ethylene-di-amine-tetra-acetic acid (EDTA) to M medium (Cleland and Briggs 1968). The compositions of M and H medium were described by Hillman (1961). The composition of NF medium is: 2.7 mM CaCl₂, 1.2 mM MgSO₄, 1 mM KH₂PO₄, 5 mM KNO₃, 18 μM MnCl₂, 46 μM H₃BO₃, 0.77 μM ZnSO₄, 0.32 μM CuSO₄, 0.49 μM MoO₃, 20 μM FeSO₄, 50 μM Na₂EDTA. The pH was adjusted to 5.0 with KOH. All strains were cultured under constant light conditions; light intensity supplied by fluorescent lamps (FLR40SEX-W/M/36-HG; NEC, <http://www.nec.com/>) was approximately 50 μE m⁻² s⁻¹. Growth temperature was maintained at 25°C ± 1°C. Colonies were grown in 75 ml of growth medium in 200 ml Erlenmeyer flasks plugged with cotton. New stock cultures were made every week, and well-grown plants were used for experiments.

Species identification

The species of the strains isolated in our laboratory were identified by morphological characteristics (Les *et al.* 1997). To confirm species identification, the *atpF-atpH* marker was sequenced (Wang *et al.* 2010). Total DNA of each strain was extracted using Extract-N-Amp Plant PCR Kits (SIGMA-ALDRICH, <http://www.sigmaaldrich.com/>). The *atpF-atpH* sequences were amplified with primers as described in Wang *et al.* (2010). PCR products were purified with GenElute PCR Clean-Up Kit (SIGMA-ALDRICH, <http://www.sigmaaldrich.com/>) and then sequenced with an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, <http://www.appliedbiosystems.com/>). The sequence of each strain was compared with the reference

sequences reported in Wang *et al.* (2010) and a phylogenetic tree was generated using ClustalW (<http://clustalw.ddbj.nig.ac.jp/>) (Fig. S1).

Luciferase reporter constructs

pUC-AtCCA1::luc+ (*AtCCA1::luc+*) was used as the circadian bioluminescent reporter (Muranaka *et al.* 2013). *pSPI-ZmUBQ1::luc+* (*ZmUBQ1::luc+*) and *pSPI-AtPRR1::luc+* (*AtPRR1::luc+*) were described previously (Miwa *et al.* 2006; Serikawa *et al.* 2008). *pUC18-CaMV35S::intron-luc+* (*CaMV35S::luc+*) is a modified version of *pUC18-CaMV35S::luc+*, which has been described previously (Muranaka *et al.* 2013), with an intron sequence inserted at 148 bp from the start codon of the luciferase gene. The inserted intron sequence was described in Ohta *et al.* (1990).

Particle bombardment experiment

Reporter constructs were introduced into plants by particle bombardment. 8 µl of prewashed gold particle suspension (1 µm diameter; Bio-Rad, <http://www.bio-rad.com/>) in 50% glycerol (60 mg ml⁻¹) was mixed with 2 µl of plasmid DNA (1 µg µl⁻¹), 3.3 µl of spermidine (0.1 M), and 8 µl of CaCl₂ (2.5 M). After incubating for 5 minutes at room temperature, the suspension was centrifuged for 10 min at 12,000 rpm at room temperature and the supernatant was discarded. DNA-coated particles were washed once with 80 µl of 80% ethanol and once with 100% ethanol and resuspended in 15 µl of 100% ethanol. The suspension of DNA-coated particles were then spread on a macrocarrier and dried. A helium gun device (PDS-1000/He; Bio-Rad) was used according to the manufacturer's instructions [vacuum, 28 mmHg; helium pressure, 450 psi (rupture disc)]. Duckweed plants were laid on a 60-mm polystyrene dish (IWAKI, <http://www.iwaki-kk.co.jp/>). The dish was set underneath the muzzle of the gun, and DNA-coated particles on the macrocarrier were fired into the plants. After bombardment, duckweed plants were divided into four 35-mm dishes with 3 ml of growth medium containing firefly luciferin (0.5 mM potassium salt; Wako, <http://www.wako-chem.co.jp/>).

Bioluminescence monitoring

Bioluminescence monitoring was performed as described previously (Miwa *et al.* 2006; Serikawa *et al.* 2008) with minor revisions. The luminescence dish-monitoring system used photomultiplier tubes (H7360-01; Hamamatsu Photonics K.K.) for bioluminescence detection. To reduce background

chlorophyll fluorescence, a short-pass filter (SVO630; Asahi Spectra) was set at the detection site of the photomultiplier tubes. Each dish was subjected to 30 s measurement every 20 min. The monitoring system was placed in an incubator (KCLP-1000I-CT; NK system, <http://www.nihonika.co.jp/>) with fluorescent lamps (FL20SSW/18; Mitsubishi Electric co., <http://www.mitsubishielectric.com/>). Temperature was maintained at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and light intensity was approximately $30 \mu\text{E m}^{-2} \text{s}^{-1}$.

FFT-NLLS analysis

In order to correctly estimate period lengths and relative amplitude errors, the recorded bioluminescence under constant conditions (48-168 h) were detrended by applying moving average filter (24-h window size) and then analyzed with the FFT-NLLS suite of programs available within the Biological Rhythms Analysis software system (BRASS, available from <http://www.amillar.org>).

RESULTS

A semi-transient bioluminescent reporter system for monitoring circadian rhythms in duckweed plants.

A bioluminescent reporter system using firefly luciferase has been successfully used to characterize circadian rhythms in plant, animal and cyanobacterial cells (Welsh *et al.* 2005; Ishiura *et al.* 1998). We employed a semi-transient bioluminescent reporter system to analyze the circadian rhythms of duckweed plants (Miwa *et al.* 2006; Serikawa *et al.* 2008). Reporter constructs were introduced into plants by particle bombardment. Transfected plants were placed in an automated bioluminescence monitoring system in which bioluminescence was measured by a sensor equipped with a photomultiplier tube every 20 minutes. During monitoring, plants were first entrained to two 12 h dark / 12 h light cycles, after which they were released into constant light or constant dark conditions. Circadian period lengths under constant conditions were estimated by fast Fourier transform–nonlinear least squares (FFT-NLLS) analysis. In FFT-NLLS analysis, the degree of confidence is described by the relative amplitude error (RAE), which ranges from 0 (complete sine wave) to 1 (arrhythmic) (Plautz *et al.* 1997).

Luciferase driven by the Arabidopsis *CCA1* promoter (*AtCCA1::luc+*) was used to measure circadian rhythms in *Lemna* plants (Miwa *et al.* 2006; Serikawa *et al.*, 2008; Muranaka *et al.*

2013). We introduced *AtCCA1::luc+* into *L. gibba* G3 and *S. polyrhiza* 7003 strains. In *L. gibba* plants, *AtCCA1::luc+* reporter activities showed diurnal rhythms under 12 h dark / 12 h light cycles and circadian rhythms under constant light conditions in both E and 0.5 x H growth media (Fig. 1A and B). However, bioluminescence traits were different in E medium and 0.5 x H medium. In E medium, the estimated period length under constant light conditions was 22.62 ± 0.07 (mean \pm SEM, n=4) and the bioluminescence level gradually decreased (Fig 1A). In 0.5 x H medium, the estimated period length was 24.26 ± 1.01 (n=4) and the bioluminescence level was maintained during monitoring (Fig 1B). Bioluminescence of *AtCCA1::luc+* in *S. polyrhiza* plants was low when cultured in 0.5 x H medium (Fig. 1C). These data suggest that bioluminescence rhythms in duckweed plants are influenced by growth medium conditions. We modified the composition of the growth medium to create NF medium, in which the bioluminescence level of *AtCCA1::luc+* in every tested duckweed plant was high enough to characterize temporal changes clearly. Every tested duckweed plant was well-grown in NF medium (Fig. S2).

Diurnal rhythms of five duckweed species across four genera

In addition to the circadian reporter, *AtCCA1::luc+*, luciferase driven by the maize *UBIQUITIN1* promoter (*ZmUBQ1::luc+*) was used as a constitutive reporter in *Lemna* plants (Miwa *et al.* 2006; Serikawa *et al.*, 2008). We introduced *AtCCA1::luc+* or *ZmUBQ1::luc+* into nine strains representing five duckweed species across four genera; *L. gibba* G3 and p8L, *L. aequinoctialis* 6746 and Nd, *L. punctata* NS and IS, *S. polyrhiza* 7003 and biwa1, *W. columbiana* 7972 (Fig S1). *AtCCA1::luc+* showed a clear diurnal rhythm in each strain under the 12 h dark / 12 h light cycle (Fig. 2A-I). Reporter activity peaked in the morning phase, as observed in *Arabidopsis* and in *Lemna* plants cultured in different medium (Nakamichi *et al.* 2004; Miwa *et al.* 2006). Peak times were different between *Lemna* species (0-2 h after dawn; Fig. 2A-D) and other genera (3-4 h after dawn; Fig2E-I) (Table 1). *ZmUBQ1::luc+* reporter activity did not show a clear diurnal rhythm in any duckweed plant (Fig. 2J-R). Peak bioluminescence intensities of either reporter were similar between strains of the same species, while they were different between strains of different species (Table 1). Note that bioluminescence in *L. aequinoctialis* Nd showed an acute increase at the dark-light transition (Fig. 2D and M). *L. aequinoctialis* Nd and *W. columbiana* 7972 strains showed high bioluminescence levels for both reporters.

Circadian rhythms of five duckweed species across four genera under constant light conditions

Reporter activities of *AtCCA1::luc+* and *ZmUBQ1::luc+* were monitored in the nine strains under constant light conditions. *AtCCA1::luc+* showed circadian rhythmicity with similar period lengths in *L. gibba* G3 and p8L (Fig. 3A and B, Table 2). *ZmUBQ1::luc+* activity showed damped circadian rhythms with four peaks (Fig. 3J and K). These peak times corresponded to the phase of subjective dawn. The estimated period lengths of *ZmUBQ1::luc+* rhythms were ~1 h longer than those of *AtCCA1::luc+* (Table 2). The circadian rhythmicity of *ZmUBQ1::luc+* was unexpected, so we tested *CaMV35S::luc+*, wherein luciferase expression is driven by the commonly used constitutive cauliflower mosaic virus 35S promoter (Leeuwen *et al.* 2000). Under constant light conditions, *CaMV35S::luc+* showed a damped circadian rhythm with three peaks, at which *ZmUBQ1::luc+* reporter activities peaked in *L. gibba* p8L (Fig. S3). The estimated period length was 25.59 ± 0.08 h (n=4), similar to that of the *ZmUBQ1::luc+* (25.63 h) in the same strain (Table 2). Thus, these reporters which typically show constitutive expression seemed to function as novel circadian reporters in our experimental conditions. It is noteworthy that *AtPRR1::luc+* reporter activities in *L. gibba* under the same conditions showed a circadian rhythm in antiphase to those of *AtCCA1::luc+*, *ZmUBQ1::luc+*, and *CaMV35S::luc+* (Fig. S4; Serikawa *et al.* 2008). This suggests that the circadian rhythmicity of them was unlikely to result from a possible genome-wide transcriptional regulation with circadian rhythmicity.

Characteristics of *AtCCA1::luc+* and *ZmUBQ1::luc+* bioluminescence rhythms were different between *L. aequinoctialis* 6746 and *L. aequinoctialis* Nd strains. In the 6746 strain, *AtCCA1::luc+* reporter activity showed a severely damped circadian rhythm with only one peak, while in the Nd strain, reporter activity showed a gradually damped rhythm with five peaks. (Fig. 3C and D). *ZmUBQ1::luc+* showed a gradual decrease without clear circadian rhythmicity in the 6746 strain (RAE = 0.27) and a low-amplitude circadian rhythm with four peaks in the Nd strain (RAE = 0.20) (Fig. 3L and M, Table 2). The estimated period lengths of the *AtCCA1::luc+* rhythm (21.55 h) and the *ZmUBQ1::luc+* rhythm (21.76 h) in the Nd strain were similar (Table 2). In both strains, bioluminescence intensity from *AtCCA1::luc+* transfected cells was relatively low under constant light conditions when compared to the intensity under the 12 h dark / 12 h light cycle.

AtCCA1::luc+ showed circadian rhythms in *Landoltia punctata* NS and IS strains (Fig. 3E and F). The estimated period length of the NS strain (22.39 h) was ~0.5 h shorter than that of the

IS strain (22.91 h) (Table 2). *ZmUBQ1::luc+* reporter activity also showed a damped circadian rhythm with five peaks in *L. punctata* NS and IS strains (Fig. 3N and O). Its period length was similar to that of *AtCCA1::luc+* in both strains (Table 2).

AtCCA1::luc+ showed damped circadian rhythms with three peaks in *S. polyrhiza* 7003 and biwa1 strains (Fig. 3G and H). The estimated period length of the 7003 strain (25.84 h) was ~1.5 h longer than that of the biwa1 strain (24.42 h) (Table 2). *ZmUBQ1::luc+* showed damped circadian rhythms with three peaks in *S. polyrhiza* 7003 and biwa1 strains (Fig. 3P and Q) with a period length ~0.5 h longer than that of the *AtCCA1::luc+* rhythm in the 7003 strain, while period lengths of both bioluminescent reporter rhythms were similar in the biwa1 strain (Table 2).

AtCCA1::luc+ showed damped circadian rhythm with four peaks in the *W. columbiana* 7972 strain (Fig. 3I). The first peak interval (~30 h) was larger than the second and third intervals (~20 h), indicating that the period length of the circadian rhythm was unstable. *ZmUBQ1::luc+* did not show a clear circadian rhythm in this strain (Fig. 3R).

Circadian rhythms of five duckweed species across four genera under constant dark conditions

AtCCA1::luc+ and *ZmUBQ1::luc+* activities were monitored in the nine strains under constant dark conditions. *AtCCA1::luc+* showed a damped circadian rhythm in each strain (Fig. 4A-I). The estimated period length was longer than that under constant light conditions in each strain except *W. columbiana* 7972, in which the estimated period length was shorter (Tables 2 and 3). However, as mentioned above, the *AtCCA1::luc+* period length in *W. columbiana* 7972 strain was unstable under constant light conditions, and peak intervals of the second and third cycles (~20 h and ~22 h, respectively) were shorter than the estimated period length under constant dark conditions (~24 h). Thus the circadian period lengths of duckweed plants were likely to be longer under constant dark conditions than under constant light conditions, as observed in *Arabidopsis* (Dalchau *et al.* 2011). *ZmUBQ1::luc+* activity gradually decreased without clear rhythmicity (RAE > 0.25) in each strain (Fig. 4J-R, Table 3). Since *ZmUBQ1::luc+* showed circadian rhythms under constant light conditions, the bioluminescence rhythm of *ZmUBQ1::luc+* appears to be light dependent.

DISCUSSION

In this study we investigated the diversity of circadian systems in duckweed plants by monitoring

circadian rhythms in nine duckweed strains representing five species (*Lemna gibba*, *Lemna aequinoctialis*, *Landoltia punctata*, *Spirodela polyrhiza*, *Wolffia columbiana*) with a semi-transient bioluminescent reporter system. Under constant conditions, circadian *AtCCA1::luc+* rhythms showed diversity in period lengths and sustainability. Period lengths were varied under both constant light (21.55-25.84 h) and constant dark conditions (23.59-29.71 h) (Tables 2 and 3). The number of observable peaks ranged from one to five (Fig. 3 and 4). This suggests that the circadian clock mechanisms are somewhat diversified among duckweed plants. The range of period lengths between strains of the same species (< 2 h) was smaller than that between strains of different species and was consistent with natural variation in *Arabidopsis* (Tables 2 and 3) (Micheal *et al.*, 2003). In contrast to period length, sustainability showed diversity even between strains of the same species. The number of observable peaks of *AtCCA1::luc+* activity in *L. aequinoctialis* was different between the 6746 strain (one peak) and the Nd strain (five peaks) under constant light conditions (Fig. 3C and D). It should be noted that both strains showed three peaks under constant dark conditions (Fig. 4 C and D). The light dependency of these differences in sustainability implies that this phenotypic diversity might be caused by differences in light signaling mechanisms between the two strains. In *L. aequinoctialis* (*L. paucicostata*) species, a short-day photoperiodic flowering species, the critical dark periods showed a large range of natural variation (0-13.5 h) (Yukawa and Takimoto 1976). Thus, *L. aequinoctialis* may show a wide range of natural variation in a time dependent manner.

Under entrainment conditions with 12 h dark / 12 h light cycles, *AtCCA1::luc+* showed diurnal rhythms with peaks in the morning in all nine strains. This suggests that the mechanisms for scheduling biological timing in the presence of the day-night cycle are conserved in duckweed plants. However, duckweeds in the *Lemna* genus showed peak times ~2 h earlier than those of the other three genera. Among these four genera, only *Lemna* species lack guard cell plastids, and it was shown that stomata of *L. gibba* plants are permanently open and do not respond to light-dark transitions (Hes *et al.* 1997; Park *et al.* 1990). One possible explanation of these peak time differences is that the lack of diurnal stomatal movements affects daily metabolic changes and the phase of morning gene expression. It should be noted that at least one peak was observed in constant conditions after the light-dark (dark-light) transition in each strain (Fig. 3A-I and 4A-I). This means that the circadian system in each strain was capable of keeping time information for at least one day after entrainment cues were removed. One of the major functions of the circadian clock system is anticipation of dawn after reset at the previous dawn (Dodd *et al.* 2014). Our study

indicates that this function was conserved in the evolution of duckweed plants.

Under constant light conditions, *ZmUBQ1::luc+* and *CaMV35S::luc+* showed circadian rhythms with the peak time in the morning in many duckweed species (Fig. 3J-R). In Arabidopsis, the promoter activity of a *UBQUITIN* gene is remarkably stable and often used as a reference gene for transcript normalization (Czechowski *et al.* 2005). The *CaMV35S* promoter is also well-known as a constitutive promoter, and the bioluminescence of *CaMV35S::luc+* did not show a clear circadian rhythm under constant conditions in Arabidopsis (Millar *et al.* 1992). Hence, the circadian rhythms of these reporters observed in duckweed plants might be generated at the level of post-transcriptional regulations (Kojima *et al.* 2011; Pal *et al.* 2013). In addition to luciferase enzyme amounts, intracellular concentrations of its substrates affect bioluminescence intensity *in vivo*. Firefly luciferase catalyzes the ATP-dependent oxidation of luciferin in a reaction that produces light (Leeuwen *et al.* 2000). In many plant species, circadian regulation of photosynthesis has been analyzed (Dodd *et al.* 2013). In *Landoltia punctata* (*Spirodela oligorrhiza*), it was reported that phosphorylation of the D1 photosystem II reaction center protein is controlled by an endogenous circadian rhythm (Booij-James *et al.* 2002). Thus, luciferase reporter activities may be influenced by changes in intracellular concentrations of ATP and oxygen, which are dependent on photosynthetic activities and may cause circadian rhythmicity of these reporters. Even though these possible rhythms would widely influence the luciferase reporter activities, bioluminescence rhythms of the *AtCCA1::luc+* reporter with similar peak time were likely to reflect its transcription behavior. It was reported that overexpression/RNAi of clock-related genes in the reporter-introduced cells of *L. gibba* changed bioluminescence traces of *AtCCA1::luc+* but it did not affect those of *ZmUBQ1::luc+* (Serikawa *et al.* 2008). Moreover, the possible rhythmic influence on the luciferase reporter would be not as large as to overcome the circadian rhythms of *AtPRR1::luc+* with peak time in the evening (Fig. S4). The physiological and molecular basis for the circadian rhythms of *ZmUBQ1::luc+* and *CaMV35S::luc+*, and the extent of this kind of phenomena across plants should be an interesting issue to understand the circadian system in plants.

In previous studies of natural variation in plant circadian systems, circadian outputs such as leaf movements and delayed fluorescence were used as markers for circadian rhythms (Micheal *et al.* 2003; Gould *et al.* 2009). A recent study using circadian luciferase reporters in various Arabidopsis accessions revealed difference in the temperature-buffering capacity between the leaf movement rhythm and the clock gene expression (Kusakina *et al.* 2014). In this study we monitored

the rhythm of clock gene expression, which directly reflects features of the circadian clock, by employing a semi-transient bioluminescent reporter system using particle bombardment. In the particle bombardment method, reporter constructs are introduced into only mature epidermal (~20%) or mesophyll (~80%) cells (Muranaka *et al.* 2013). This allowed us to characterize the circadian oscillator in plant cells without complexity due to cell proliferation and developmental processes (Fukuda *et al.* 2007; Yakir *et al.* 2009; Fukuda *et al.* 2012). The flat, smooth surface of the duckweed frond is suitable for the particle bombardment method and the tiny body allowed us to keep the whole plant in small dishes under normal growth conditions throughout the experiments. These characteristics of duckweed plants and the reporter transfection technique give advantages to compare circadian rhythms of many plants in various species and strains. Thus, duckweed plants are a good model for research on the divergence and evolution of circadian clock systems in higher plants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree (Neighbor-Joining method) based on *atpF-atpH* sequences.

Figure S2. Nine strains of five duckweed species in four genera cultured in NF medium.

Figure S3. Bioluminescence of *CaMV35S::luc+* in *L. gibba* p8L under constant light conditions.

Figure S4. Bioluminescence of *AtPRR1::luc+* in *L. gibba* p8L under constant light conditions.

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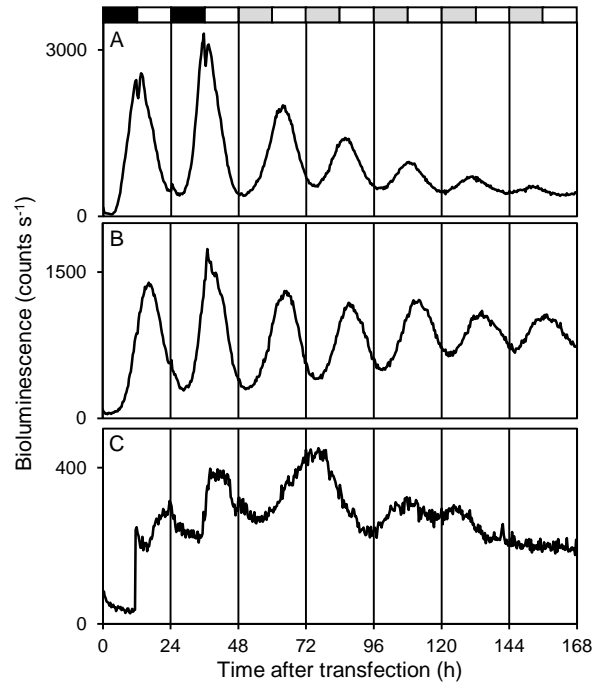


Fig. 1 Temporal bioluminescence changes of transfected duckweed plants. The circadian reporter *AtCCA1::luc+* was introduced into *L. gibba* G3 cultured in E medium (A), *L. gibba* G3 cultured in $0.5 \times$ H medium (B), *S. polyrhiza* 7003 cultured in $0.5 \times$ H medium (C). Plants cultured under constant light conditions were subjected to gene transfection by particle bombardment and then entrained to two 12 h dark / 12 h light cycles and released into constant light conditions. Black, open, and gray bars indicate dark, light, and subjective night, respectively. Data are representative of $n=4$ replicate dishes.

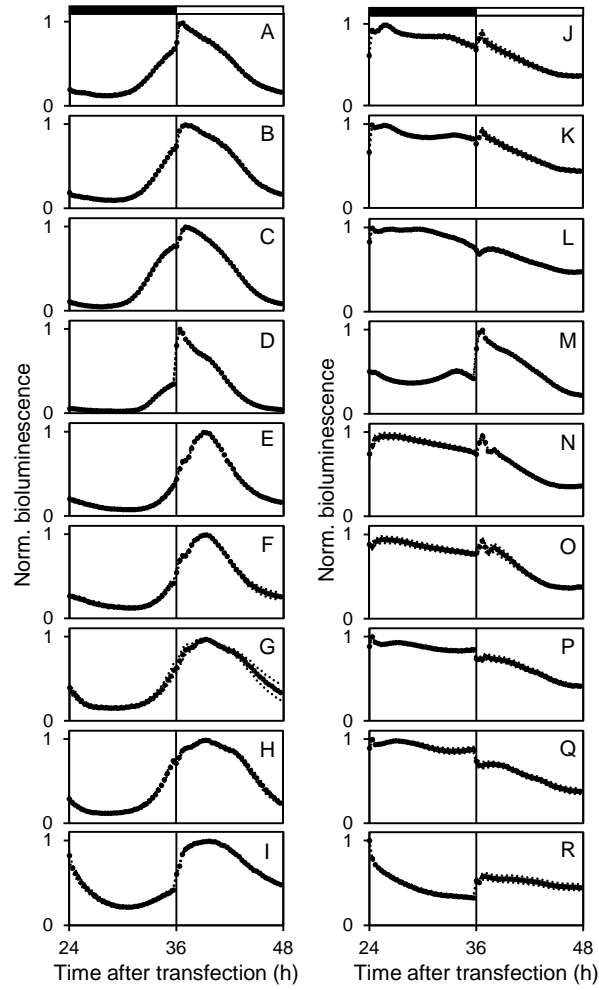


Fig. 2 Bioluminescence of *AtCCA1::luc+* and *ZmUBQ1::luc+* under the 12 h dark / 12 h light cycle. *AtCCA1::luc+* was introduced into *L. gibba* G3 (A) and p8L (B), *L. aequinoctialis* 6746 (C) and Nd (D), *L. punctata* NS (E) and IS (F), *S. polyrhiza* 7003 (G) and biwa1 (H), *W. columbiana* 7972 (I). *ZmUBQ1::luc+* was introduced into *L. gibba* G3 (J) and p8L (K), *L. aequinoctialis* 6746 (L) and Nd (M), *L. punctata* NS (N) and IS (O), *S. polyrhiza* 7003 (P) and biwa1 (Q), *W. columbiana* 7972 (R). Plants cultured in NF medium under constant light conditions were subjected to gene transfection and then transferred to 12 h dark / 12 h light conditions. Bioluminescence intensities 24-48 h after gene transfection were plotted. Bioluminescence intensities were normalized by dividing by their 24-48 h maximum. Mean values (black circle) \pm SEM (dotted line) of $n = 8$ replicate dishes were plotted. Black and open bars indicate dark and light, respectively.

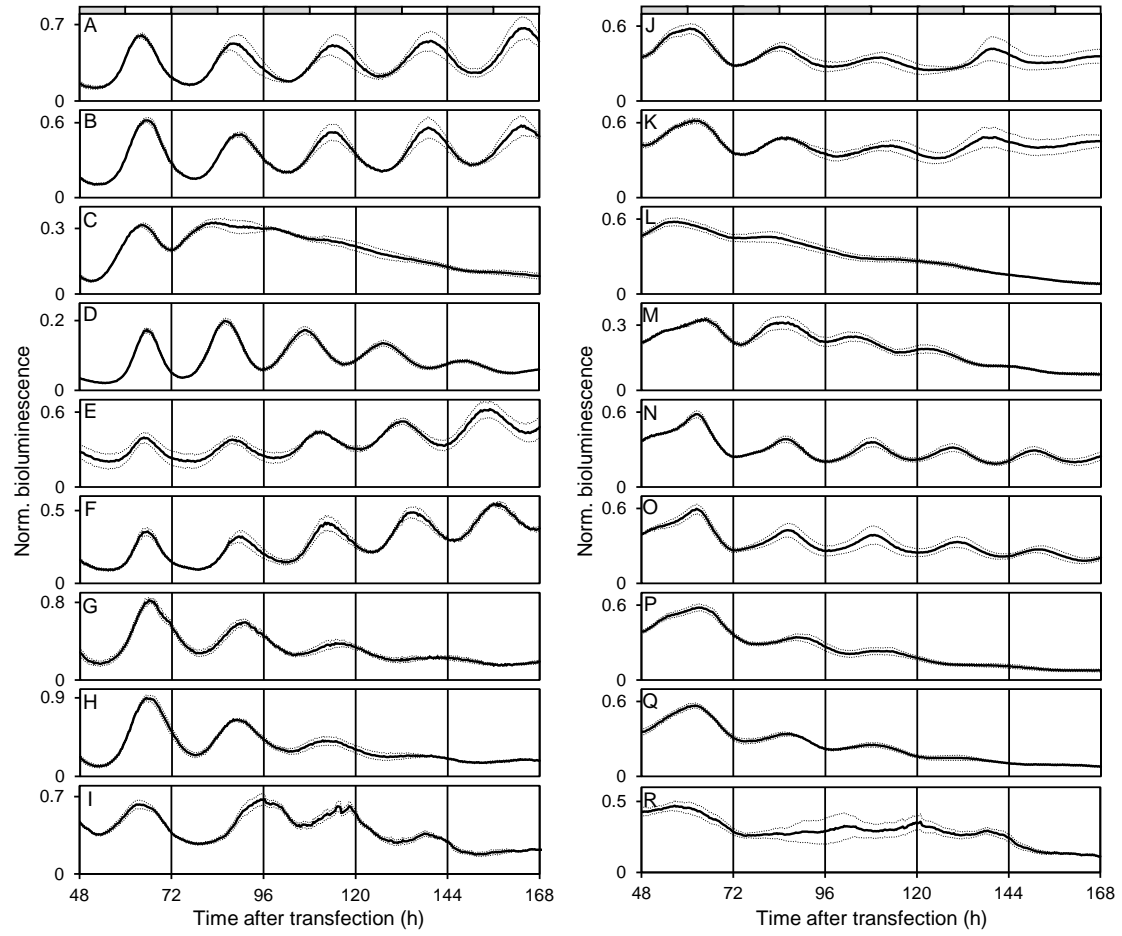


Fig. 3 Bioluminescence of *AtCCA1::luc+* and *ZmUBQ1::luc+* under constant light conditions. *AtCCA1::luc+* was introduced into *L. gibba* G3 (A) and p8L (B), *L. aequinoctialis* 6746 (C) and Nd (D), *L. punctata* NS (E) and IS (F), *S. polyrhiza* 7003 (G) and biwa1 (H), *W. columbiana* 7972 (I). *ZmUBQ1::luc+* was introduced into *L. gibba* G3 (J) and p8L (K), *L. aequinoctialis* 6746 (L) and Nd (M), *L. punctata* NS (N) and IS (O), *S. polyrhiza* 7003 (P) and biwa1 (Q), *W. columbiana* 7972 (R). Plants cultured in NF medium under constant light conditions were subjected to gene transfection and then entrained to two 12 h dark / 12 h light cycles and released into constant light conditions. Bioluminescence intensities 48-168 h after gene transfection were plotted. Bioluminescence intensities were normalized by dividing by their 24-48 h maximum. Mean values (solid line) \pm SEM (dotted line) of $n = 4$ replicate dishes were plotted. Open and gray bars indicate light and subjective night, respectively.

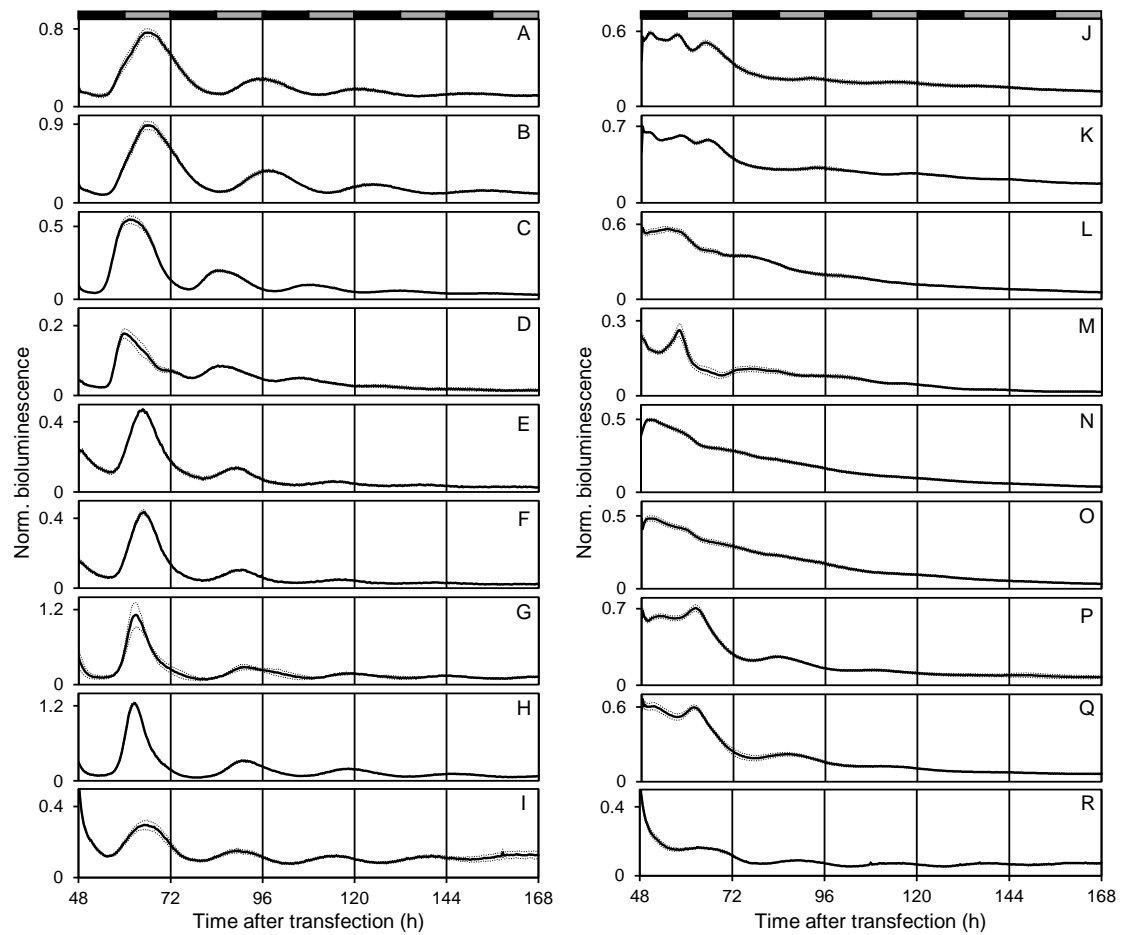
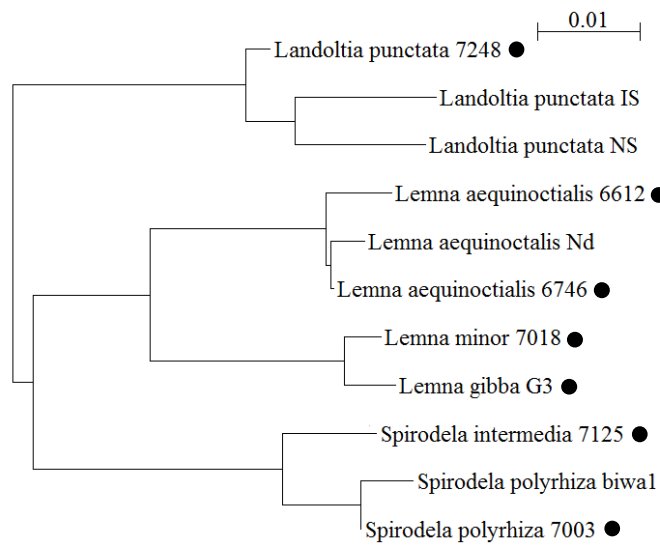
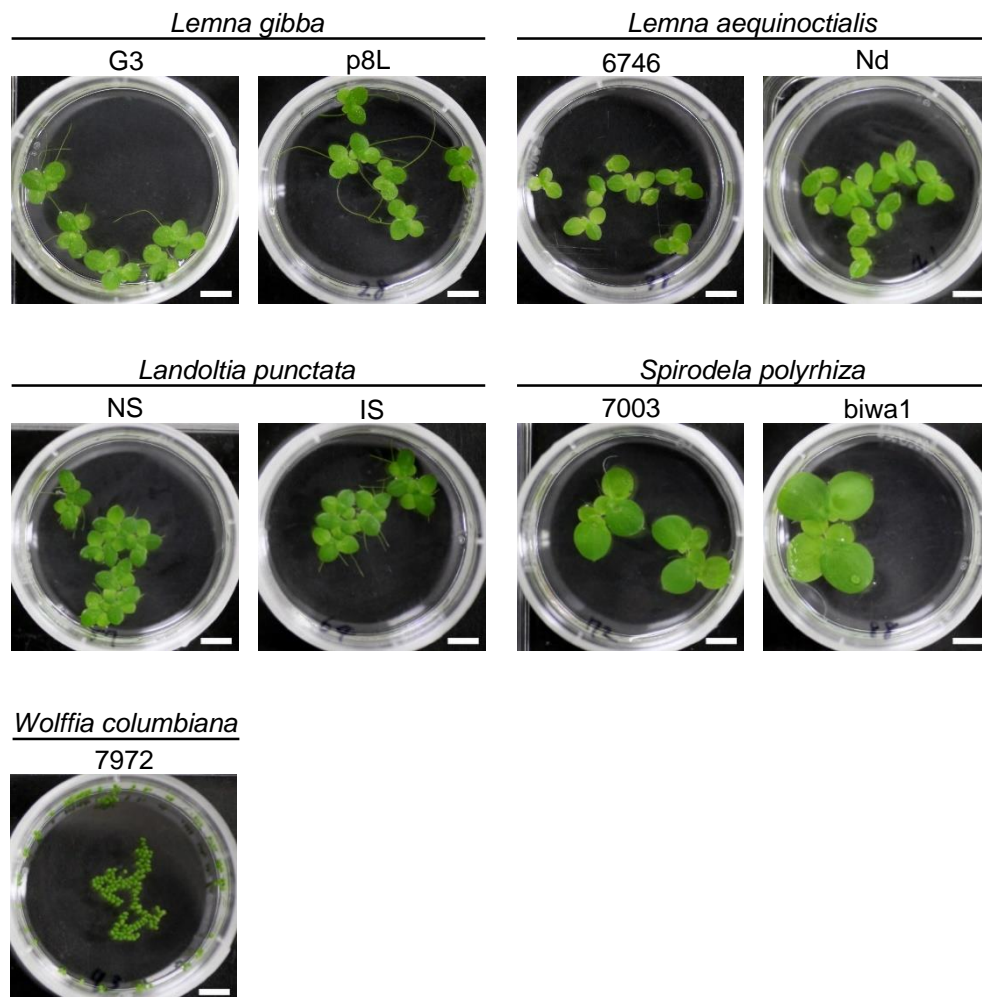


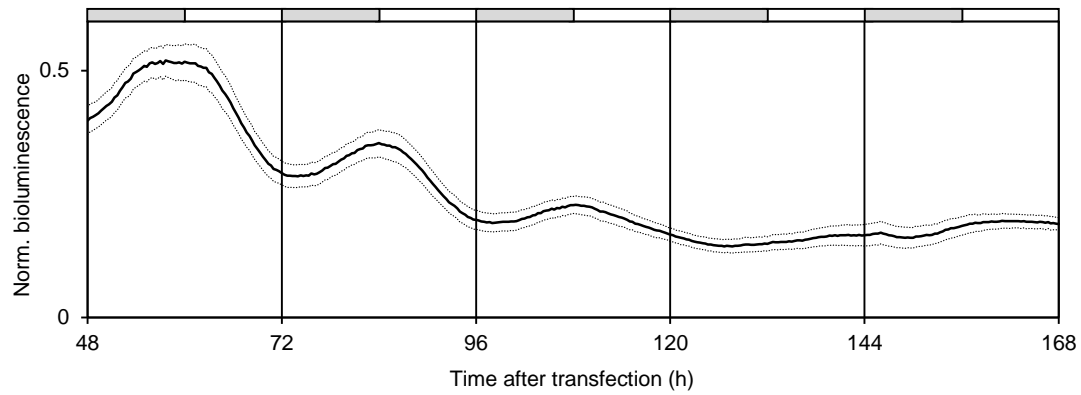
Fig. 4 Bioluminescence of *AtCCA1::luc+* and *ZmUBQ1::luc+* under constant dark conditions. *AtCCA1::luc+* was introduced into *L. gibba* G3 (A) and p8L (B), *L. aequinoctialis* 6746 (C) and Nd (D), *L. punctata* NS (E) and IS (F), *S. polyrhiza* 7003 (G) and biwa1 (H), *W. columbiana* 7972 (I). *ZmUBQ1::luc+* was introduced into *L. gibba* G3 (J) and p8L (K), *L. aequinoctialis* 6746 (L) and Nd (M), *L. punctata* NS (N) and IS (O), *S. polyrhiza* 7003 (P) and biwa1 (Q), *W. columbiana* 7972 (R). Plants cultured in NF medium under constant light conditions were subjected to gene transfection and then entrained to two 12 h dark / 12 h light cycles and released into constant dark conditions. Bioluminescence intensities of 48-168 h after gene transfection were plotted. Bioluminescence intensities were normalized by dividing by their 24-48 h maximum. Mean values (solid line) \pm SEM (dotted line) of $n = 4$ replicate dishes were plotted. Black and gray bars indicate dark and subjective day, respectively.



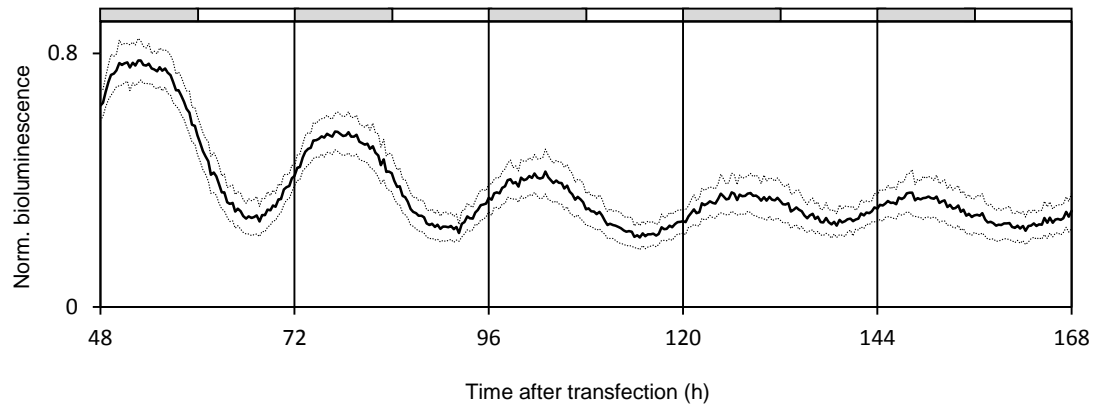
Supplementary Fig. S1 Phylogenetic tree (Neighbor-Joining method) based on *atpF-atpH* sequences. Black dots indicate reference strains reported in Wang *et al.* (2010).



Supplementary Fig. S2 Nine strains representing five duckweed species across four genera cultured in NF medium. Pictures were taken just after gene transfection. Plants were placed in 35-mm dishes for bioluminescence monitoring. Scale bars indicate 5 mm.



Supplementary Fig. S3 Bioluminescence of *CaMV35S::luc+* in *L. gibba* p8L under constant light conditions. Plants cultured in NF medium under constant light conditions were subjected to gene transfection and then entrained to two 12 h dark / 12 h light cycles and released into constant light conditions. Bioluminescence intensities of 48-168 h after gene transfection were plotted. Bioluminescence intensities were normalized by dividing by their 24-48 h maximum. Mean values (solid line) \pm SEM (dotted line) of $n = 4$ replicate dishes were plotted. Open, gray bars indicate light, and subjective night, respectively.



Supplementary Fig. S4 Bioluminescence of *AtPRR1::luc+* in *L. gibba* p8L under constant light conditions. Plants cultured in NF medium under constant light conditions were subjected to gene transfection and then entrained to two 12 h dark / 12 h light cycles and released into constant light conditions. Bioluminescence intensities of 48-168 h after gene transfection were plotted. Bioluminescence intensities were normalized by dividing by their 24-48 h maximum. Mean values (solid line) \pm SEM (dotted line) of $n = 4$ replicate dishes were plotted. Open, gray bars indicate light, and subjective night, respectively.