Conserved Expression Profiles of Circadian Clock-related Genes in Two *Lemna* Species Showing Long-day and Short-day Photoperiodic Flowering Responses

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The *Lemna* genus is a group of monocotyledonous plants with tiny, floating bodies. *Lemna gibba* G3 and *L. paucicostata* 6746 were once intensively analyzed for physiological timing systems of photoperiodic flowering and circadian rhythms since they showed obligatory and sensitive photoperiodic responses of a long-day and a short-day plant, respectively. We attempted to approach the divergence of biological timing systems at the molecular level using these plants. We first employed molecular techniques to study their circadian clock systems. We developed a convenient bioluminescent reporter system to monitor the circadian rhythms of *Lemna* plants. As in Arabidopsis, the Arabidopsis *CCA1* promoter produced circadian expression in *Lemna* plants, though the phases and the sustainability of bioluminescence rhythms were somewhat diverged between them. *Lemna* homologs of the Arabidopsis clock-related genes *LHY/CCA1*, *GI*, *ELF3* and *PRRs* were then isolated as candidates for clock-related genes in these plants. These genes showed rhythmic expression profiles that were basically similar to those of Arabidopsis under light–dark conditions. Results from co-transfection assays using the bioluminescence reporter and overexpression effectors suggested that the *LHY* and *GI* homologs of *Lemna* can function in the circadian clock system like the counterparts of Arabidopsis. All these results suggested that the frame of the circadian clock appeared to be conserved not only between the two *Lemna* plants but also between monocotyledons and dicotyledons. However, divergence of gene numbers and expression profiles for *LHY/CCA1* homologs were found between *Lemna*, rice and Arabidopsis, suggesting that some modification of clock-related components occurred through their evolution.

**Keywords**: Bioluminescent reporters — Circadian rhythm — Clock-related genes — *Lemna* — *LHY/CCA1* — Photoperiodic flowering.

Abbreviations: CT, circadian time; LD, long-day; PRR, pseudo-response regulator; RT–PCR, reverse transcription–PCR; SD, short-day; ZT, Zeitgeber time.

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Introduction

Various biological phenomena are temporally coupled to environmental changes with various time scales. Environmental periodicities such as day–night cycles and seasonal illumination/temperature variations are especially important factors to which many organisms regularly adapt themselves. Endogenous circadian clocks are important devices for the timing system in the daily cycle. Some of the developmental processes are strongly affected by environmental periodicities. Photoperiodic flowering is one example. The changing photoperiods are used as an environmental cue for seasonal information, allowing plants to flower at appropriate times of the year (Bünning 1967, Thomas 1998). These timing machineries have long been of interest, and various physiological and molecular analyses have been carried out. Recent molecular genetic studies using Arabidopsis and rice have gradually revealed their mechanisms. *LHY/CCA1* and *TOC1* were shown to be major components of the circadian oscillator in Arabidopsis (Wang and Tobin 1998, Schaffer et al. 1998, Alabadi et al. 2001, Mizoguchi et al. 2002, Yanovsky and Kay 2002). *LHY/CCA1* are Myb-related transcription factors that regulate their own expression, and their expression levels oscillate in a circadian manner. *LHY/CCA1* also negatively regulate the expression of *TOC1*, which in turn positively affects the expression of *LHY/CCA1*. This loop is proposed to be the basis of the circadian oscillations (Alabadi et al. 2001). *TOC1* encodes a pseudo-response regulator (PRR) that shows circadian expression in antiphase to *LHY/CCA1*. In Arabidopsis, the *TOC1*-type PRRs form a gene family of five members. Each member shows a rhythmic expression pattern with its specific phase, and all of them are shown to be involved in biological timing systems of the circadian clock (Mizuno and Nakamichi 2005). *GI* and *ELF3* also play important roles in both the clockwork and the flowering time (Hicks et al. 1996, Fowler et al. 1999, Park et al. 1999, Hicks et al. 2001, Covington et al. 2001, Liu et al. 2001, Mizoguchi et al. 2005). They are involved in the *LHY/CCA1–TOC1* loop and their expression levels oscillate in a circadian manner. The ELF3 protein associates with PHYB and functions as a time-dependent gating mechanism that responds to light input. All of
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Circadian rhythms of bioluminescent reporter activities in *Lemna* plants

Bioluminescent reporter systems using firefly luciferase (luc) have been successfully used in plants, animals and cyanobacteria to detect circadian rhythms in living cells (Welsh and Kay 2005). For example, an Arabidopsis *CCA1* promoter–luc construct was recently used as a circadian expression marker (Nakamichi et al. 2004). We employed semi-transient bioluminescence monitoring using a particle bombardment method. The luminescence dish monitoring system automatically monitored the bioluminescence from sample dishes containing cyanobacterial cells, Arabidopsis seedlings and cultured Arabidopsis cells raised under normal growth conditions for circadian rhythms (Kutsuna et al. 1998, Kitayama et al. 2004, Nakamichi et al. 2004). *Lemna gibba* G3 plants into which the reporter construct was introduced clearly showed circadian rhythms of bioluminescence for 4 d under constant light conditions (Fig. 1A). The period length was ∼24 h and the peak of the bioluminescent signals occurred around circadian time 4 (CT 4). The phase property of the rhythms was similar to that of cultured Arabidopsis cells (Nakamichi et al. 2004). Under constant dark conditions, at least two peaks of bioluminescence circadian rhythms were observed (Fig. 1B). We tried to observe circadian rhythms in *L. paucicostata* by the same method. The bioluminescence traces usually showed a circadian rhythmicity in a couple of days after the light was turned on, followed by gradual changes in the signals without clear rhythmicity under constant light conditions (Fig. 1C). Thus the sustainability of circadian rhythm for the bioluminescent reporter activities in this species seemed to be inferior to that in *L. gibba*. However, the first and second peaks of the rhythm occurred at similar times to those of *L. gibba*, suggesting a common regulatory mechanism for circadian expression by this Arabidopsis *CCA1* promoter. Under constant dark conditions, the bioluminescence severely decreased after the first peak (around 26 h) and then a faint trace of bioluminescent rhythm was observed (Fig. 1D).

Diurnal oscillations of the bioluminescent reporter were observed under long-day (15 h light/9 h dark) and short-day (9 h light/15 h dark) conditions in both *Lemna* plants. Under these conditions, these two species clearly showed opposite flowering responses; the flowering of *L. gibba* G3 and *L. paucicostata* 6746 only occurred under long-day and short-day conditions, respectively (Hillman 1961b). The properties of the rhythms of both species were similar to each other. Under long-
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day conditions, peaks of the rhythmic bioluminescence in both species occurred approximately 3 h after the lights were turned on [Zeitgeber time (ZT) ~3] (Fig. 1E, F). For *L. paucicostata* under short-day conditions, the peak signal was observed around dawn (ZT 0) followed by a subsequent peak of apparent light-induced bioluminescence (Fig. 1F), i.e. the phase of the short-day cycles was advanced as compared with that of the long-day cycles. The phase advance in *L. paucicostata* was clearly observed during the trough times (~ZT 13 and ~ZT 18 for the short-day and long-day cycles, respectively). In Arabidopsis, the phase of the rhythmic promoter activity of the *LHY* gene was also shown to be delayed under long-day conditions as compared with short-day conditions (Roden et al. 2002). In *L. gibba*, the phase difference between long-day and short-day conditions was ~2 h and was smaller than that observed in *L. paucicostata* (~5 h) (Fig. 1E). In spite of the small difference in the *AtCCA1* promoter activity in the two species, it was clear that *Lemna* plants possess circadian rhythm systems that are similar to that of Arabidopsis, at least at the level of the day–night transcriptional regulation of the *LHY/CCA1* promoters. Thus, transcription regulators and promoter cis-elements for circadian gene expression are likely to be conserved among monocotyledons and dicotyledons.

**Isolation of *Lemna* homologs of *LHY/CCA1*, *GI* and *ELF3***

We next cloned *Lemna* genes that were likely to be involved in the circadian clock systems of these plants. We targeted the homologs of Arabidopsis *LHY/CCA1*, *GI* and *ELF3*. These genes play important roles in the circadian clock system, and their gene expression profiles show circadian rhythmicity (Hayama and Coupland 2003). Two homologs of *LHY/CCA1* and a single homolog of *GI* and *ELF3* were isolated from each *Lemna* species. DNA sequence variation was observed among the clones of each gene from *L. paucicostata*, whereas the predicted protein sequences were highly conserved (data not shown). The variation was probably caused by the polyploidy of this plant (Urbanska-Worytkiewicz 1975). We have named the homologs by the following rules: Lg or Lp is used as the prefix for *L. gibba* or *L. paucicostata* homologs, whereas the H1 or H2 suffix indicates the number of homologs isolated. Homologous proteins from the two *Lemna* species that are highly similar to each other were assigned the same suffix.

**LHY/CCA1.** Two homologs of *LHY/CCA1* were isolated from each *Lemna* species (LgLHYH1, LgLHYH2, LpLHYH1 and LpLHYH2). The lengths of their deduced amino acid sequences range from 443 to 581 residues (Fig. 2A), making these proteins shorter than Arabidopsis LHY (645 amino acids) and CCA1 (608 amino acids). The overall primary structures of the *Lemna* LHYHs, however, are conserved. The Myb-related regions are located at the N-termini, and five other regions that are highly conserved between LHY and CCA1 are also found in these *Lemna* homologs (Schaffer et al. 1998; Supplementary Fig. S1). The 47 amino acid Myb-related region is characteristic of the LHY/CCA1 family. In Arabidopsis LHY and CCA1, this region differs by only six amino acid residues. The numbers of substitutions between this region in Arabidopsis LHY and the *Lemna* homologs are four (LgLHYH1), six (LgLHYH2), four (LpLHYH1) and nine (LpLHYH2), demonstrating the close relationship between these proteins and other members of the LHY/CCA1 family. A phylogenetic tree, which was built on the basis of the poly-

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**Fig. 1** Bioluminescence rhythms of *Lemna* plants. The bioluminescent reporter gene, firefly luciferase driven by the Arabidopsis *CCA1* promoter, was introduced into plants by a particle bombardment method. The bioluminescence circadian rhythm of *L. gibba* (A and B) and of *L. paucicostata* (C and D). Plants cultured under 12 h light/12 h dark conditions were (open circles) or were not (filled circles) subjected to bombardment, and then entrained by a 12 h light/12 h dark cycle. Bioluminescence of the entrained plants was then measured under constant light conditions (A and C) or under constant dark conditions (B and D). Bioluminescence rhythms of *L. gibba* (E) and *L. paucicostata* (F) under 15 h light/9 h dark conditions (LD, open squares) and 9 h light/15 h dark conditions (SD, filled squares). Typical bioluminescence traces of plants 3 d after the bombardment are shown. Bioluminescence levels and the amplitudes of the rhythms differed among samples.
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peptide sequences of the six conserved regions in each protein, clearly shows that the *Lemna* LHY homologs can be classified into two groups: LgLHYH1/LpLHYH1 and LgLHYH2/LpLHYH2 (Fig. 2B). Although the topology is uncertain, the two kinds of *Lemna* LHY, like those of rice, appear to belong to the LHY/CCA1 family. These data from phylogenetic analysis suggest that the LHY homologs of *Lemna* and Arabidopsis LHY/CCA1 are closely related to each other and may have similar functions in the circadian machinery.

**GI.** A homolog of GI was isolated from each *Lemna* species (Supplementary Fig. S2). The lengths of LgGIH1 and LpGIH1 are 1,116 and 1,115 amino acid residues, respectively, and they are comparable with that of Arabidopsis GI (1,173 amino acids). Their sequences are 90% identical to each other. This high degree of similarity suggests that these genes have similar functions in both *Lemna* species. Moreover, the high level of identity between these *Lemna* GI homologs and the GI from Arabidopsis (~50% identity) suggests that these proteins perform analogous functions.

**ELF3.** A homolog of ELF3 was isolated from each *Lemna* species. The lengths of LgELF3H1 and LpELF3H1 are 470 and 492 amino acid residues, respectively, and they are approximately 200 amino acid residues shorter than Arabidopsis ELF3 (695 amino acids; Fig. 2C). Their sequences are 57% identical. Although the sizes of the *Lemna* homologs differ from that of Arabidopsis ELF3, four highly conserved regions of the ELF3 family are strictly conserved in the *Lemna* homologs (Liu et al. 2001; Fig. 2C; Supplementary Fig. S3), implying that these proteins function in similar ways. A phylogenetic tree, which was built on the basis of the polypeptide sequences of the four conserved regions in each protein, shows a cluster of gene family that include the Arabidopsis ELF3 and the *Lemna* homologs as well as rice (Fig. 2D). This sequence similarity suggests the relationship of the functions among them.

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**Fig. 2** Sequence characterization of the *Lemna* homologs of LHY/CCA1 and ELF3. (A) Schematic diagrams of the *Lemna* LHY homologs, AtLHY and AtCCA1. Six highly conserved regions and their amino acid positions are indicated. (B) A phylogenetic tree of the *Lemna* LHYs, AtLHY, AtCCA1, CsLHY (Ramos et al. 2005), MccCCA1 (Boxall et al. 2005), PvLHY (Kaldis et al. 2003) and OsLHY (Izawa et al. 2002). Amino acid sequences of the MYB and the five conserved regions were aligned with the ClustalW method (http://www.ddbj.nig.ac.jp/search/clustalw-j.html), and the unrooted tree was made using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treewiew.html). The bootstrap values are indicated at the branching points. (C) Schematic diagrams and comparisons of LgELF3H1, LpELF3H1 and AtELF3. Four highly conserved regions and their amino acid positions are indicated. (D) A phylogenetic tree of the *Lemna* ELF3 homologs, AtELF3, AeEC (Liu et al. 2001), McELF3 (Boxall et al. 2005), OsELF3 and OsELF3-2. Amino acid sequences of the four conserved regions were aligned and the unrooted tree was produced by the same methods as described in (B). Detailed sequence information for the conserved regions is listed in Tables S1 (LHY/CCA1) and S2 (ELF3) in the Supplementary material.
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**Temporal expression profiles of the LHY, GI and ELF3 homologs**

LHY/CCA1, GI and ELF3 in Arabidopsis show circadian expression profiles with different phases; the peak levels of expression of these genes occur at dawn, dusk and night, respectively (Schaffer et al. 1998, Wang and Tobin 1998, Fowler et al. 1999, Hicks et al. 2001). Their oscillatory expression appears to be tightly coupled with the core oscillation of the circadian clock (Alabadí et al. 2001, Alabadí et al. 2002, Mizoguchi et al. 2002). The OsLHY and OsGI genes from rice show similar temporal expression patterns to their Arabidopsis homologs (Hayama et al. 2002, Izawa et al. 2002). We examined the temporal gene expression of the *Lemna* homologs of these genes using real-time quantitative reverse transcription–PCR (RT–PCR). RNA was extracted from *Lemna* plants grown under long-day or short-day conditions and the levels of gene expression were analyzed. Each *Lemna* homolog showed mRNA cycling profiles that were similar to those of the Arabidopsis genes. In both *Lemna* species, LHYH1 and LHYH2 were most highly expressed at around dawn (Fig. 3A–D). Therefore, as for LHY/CCA1 from Arabidopsis, these *Lemna* homologs are morning genes. Some differences between the expression profiles of LHYH1 and LHYH2 and also between

![Graphical representation of rhythmic expression of circadian clock-related homologs in *L. gibba* G3 and *L. paucicostata* 6746 under long-day or short-day conditions.](https://academic.oup.com/pcp/article-abstract/47/5/601/1844162)
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The results obtained in the long- and short-day conditions were observed. The level of *LpLHYH1* gene expression was highest before dawn when the plants were grown under short-day conditions, whereas the peak expression level was seen just at dawn when the plants were grown under long-day conditions (Fig. 3B). It was also clearly observed that the expression level of *LpLHYH1* began increasing at ZT 14 under the short-day conditions but did not increase until ZT 18 under the long-day conditions. In *L. gibba*, a phase difference of *LgLHYH1* rhythmic expression was also observed, but the shift appeared to be ∼2 h smaller than that seen in *L. paucicostata* (Fig. 3A). This difference between the two species seemed to correspond to that observed in the *AtCCA1* promoter–luc rhythms (Fig. 1E, F). In both species, the peak levels of *LHYH2* gene expression came 2–6 h later than those of *LHYH1* (Fig. 3C, D), implying that the physiological functions of these proteins may have diverged.

The expression profiles of *LHYH* genes under constant light conditions were analyzed for the circadian rhythmicity of their mRNA accumulation. The mRNA levels of each gene fluctuated with a circadian period for 3 d in both *Lemna* plants (Fig. 4A–D). However, in *L. gibba*, the second peaks of *LgLHYH1* and *LgLHYH2* expression occurred ∼4 h later than the expected phases (subjective morning) (Fig. 4A, B).

The Arabidopsis *GI* gene shows light-induced diurnal rhythmic expression with a peak at around dusk (Fowler et al. 1999). The *Lemna GI* homologs from both species showed similar expression patterns to that of Arabidopsis *GI*. The expression levels were lowest at dawn and then increased to a peak around dusk before decreasing again (Fig. 3E, F). These properties of *GI* gene expression were also observed in rice (Hayama et al. 2002). The conservation among various angiosperms suggested that *GI* functions and the circadian clock mechanisms that involve *GI* are similar in the different species.

In Arabidopsis, the *ELF3* gene is an evening gene that shows diurnal rhythmic expression with a peak at around dusk (Hicks et al. 2001). The expression pattern is similar to that of *GI* but the phase of the *ELF3* rhythm is delayed ∼4 h. Namely, the expression level of *ELF3* remains high during the early part of the night, whereas the trough level occurs ∼4 h after the
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Lights are turned on. *ELF3* and its homolog from *L. gibba* showed similar expression patterns. The expression levels were lowest in the early daytime and then increased to a peak at around dusk under both long- and short-day conditions (Fig. 3G). We applied real-time quantitative RT–PCR for the *ELF3* homolog in *L. paucicostata* using several sets of primers, but failed to generate reproducible results (data not shown). This might be due to a low level of the transcript.

Thus, all of the *Lemna* homologs to the three clock-related genes showed expression profiles that were comparable with those of the corresponding genes in Arabidopsis, suggesting that the *Lemna* plants use a similar circadian clock machinery. The results also suggest that a similar circadian system is used by long-day and short-day *Lemna* plants.

**Effects of overexpression of LHY and GI homologs on the circadian rhythmicity of the AtCCA1 promoter activity in L. gibba**

In order to examine functional implications of the *Lemna* homologs in the circadian oscillation, we tried to observe the effects of their overexpression on the rhythmic activities of the *CCA1* promoter. Overexpression of *CCA1/LHY* and *GI* was shown to result in abnormalities in the circadian system in Arabidopsis (Schaffer et al. 1998, Wang and Tobin 1998, Mizoguchi et al. 2005). We employed a co-transfection assay using the semi-transient bioluminescence monitoring system. The bioluminescent reporter (*AtCCA1–luc*) was introduced together with an effector construct that expressed a *Lemna* homolog under the cauliflower mosaic virus (CaMV) 35S promoter. The *LgLHYH1* overexpression effector (*LgLHYH1*-ox) damped the circadian rhythms of the bioluminescence (Fig. 5A). The difference between the maximum and the minimum of bioluminescence was smaller when the *LgLHYH1*-ox was introduced than when the reference was introduced. The phase of the dampened rhythm was advanced by ~3 h when the second peak was compared with that of the reference. The *LgLHYH2*-ox showed a more severe effect on the rhythmicity than the *LgLHYH1*-ox; the rhythmic trace almost disappeared (Fig. 5C). These effects of overexpression of *Lemna* LHY homologs were reminiscent of the phenotype of Arabidopsis *LHY/CCA1* overexpressing transformants that showed circadian arrhythmia (Schaffer et al. 1998, Wang and Tobin 1998). The *LgGIH1*-ox showed a low amplitude phenotype that was similar to that of *LgLHYH1*-ox (Fig. 5E). The slight difference between them was found in the first cycles of bioluminescent traces. The *LgGIH1*-ox mildly affected the trace; the first peak and the following sharp decline of bioluminescence were sustained like the reference. The *LgLHYH1*-ox, however, diminished the fluctuation in this cycle. The low amplitude phenotype of *LgLHYH1*-ox appeared comparable with that observed in the *GI*-overexpressing transformatant of Arabidopsis, suggesting a similarity in their molecular functions (Mizoguchi et al. 2005). We also examined the effects of the overexpression of those homologs on the non-circadian promoter activity of maize *UBIQUITIN1* (Christensen et al. 1992). Bioluminescence of the reporter (*ZmUBQ1–luc*) gradually decreased without apparent rhythmicity under constant light conditions (Fig. 5B). The bioluminescence traces appeared to be unaffected by co-transfection with any of the overexpression effectors, suggesting that they were unlikely to affect reporter activities in general (Fig. 5B, D, F).

**Fig. 6** Sequence characterization of the *Lemna* PRR homologs. (A) Schematic diagrams of the *Lemna* PRR homologs and Arabidopsis TOC1/PRR1. Pseudo-receiver domains and CCT motifs are indicated. The critical amino acid residues (glutamate, E) are also indicated. (B) A phylogenetic tree of the PRRs of *L. gibba*, *L. paucicostata*, Arabidopsis and rice. Amino acid sequences of the pseudo-receiver domain and CCT motif were aligned and the diagram was produced as described in Fig. 2. Detailed sequence information for the conserved regions is listed in Table S3 in the Supplementary material.
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An intriguing phenomenon found in a group of Arabidopsis clock-related genes is the ‘circadian waves’ of *PRR* gene expression (Matsushika et al. 2000, Mizuno and Nakamichi 2005). The five members of this family encode pseudo-response regulators that are involved in biological timing systems of the circadian clock and photoperiodism. Each member shows a rhythmic expression pattern with its specific phase. Rice also has these five members in this group, and shows expression profiles related to those of Arabidopsis (Murakami et al. 2003). We tried to clone *PRR* homologs in *Lemna* plants and succeeded in isolating three homologs in each species (Fig. 6A, Supplementary Fig. S4). Every homolog encodes a pseudo-receiver domain and a CCT motif that are characteristics of this gene family. A phylogenetic analysis showed that two of them were similar to OsPRR59 and OsPRR95 of rice. Thus, we designated their *Lemna* homologs with the same numbers. The other homolog was classified into the clade of AtPRR3, AtPRR7, OsPRR37 and OsPRR73; however, the relationship among them was not clear (Fig. 6B). Thus we tentatively designated it *PRRH37*.

The expression patterns of each gene of *L. gibba* under long-day and short-day conditions were examined. All of them showed diurnal rhythms with somewhat different waveforms (Fig. 7). *LgPRRH37* showed a sharp increase in the transcript level just after the onset of light, and a gradual decrease after the onset of darkness (Fig. 7A). In contrast, both *LgPRRH59* and *LgPRR95* showed sharp increases about 4 h after the onset of light (Fig. 7B, C). The peak levels of these three transcripts occurred at around dusk under both photoperiodic conditions, i.e. the peak times in long-day conditions were delayed for 2–6 h compared with those in short-day conditions. These expression profiles of *LgPRR* homologs seemed to be related to those of *OsPRRs*. The *OsPRR37/OsPRR73* transcripts sharply increased after the onset of light, but *OsPRR59/OsPRR95* did not show such an acute response (Murakami et al. 2003). This appeared parallel to the sharp increase of the *LgPRRH37* transcript but not of *LgPRRH59/LgPRRH95* in the light. These results suggested that the regulatory mechanisms for the expression of *PRR* genes are likely to be conserved between *Lemna* and rice.

Discussion

In this work, we analyzed the circadian clocks of two closely related monocotyledonous plants, the long-day plant *L. gibba* and the short-day plant *L. paucicostata*, at the molecular level. The circadian clock is a basic element for photoperiodic time measurement. *Lemna* homologs of Arabidopsis clock-related genes were isolated from both plants and their expression patterns were examined. The Arabidopsis *LHY/CCA1* genes play a pivotal role in the mechanism underlying circadian oscillation. Their gene products regulate their own expression and that of other clock-related genes, including *GI*,

![Fig. 7 Diurnal rhythmic expression of PRR homologs in L. gibba G3. Expression levels for LgPRRH37 (A), LgPRRH59 (B) and LgPRRH95 (C) in L. gibba plants grown under long-day or short-day conditions are plotted. Experimental procedures and annotations are the same as described in Fig. 3.](https://academic.oup.com/pcp/article-abstract/47/5/601/1844162/2)
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ELF3 and TOC1/PRR1 (Fowler et al. 1999, Alabadi et al. 2001, Hicks et al. 2001, Alabadi et al. 2002, Mizoguchi et al. 2002, Mizoguchi et al. 2005). The transcriptional regulation of clock-related genes in Arabidopsis is highly linked, and this regulatory network is likely to be important for the robust circadian oscillation. We characterized the homologs of _LHY/CCA1, GI, ELF3_ and _PRR5/PRR9_ in two _Lemna_ species. Attempts to isolate _TOC1/PRR5_ orthologs yielded three genes that showed higher similarities to _PRR5/PRR7_ and _PRR5/PRR9_ than to _TOC1/PRR1_ in this family (Matsushika et al. 2000, Murakami et al. 2003; Fig. 6). Other than _TOC1/PRR1_, we succeeded in identifying those clock-related homologs that showed high sequence similarities between _L. gibba_ and _L. paucicostata_. These similarities suggest that they are likely to be orthologous genes between the two species. The similarities between the amino acid sequences of the _Lemna_ homologs and the Arabidopsis counterparts suggest similar molecular functions of these proteins. Moreover, the transcriptional regulatory mechanisms for these genes appear to be conserved between the _Lemna_ plants and Arabidopsis since their temporal expression profiles were similar (Fig. 3, 4, 7). This idea was supported by the fact that an Arabidopsis _CCA1_ promoter produced similar results in the _Lemna_ plants and Arabidopsis (Fig. 1), i.e. transcriptional cis-elements for circadian expression in the _CCA1_ promoter can be similarly used by clock-related transcription factors in _Lemna_.

Characterization of _Lemna LHY_ and _GI_ homologs using cotransfection assays of a bioluminescent reporter and overexpression effectors strongly suggested that these homologs can function in the circadian systems in ways similar to their Arabidopsis counterparts (Fig. 5). All of these results suggested that the structure of the transcriptional networks underlying circadian rhythm is conserved between monocotyledons (_Lemna_) and dicotyledons (Arabidopsis). This means that the circadian systems for the regulation of clock-related genes are likely to be sustained without regard to the evolutionary divergence in higher plants. The conservation of the circadian systems, even in the face of developmental and environmental disturbances, was recently suggested in studies of _Mesembryanthemum crystallinum_ (Boxall et al. 2005).

Although the overall expression profiles of the clock-related genes in _Lemna_ plants were similar to those of their Arabidopsis counterparts, slight but interesting divergence was observed in the two _LHY_ homologs (_LHYH1_ and _LHYH2_). In Arabidopsis plants under light–dark conditions, mRNA levels of both _LHY_ and _CCA1_ oscillated similarly, with a peak around the time the light came on (Kim et al. 2003). Although _LHYH1_ showed a similar expression pattern, the expression level of _LHYH2_ peaked 2–6 h later than that of _LHYH1_. Thus, the transcription of the two _LHY_ homologs appeared to be regulated differently in _Lemna_ plants. This also suggests that these proteins may have distinct physiological functions. Interestingly, a phylogenetic analysis showed an almost parallel relationship between _LHYH1_, _LHYH2_, _AtLHY_ and _AtCCA1_ (Fig. 2B). Together with the fact that the Arabidopsis _LHY_ and _CCA1_ have similar molecular functions, this suggests comparable molecular functions for these Arabidopsis and _Lemna_ proteins. It should be noted that the genome sequence of rice, a model monocotyledon, contains only one _LHY_ locus, _OsLHY_, suggesting gene duplication/deletion through the evolution of rice and _Lemna_ (Izawa et al. 2003). The expression pattern of this rice gene under light–dark conditions is similar to those of Arabidopsis _LHY/CCA1_ and _Lemna LHYH1_ (Izawa et al. 2002). These results suggest that _Lemna LHYH2_ may be involved in the clock in a way different from the _LHY/CCA1_ proteins.

Circadian systems of _Lemna_ plants were analyzed previously using the physiological rhythms of _CO₂_ output and potassium uptake (Hillman 1970, Kondo and Tsudzuki 1978). The flat, tiny plant bodies and aseptic culture on liquid media in small containers enabled precise monitoring of these physiological fluctuations. These advantages of the _Lemna_ system are still true for the present studies. We developed an alternative method to monitor the circadian rhythm using a bioluminescent reporter gene introduced into the plants by particle bombardment. The size and shape of the _Lemna_ plants allowed us to manipulate whole plants in the bombardment machine. We have also succeeded in the analysis of gene functions using cotransfection of the reporter and effectors. This is an easy and powerful way to test molecular functions since the genetic transformation is dispensable. The reporter activity could be sustained for >1 week (data not shown), long enough to examine circadian rhythms and also to cover the whole course of floral induction, which can be visually observed within a week after photoperiodic transitions (Hillman 1959, Umemura et al. 1963). Thus, this method could be applied for continuous, in-depth bioluminescence monitoring of photoperiodic responses.

Our bioluminescent reporter experiments and temporal expression analyses using both _L. gibba_ and _L. paucicostata_ showed some differences between them in rhythmic phenomena. Whereas the _L. gibba_ plants showed robust circadian rhythms under constant light conditions, _L. paucicostata_ did not (Fig. 1A, B). This phenomenon was also observed in previous studies (Hillman 1970, Kondo and Tsudzuki 1978). Phase differences of the _AtCCA1–luc_ bioluminescence rhythm and the _LHY_ expression rhythm between short-day and long-day conditions were larger in _L. paucicostata_ than in _L. gibba_ (Fig. 1, 3). These differences may be caused by different light signaling systems. Recent studies on natural variation in the Arabidopsis circadian clock have shown that the phases, period lengths and amplitudes of the oscillations are likely to vary considerably depending on the habitat (Michael et al. 2003). The difference of circadian traits between _L. gibba_ and _L. paucicostata_ was not larger than the natural variation observed among Arabidopsis accessions, suggesting a close relationship of the circadian systems in _Lemna_ plants. Such a small divergence of the circadian systems could affect the photoperiodic flowering of these plants, but it is unlikely to direct the difference between long-day and short-day responses.
A plausible model in which the long-day and short-day flowering are due to the opposite effect of CO (Hd1) on FT gene expression has been proposed on the basis of comparative molecular genetic studies between Arabidopsis and rice (Yano et al. 2000, Suárez-López et al. 2001, Izawa et al. 2002, Kojima et al. 2002, Hayama et al. 2003). The circadian clock is involved in the circadian expression of CO (Hd1), which is critical for daylength measurement. Since the regulatory mechanisms for circadian gene expression are likely to be highly conserved, the variation between long-day and short-day flowering might have arisen via the modification of flowering-related gene functions or the generation of additional genes with flowering-related functions. The example of CO and Hd1 fits with the former hypothesis. On the other hand, Eth1, an apparent rice-specific photoperiodic flowering gene, may fit with the latter idea (Doi et al. 2004). Since the genetic distance between L. gibba and L. paucicostata is much smaller than that between Arabidopsis and rice, there seems to be smaller functional divergence of the photoperiodic flowering-related genes in these Lemna plants. The puzzle of how the opposite photoperiodic responses are realized in these closely related species will be investigated by means of functional gene analyses using the Lemna plants.

Materials and Methods

Plant materials and growth conditions

The L. gibba G3 and L. paucicostata 6746 strains have been maintained in our laboratory for >40 years by vegetative reproduction. Lemna gibba plants were kept in M medium with 1% sucrose under short-day (9 h light/15 h dark) conditions. Lemna paucicostata plants were kept in 0.5 × H medium with 1% sucrose under constant light conditions. The compositions of M and H media were described previously (Hillman 1961b). For both Lemna species, the growth temperature was maintained at 25 ± 1 °C and the light intensity supplied by fluorescent lamps (FLR40SW/M36 or FL20SSW18; Mitsubishi/Osram, Kakegawa, Shizuoka, Japan) was approximately 25 µmol m⁻² s⁻¹. Colonies were grown in 100 ml of medium in 200 ml Erlenmeyer flasks plugged with cotton. New stock cultures were made every week, and well-grown plants were used for experiments.

Particle bombardment

pSP1-CCA1::LUC-B was used as the bioluminescent reporter construct (Nakamichi et al. 2004). This plasmid vector was introduced using particle bombardment. A 25 µl aliquot of pre-washed gold particle suspension (1µm diameter; Bio-Rad, Hercules, CA, USA) in 50% glycerol (60 mg ml⁻¹) was mixed with 10 µl of plasmid DNA solution (0.5 µg ml⁻¹), 25 µl of CaCl₂ (2.5 M) and 1 µl of spermidine (1 M). After vortexing for 3 min, the tube was briefly centrifuged. The supernatant was discarded and 200 µl of 70% ethanol was added and mixed well. The suspension was briefly centrifuged and the supernatant was discarded. The DNA-coated particles were washed again with 100% ethanol and resuspended in 30 µl of 100% ethanol. A helium gun device (GIE-III IDERA; Tanaka Co., Sapporo, Japan) was used for particle bombardment according to the manufacturer’s instructions (vacuum, 800 hектoPa; helium gauge pressure, 5.0 hектoPa). About a dozen Lemna colonies were set on a 35 mm polystyrene dish (Asahi Techno Glass, Tokyo, Japan) and covered with a small piece of plastic mesh. The dish was set underneath the muzzle of the gun and 8 µl of DNA-coated particle suspension was fired into the sample. After the bombardment, 3 ml of medium containing firefly luciferin (1 mM potassium salt; Biosynth AG, Switzerland) was added to the dish. The samples were cultured under light–dark entrainment conditions for at least 1 d before bioluminescence measurements began.

Bioluminescence monitoring

The monitoring of bioluminescence of Lemna plants was basically done as described previously (Kutsuna et al. 1998, Kitayama et al. 2004). The luminescence dish monitoring system used photomultiplier tubes (R329P; Hamamatsu Photonics K.K., Toyooka, Shizuoka, Japan) for bioluminescence detection. To reduce the fluorescence signals from chlorophyll, a short-pass filter (SV0630; Asahi Spectra Co. Ltd, Nishinasuno, Tochigi, Japan) was set at the detection site of the photomultiplier tubes. Each dish was subjected to 30 s measurements of bioluminescence every 30 min.

Total RNA isolation and cDNA synthesis

Lemna plants on medium were harvested in mesh tea bags and immediately frozen in liquid nitrogen. After dark-grown plants were harvested, the sampling procedure was carried out under a green safety light. The frozen samples were ground to a frozen powder using a mortar and pestle. Total RNA was isolated from the powder using an RNaseasy Plant Mini Kit (Qiagen, Valencia, CA, USA) for small-scale preparations or TRIReagent (Sigma, St Louis, MO, USA) for large-scale preparations. cDNA synthesis reactions (20 µl) were carried out using 3 µg of total RNA and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) by following the manufacturer’s instructions. The reverse transcription reactions were treated with RNase H (TAKARA BIO INC., Otsu, Japan).

Molecular cloning of clock-related gene homologs

We cloned clock-related genes from Lemna using a degenerate primer-mediated PCR method. We tried several sets of degenerate primers for each target gene, and those listed in Table S4 in the Supplementary material were used for successful amplifications of the proper PCR fragments. cDNA that was synthesized as described above was used for the template, and TAKARA ExTaq was used for the PCRs (TAKARA BIO INC.). We performed touchdown PCR with several cycling conditions for each target. Amplified PCR fragments were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and the nucleotide sequences were determined. Putative full-length cDNA sequence was obtained with 5’- and 3’-RACE (SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA, USA).

RT–PCR-based quantification of gene expression

Two RT–PCR-based quantification methods were used to evaluate gene expression levels: real-time PCR with TaqMan probes and with SYBR Green fluorescent dye. cDNA templates were synthesized as described above. A 1 : 100 dilution of the reverse transcription reaction was used for the real-time PCRs. The primer sequences and TaqMan probe sequences are shown in Table S4 in the Supplementary material. Real-time PCR with TaqMan probes was done using a TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA, USA) and a real-time thermal cycler (ABI PRISM 7700 Sequence Detector; Applied Biosystems) according to the manufacturer’s recommendations. Real-time quantitative detection of SYBR Green fluorescence was carried out using a SYBR Green PCR Master Mix Kit (Applied Biosystems) and the real-time thermal cycler according to the manufacturer’s recommendations. Reverse transcription reactions to which the reverse transcriptase was not added were used as control samples to check for contamination by genomic DNA. Raw expression data were normalized using ubiquitin gene expression data that
were obtained by the real-time PCR with a TaqMan probe. Microsoft Excel X for Mac was used for statistical analyses.

Co-transfection assays
Coding regions for clock-related genes were amplified using PCR and they were cloned into pBI221 (Clontech) at the Xhol/Sacl sites for LgLUH1 and LHY2 or at the Smal/Escl/136II sites for LgGHI1. pBI221 was the plasmid containing the β-glucuronidase (GUS) gene under the CaMV 35S promoter, and the GUS region was replaced with the coding region in the overexpression effectors. In the pSP1(Promega)-based ZnUBQ1 promoter–luc construct, an ~0.9 kbp promoter and ~1.0 kbp first intron region of ZnUBQ1 (Christensen et al. 1992) was connected to the 5′ end of the luc gene, and the Nos terminator region from pBI221 was at the 3′ end. A 3 μg aliquot of effector plasmid and 1 μg of pBI221 were mixed into 6 μl of reporter plasmid DNA solution (0.5 mg ml⁻¹). A reference plasmid that was made by removing the GUS coding region from pBI221 was used as the reference sample. The particle bombardment and bioluminescence monitoring procedures were the same as described above. After the monitoring, plants were stained with GUS staining solution to check the transfection efficiencies roughly.

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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References
Expression of clock-related genes in *Lemna* plants


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