Plant Biotechnology 31, 319–328 (2014)
DOI: 10.5511/plantbiotechnology.14.1111a

Original Paper

Overexpression of a *CO* homologue disrupts the rhythmic expression of clock gene *LgLHYH1* in *Lemna gibba*

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Received June 12, 2014; accepted November 11, 2014 (Edited by T. Mizoguchi)

Abstract In plants, the circadian clock is involved in the regulation of various physiological phenomena. The Arabidopsis *CONSTANS* gene (*AtCO*), a central component in photoperiodic flowering, as well as the *CONSTANS-LIKE 1* gene (*AtCOL1*), which has been suggested to be involved in the light input pathway, belong to the *CONSTANS-LIKE* gene family (*COL* family). To investigate the function of the *COL* family in the circadian system, we characterized *CO* homologues in two *Lemna* plant species (*LgCOH1* in *L. gibba* G3 and *LaCOH1* in *L. aequinoctialis* 6746). The expression of *LgCOH1* was upregulated by light, showing diurnal rhythmic expression with peak expression during daytime. We examined the effect of *LgCOH1* overexpression (*LgCOH1-ox*) on a circadian bioluminescent reporter. *LgCOH1-ox* damped the circadian bioluminescence rhythm, suggesting that it disturbed the circadian system. The overproduction of the N-terminal region including the zinc finger regions, or the C-terminal region including the CCT domain of *LgCOH1* damped the circadian bioluminescence rhythm, suggesting that both regions were involved in the phenotypic abnormalities found in the full-length *LgCOH1-ox* mutant. The overexpression of *AtCO* also damped the circadian bioluminescence rhythm in our co-transfection assay using *Lemna* plants, while its effect on the circadian rhythm was weaker than that of *LgCOH1-ox*. Based on these results, we suggest that some *COL* family genes may function in the regulation of the circadian system including the light input pathway.

Key words: Circadian rhythm, *CONSTANS-LIKE* gene family, bioluminescent reporter, *Lemna*.

Environmental conditions on the earth diurnally vary in physical conditions such as light and temperature. Endogenous circadian clocks are important devices to allow organisms to adapt to periodic environmental changes. Circadian rhythms persist with a period of approximately 24 h even in the absence of external cues, and can be entrained to periodic environment changes. Circadian clocks in plants are involved in various physiological behaviors such as cell growth, changes in stomata aperture, metabolism, and photoperiodic flowering (Más 2005; Sweeney 1987). Recent molecular genetic studies using Arabidopsis have revealed the molecular mechanisms of the circadian clock. *LATE ELONGATED HYPOCOTYL* (*LHY*), *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and the *PSEUDO-RESPONSE REGULATOR* (*PRR*) family [including *TIMING OF CAB EXPRESSION1* (*TOC1*)/*PRR1*] were shown to be major components of the circadian oscillator in Arabidopsis (Nagel and Kay 2012). *LHY* and *CCA1* encode Myb-related transcription factors with peak circadian expression at dawn. *TOC1* encodes a pseudo-response regulator with circadian expression in antiphase to *LHY* and *CCA1*. LHY and CCA1 act as repressors of *TOC1* expression during the day, and TOC1 directly represses *LHY* and *CCA1* expression by binding their promoters (Gendron et al. 2012; Huang et al. 2012). Other evening-peaking components, including *GIGANTIA* (*GI*) and *EARLY FLOWERNG 3* (*ELF3*), interconnect with the core oscillator to form multiple feedback loops and a complex clock network (Alabadí et al. 2001; McClung 2014; Nagel and Kay 2012).

The circadian clock is involved in the measurement of day length in photoperiodic flowering. In Arabidopsis, the *CONSTANS* (*AtCO*) gene is the key component in control of day length-dependent flower induction (Hayama and Coupland 2003; Suárez-López et al. 2001). The expression of *CO* mRNA is under circadian control, showing two peaks at late daytime and night time, under long day conditions. Light signals stabilize and activate the CO protein, and the stabilized CO protein

Abbreviations: PCR, polymerase chain reaction; C-terminus, carboxy-terminus; N-terminus, amino-terminus; qRT-PCR, real-time quantitative reverse transcription-PCR; LUC, firefly luciferase; RNAi, RNA interference; CaMV, cauliflower mosaic virus; *CAB*, *chlorophyll a/b-binding proteins*.
This article can be found at http://www.jspcmb.jp/
Published online December 4, 2014

directly induces the expression of the floral integrator *FLOWERING LOCUS T* (*FT*) gene (Suárez-López et al. 2001; Valverde et al. 2004).

CO belongs to the CONSTANS-LIKE (COL) family, which is characterized by two conserved domains (Putterill et al. 1995). The first is the B-box, which is composed of two zinc finger domains near the N-terminus (Putterill et al. 1995). The B-box is related to a motif in a group of transcription factors found in animals and other organisms. This motif is presumably involved in protein–protein interactions (Khanna et al. 2009). The second characteristic domain is a CCT (CO, CO-like, TOC1) domain near the C-terminus which is involved in nuclear localization and is presumably related to DNA-binding (Ben-Naim et al. 2006; Robson et al. 2001; Tiwari et al. 2010; Wenkel et al. 2006). The CCT domain is also found in the Arabidopsis PRR family whose members function as clock components. In Arabidopsis, *toc1-1* mutants with a mutation in the CCT domain show a short period phenotype (Strayer et al. 2000). COL family genes are conserved in land plants as well as green algae such as *Chlamydomonas* (Matsuo et al. 2008; Serrano et al. 2009). Arabidopsis and rice contain 17 and 16 COL genes in their genomes, respectively (Griffiths et al. 2003; Lagercrantz and Axelesson 2000).

Several expression profiling studies on members of the *COL* family have been shown that many members of the *COL* family are under circadian control and are involved in light-dependent processes (Kikuchi et al. 2012; Kim et al. 2003; Ledger et al. 2001; Shimizu et al. 2004; Shin et al. 2004). In Arabidopsis, overexpression of *CONSTANS-LIKE 1* (*COL1*) shortened the circadian period, suggesting that *COL1* could affect light input to the circadian clock system (Ledger et al. 2001). Loss-of-function of *col3* in Arabidopsis led to longer hypocotyls in red light, suggesting that COL3 is a positive regulator of red light signaling (Datta et al. 2006). In *Chlamidomonas*, an insertional mutant of *ROC66*, a *COL* gene, exhibited a longer circadian period as compared to the wild-type when measured by choloroplast bioluminescent reporter activity (Matsuo et al. 2008). These studies suggest that *COL* family members are closely related to the circadian system, although the functions of many *COL* family members remain to be understood.

The *Lemna* genus (duckweeds) is a group of monocotyledonous plants with tiny, floating bodies. The physiological photoperiodic flowering timing systems of and circadian rhythms of *Lemna gibba* G3 and *Lemna aequinoctialis* (also called *L. paucicostata* and *L. perpusilla*) 6746 strains have been intensively analyzed because these strains show obligatory and sensitive long-day and short-day photoperiodic responses, respectively (Hillman 1961a). Tiny floating plant bodies, rapid growth rates, and strictly controllable aseptic culture conditions make these *Lemna* plants experimentally attractive (Hillman 1961a). Various techniques to monitor luciferase activities have been developed in plants to allow quantitative analyses of gene expression and time-series data collection (Hayakawa et al. 2012; Muranaka et al. 2013; Watanabe et al. 2011). A transient gene expression system to monitor circadian expression of bioluminesent reporters has also been developed in *Lemna* plants (Miwa et al. 2006; Muranaka et al. 2013). Clock-related gene homologues of *LHY*, *GI*, *ELF3* and the *PRR* family have been isolated from both *Lemna* species, and their expression profiles were shown to be similar to those of Arabidopsis (Miwa et al. 2006). Furthermore, functional analyses using transient gene expression systems with overexpression and RNA interference (RNAi) have shown that *Lemna* homologues of *LHY*, *GI*, and *ELF3* are important in maintaining the rhythmicity of bioluminescent reporters (Serikawa et al. 2008). These results suggest that essential components of the circadian clock may be conserved between *Lemna* (monocotyledons) and Arabidopsis (dicotyledons).

To investigate the function of *COL* family members in the circadian system, we isolated the *Lemna CO* homologues *LgCOH1* and *LaCOH1* from *L. gibba* and *L. aequinoctialis*, respectively. We found that *LgCOH1* expression was upregulated by light, showing diurnal rhythmic expression. Using transient gene expression system of a bioluminescent reporter, we showed that overexpression of *LgCOH1* disrupted the circadian rhythm of *Lemna*, suggesting that some *COL* family members are involved in circadian systems.

Materials and methods

Plant materials and growth conditions

The *Lemna gibba* G3 and *Lemna aequinoctialis* (also called *L. paucicostata* and *L. perpusilla*) 6746 strains have been maintained in our laboratory for more than 50 years by vegetative reproduction. *L. gibba* plants were kept in M medium with 1% sucrose under short day (9 h light/15 h dark) conditions. *L. aequinoctialis* plants were kept in 0.5× H medium with 1% sucrose under constant light conditions. M and H media were formulated as described previously (Hillman 1961b). For both *Lemna* species, growth temperature was maintained at 25±1°C and light intensity supplied by fluorescent lamps (FLR40SW/M/36 or FL20SSW18; Mitshubishi/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.

Genomic DNA isolation, total RNA isolation, and cDNA synthesis

Lemna plants were harvested in mesh tea bags and immediately frozen in liquid nitrogen. Dark-grown plants were harvested under a green safelight. Frozen samples were ground to powder using a mortar and pestle. Genomic DNA was isolated from the powder by the CTAB-CsCl method (Murray and Thompson 1980), and total RNA isolation and cDNA synthesis were performed as described previously (Miwa et al. 2006). Total RNA was isolated from the powder using an RNeasy 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) following the manufacturer's instructions. Following the manufacturer's protocol, reverse transcription reactions were treated with 12 U RNase H (TAKARA BIO INC., Otsu, Japan).

Molecular cloning of Lemna CO homologues

We cloned *Lemna CO* homologues from using a degenerate primer-mediated PCR method. We tried several sets of degenerate primers for each target gene and those listed in Table S1 (Supplementary Data) were used for successful amplifications of the proper PCR fragments. cDNA of whole *Lemna* plants was used as template for PCR using a TaKaRa ExTaq (TAKARA BIO INC). We performed touchdown PCR with several cycling conditions for each target (Don et al. 1991). Amplified PCR fragments were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and nucleotide sequences were determined. A putative full-length cDNA sequence was obtained by 3′- and 5′-RACE (SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA, USA). We amplified full-length regions for *LgCOH1* and *LaCOH1* using PCR from cDNA of *L. gibba* and *L. aequinoctialis*, cloned amplicons into pBI221 (Clontech), and confirmed the nucleotide sequences. Sequences were submitted to the DDBJ under accession numbers *AB897788* and *AB897789*, for LgCOH1 and LaCOH1, respectively.

Real-time quantitative reverse transcription-PCR technology (qRT-PCR) based quantification of gene expression

A qRT-PCR-based quantification method with TaqMan probes was performed to evaluate gene expression levels 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 instructions. A 1 : 100 dilution of the reverse transcription reaction was used for qRT-PCR reactions. The primer sequences and TaqMan probe sequences are shown in Table S1 (Supplementary Data). Reactions to which reverse transcriptase was not added were used as control samples to check for contamination of genomic DNA. Raw expression data were normalized using ubiquitin gene expression data obtained by qRT-PCR with a TaqMan probe. Microsoft Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

Reporter and effector constructs

For the pSP1 (Promega)-based *LgLHYH1* promoter-*luc* (*LgLHYH1pro::LUC*) construct, the 786-bp region including the 5′-upstream sequence from the *LgLHYH1* start codon was amplified using PCR of *L. gibba* total genomic DNA and connected to the 5′ end of the *luc+* gene at *Hind*III/*Nco*I site, and to the *NOS* terminator region. Primer sequences are shown in Table S1 (Supplementary Data). The sequence of *LgLHYH11* promoter was submitted to the DDBJ under accession numbers AB909493.

The pSP1-based *ZmUBQ1* promoter-*LUC* (*ZmUBQ1pro::LUC*) construct was described previously as *ZmUBQ1* promoter-*luc* (Miwa et al. 2006). The *AtPRR1pro::LUC* construct was described previously as *AtPRR1::luc* (Serikawa et al. 2008)

For full-length (*LgCOH1*-ox and *AtCO*-ox) and truncated (*LgCOH1-Zf-ox*, *LgCOH1-CCT-ox*, *AtCO-Zf-ox* and *AtCO-CCT-ox*) overexpression constructs, coding regions for *LgCOH1* and *AtCO* were amplified using PCR from cDNA of *L. gibba* and *Arabidopsis thaliana* (Col), and primer sequences were shown in Table S2 (Supplementary Data). The 1.06 kb (LgCOH1), 0.86 kb (LgCOH1-Zf), 0.2 kb (LgCOH1-CCT), 1.13 kb (AtCO), 0.92 kb (AtCO-Zf) and 0.21 kb (AtCO-CCT) DNA fragments were cloned into pBI221 (Clontech) at the *Sma*I/*Ecl*136II sites. The pBI221 plasmid containing the GUS gene under the CaMV 35S promoter was used, with the GUS region replaced with the appropriate coding region in the overexpression effectors.

Particle bombardment

LgLHYH1pro::LUC and *ZmUBQ1pro::LUC* were used as the bioluminescent reporter constructs and were introduced into *L. gibba* by particle bombardment. Particle bombardment was performed as described previously (Miwa et al. 2006; Serikawa et al. 2008).

Bioluminescence monitoring

The monitoring of bioluminescence of *Lemna* plants was performed as described previously (Miwa et al. 2006) with minor revisions. The luminescence dish monitoring system used photomultiplier tubes (R329P; Hamamatsu Photonics K.K., Iwata, Shizuoka, Japan) for bioluminescence detection. To reduce chlorophyll background fluorescence signals, a short-pass filter (SVO630; Asahi Spectra. Co., Ltd., Nishinasuno, Tochigi, Japan) was placed at the detection site of the photomultiplier tubes. Each dish was subjected to 30-s bioluminescence measurements every 30 min.

Results

Isolation of CO homologues from Lemna plants

We isolated *CO* homologues from *L. gibba* G3 and *L. aequinoctialis* 6746 using a degenerate primer-mediated PCR method (*LgCOH1* for *L. gibba* G3 and *LaCOH1* for *L. aequinoctialis* 6746). The lengths of the deduced amino

acid sequences of both genes were 352 residues. The zinc finger region, with two B-boxes near the N-terminus, and the CCT domain, near the C-terminus, that are conserved in the COL family were found in *LgCOH1* and *LaCOH1* (Figure 1A). At the overall amino acid sequence level, the LgCOH1 protein is 70%, 31% and 32% identical to LaCOH1, Arabidopsis CO (AtCO), and rice Hd1 (OsHd1), respectively. A phylogenic tree built on the basis of the polypeptide sequences of the concatenated B-boxes and CCT domain of each protein, showed a cluster including AtCO, OsHd1, and the *Lemna* CO homologues (Figure 1B). *COL* genes in Arabidopsis are subdivided into three classes, termed Groups I to III, and *CO* belongs to Group I (Griffiths et al. 2003; Robson et al. 2001; Valverde 2011). AtCO and OsHd1 are included in Group Ia and members in this group have four small motifs (M1 to M4) in the middle region in addition to the two B-boxes and the CCT domain (Griffiths et al. 2003). The middle region of LgCOH1 has all four motifs, and LaCOH1 has M2, M3 and M4 motifs (Figure 1A). Despite the lack of the M1 motif in LaCOH1, both LgCOH1 and LaCOH1 were grouped with Group Ia in the phylogenetic tree.

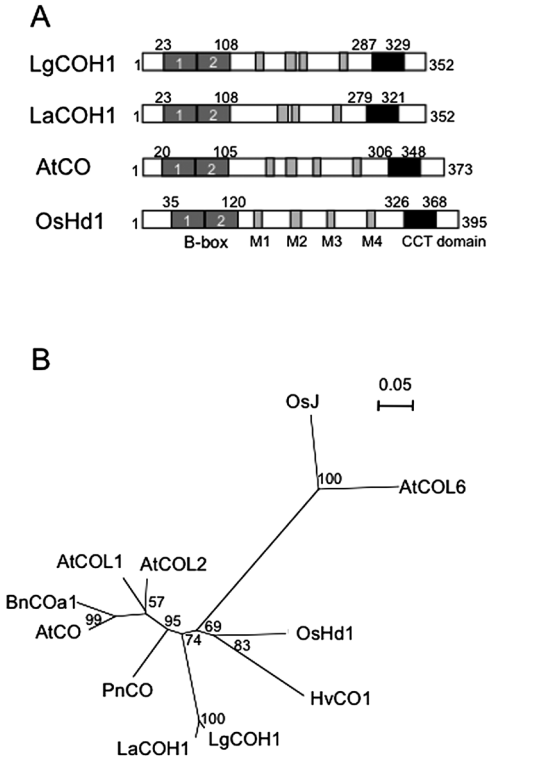

Figure 1. Sequence characterization of *Lemna* CO homologues. (A) Schematic diagrams of *Lemna* CO homologues, AtCO and OsHd1. Two B-box domains, a CCT domain, and four conserved middle region motifs are indicated (Griffiths et al. 2003). Detailed sequence information is shown in Figure S1 (Supplementary Data). (B) Phylogenetic tree of LgCOH1 (AB897788), LaCOH1 (AB897789), AtCO (CAC01783), AtCOL1 (AED92215), AtCOL2 (AAF32446), BnCOa1 (AAC27694), AtCOL6 (Q8LG76), OsJ (AC087181), HvCO1 (AAM74062), PnCO (AF300700) and OsHd1 (BAB17628). Amino-acid sequences of the two B-box domains and the CCT domain were aligned with a multiple sequence alignment tool, ClustalW (http://clustalw.ddbj.nig.ac.jp/), and the unrooted tree was drawn using NJplot (http://pbil.univ-lyon1.fr/software/njplot.html). Bootstrap values are indicated at branch points.

Temporal expression profiles of LgCOH1

In Arabidopsis, *AtCO* is transcribed in a circadian fashion showing a peak at night (Suárez-López et al. 2001). Both the phase and level of *AtCO* mRNA expression is important for photoperiodic induction of flowering. We examined the temporal expression of *LgCOH1* using qRT-PCR. RNA was extracted from *Lemna* plants grown under long day (15 h light/9 h dark) or short day (9 h light/15 h dark) conditions and the levels of gene expression were analyzed. We succeeded in measuring the expression of *LgCOH1* but failed to measure that of *LaCOH1* by qRT-PCR. This might be due to a low level of the *LaCOH1* transcript. *LgCOH1* showed diurnal rhythmic expression at a high level during daytime and a low level during nighttime. Under long day conditions, the abundance of the *LgCOH1* transcript was high at 3–15 h, and low during nighttime (Figure 2A). Under short day conditions, the abundance of the *LgCOH1* transcript was high at 3–9 h, and low during nighttime (Figure 2A). This suggests that the expression of *LgCOH1* is upregulated by light.

To determine if the rhythmic expression of *LgCOH1* was under circadian control, *Lemna* plants entrained under light and dark (9 h light/15 h dark) conditions were transferred to constant light or constant dark conditions. Under constant light conditions, the expression levels of *LgCOH1* rapidly increased during the first 6 h and then remained at a high expression level, and we did not detect circadian rhythmicity of *LgCOH1* expression (Figure 2B, upper). Under constant dark conditions, the expression levels of *LgCOH1* remained at a low level, with a slight increase from 24–48 h (Figure 2B, lower). Thus, we did not detect a robust circadian rhythm in *LgCOH1* expression. These results indicate that the expression of *LgCOH1* was regulated primarily by light signaling rather than the circadian clock.

In Arabidopsis, the expression of *AtCO* is under circadian control, showing a peak at late daytime and nighttime, under long day conditions (Suárez-López et al. 2001; Valverde 2011). The peak time and the pattern of the *LgCOH1* expression differed from that of Arabidopsis *AtCO*. Therefore, it seems reasonable to conclude that *LgCOH1* has a different role than *AtCO*.

Effects of overexpression of *LgCOH1* on circadian rhythmicity of *LgLHYH1* promoter activity in *L. gibba*

In our previous study, we introduced a bioluminescent reporter using a particle bombardment method and

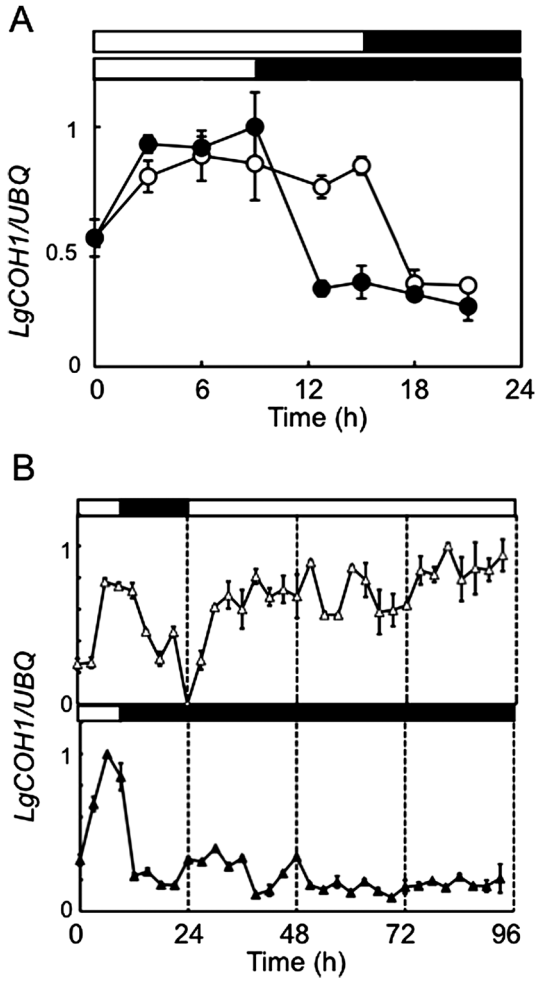

Figure 2. Expression of *LgCOH1* under long day, short day, constant light, and constant dark conditions. (A) *LgCOH1* expression under 15 h light/9 h dark (LD) or 9 h light/15 h dark (SD) conditions. *L. gibba* plants cultured under LD or SD conditions were collected every 3 h. Relative gene expression levels in plants grown under long day and short day conditions are indicated with open and closed circles, respectively. (B) *LgCOH1* expression under constant light (upper) and constant dark (lower) conditions. *Lemna* plants cultured under SD conditions were collected every 3 h for 1 day and then transferred into constant light or constant dark conditions for 3 days. Relative expression levels are plotted. The expression levels of *LgCOH1* were normalized to ubiquitin expression levels. Relative expression levels are the ratios of the expression levels to the maximum expression levels measured in each condition. Two independent samples were processed. qRT-PCR experiments were performed at least two times for each sample. Standard deviations at each time point are indicated. Black and open bar indicate dark and light, respectively.

monitored circadian rhythms in *Lemna* plants (Miwa et al. 2006; Muranaka et al. 2013). *L. gibba* plants into which a construct bearing the Arabidopsis *CCA1* promoter fused to the firefly luciferase (*LUC*) gene (*AtCCA1pro::LUC*) was introduced showed a diurnal bioluminescence rhythm under light and dark conditions and also showed a circadian rhythm under constant light and constant dark conditions (Miwa et al. 2006). We also functionally analyzed the circadian genes of *L. gibba* by co-transfection of the *AtCCA1pro::LUC* construct and overexpression- or RNAi knockdown of various effectors (Miwa et al. 2006; Serikawa et al. 2008). Using this method, we examined whether the overexpression of *LgCOH1* affected circadian rhythms.

LgLHYH1 is the *LHY* homologue in *L. gibba* and has been suggested to be a component of the circadian clock (Miwa et al. 2006; Serikawa et al. 2008). We isolated a part of the *LgLHYH1* promoter and fused it to the *LUC* gene (*LgLHYH1pro::LUC*). *L. gibba* plants into which the *LgLHYH1pro::LUC* construct was introduced showed a clear diurnal bioluminescence rhythm under 12 h light/12 h dark conditions (Figure 3A). Under constant light conditions, a circadian bioluminescence rhythm was observed with a peak at dawn (Figure 4A). This is consistent with the expression profile of *LgLHYH1* and similar to the bioluminescence profile of *AtCCA1pro::LUC* (Miwa et al. 2006).

To test the effect of disturbance of *LgCOH1* expression on circadian rhythm, we introduced an effector construct that overexpressed *LgCOH1* under the cauliflower mosaic virus (CaMV) 35S promoter with the bioluminescent reporter, *LgLHYH1pro::LUC*. The *LgCOH1* overexpression effector (*LgCOH1-ox*) damped the circadian bioluminescence rhythm under constant-light conditions (Figures 4C, S3). Under 12 h light/12 h dark conditions the diurnal rhythm of the bioluminescent reporter was damped and the increase of bioluminescence intensity before dark-to-light transitions (anticipation of the dawn) disappeared (Figures 3C, S2).

The effect of *LgCOH1-ox* on circadian rhythmicity appeared to be much more severe than that of Arabidopsis *COL1* reported previously (Ledger et al. 2001). We also considered the possibility that overexpression of *LgCOH1* generally affected a broad range of promoter activities by examining the non-circadian promoter activity of maize *UBIQUITIN1* (Christensen et al. 1992; Miwa et al. 2006). Bioluminescence traces appeared to be unaffected by co-transfection with *LgCOH1-ox*, suggesting that this overexpression effector was unlikely to affect bioluminescent reporter activity in general (Figures 3D, 4D, S2, S3).

Effects of overexpression of *AtCO* on the circadian rhythmicity in *L. gibba*

In Arabidopsis it was reported that an *AtCO* overexpressing line displayed no defect in the period length of leaf movement circadian rhythm, and a *co* loss of function allele also showed no changes in period length of *luc*-reported *CAB* expression (Ledger et al. 2001). On the other hand, it was reported that *AtCO* overexpression affected leaf and shoot development (Onouchi et al. 2000; Simon et al. 1996). Because *LgCOH1-ox* in *Lemna* plants showed a clear disruption of circadian rhythm, we checked the possibility that

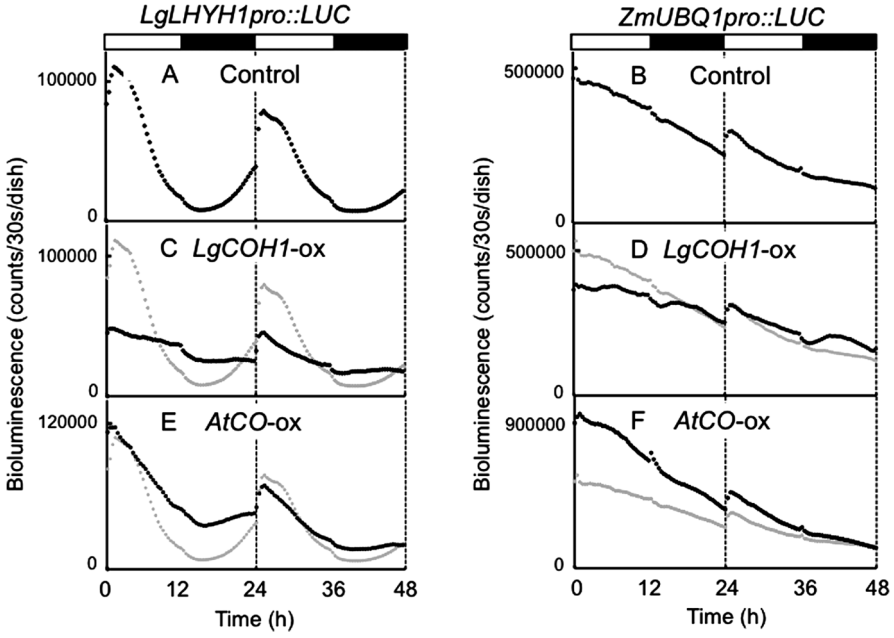

Figure 3. Aberrant bioluminescence rhythms caused by overexpression of *LgCOH1* and *AtCO* in *L. gibba* under 12 h light/12 h dark conditions. Luminescent data for *LgLHYH1pro::LUC* and *ZmUBQ1pro::LUC* expression patterns are shown in the left and right panels, respectively. Control vector (*pBI221ΔGUS*; A and B), *LgCOH1-ox* (C and D), or *AtCO-ox* (E and F) were introduced in the plants with each reporter. Bioluminescence profiles are shown as black dots. Traces for the control vector are also superimposed on C–F (gray dots). *L. gibba* plants cultured under 12 h light/12 h dark conditions were subjected to bombardment, and then entrained by a 12 h light/12 h dark cycle. The bioluminescence of the entrained plants was then measured for two cycles of the 12 h light/12 h dark cycle. Co-transfection assays were repeated at least three times. Typical bioluminescence traces are shown. Bioluminescent traces of duplicated experiments are shown in Figure S2. Black and open bar indicate dark and light, respectively.

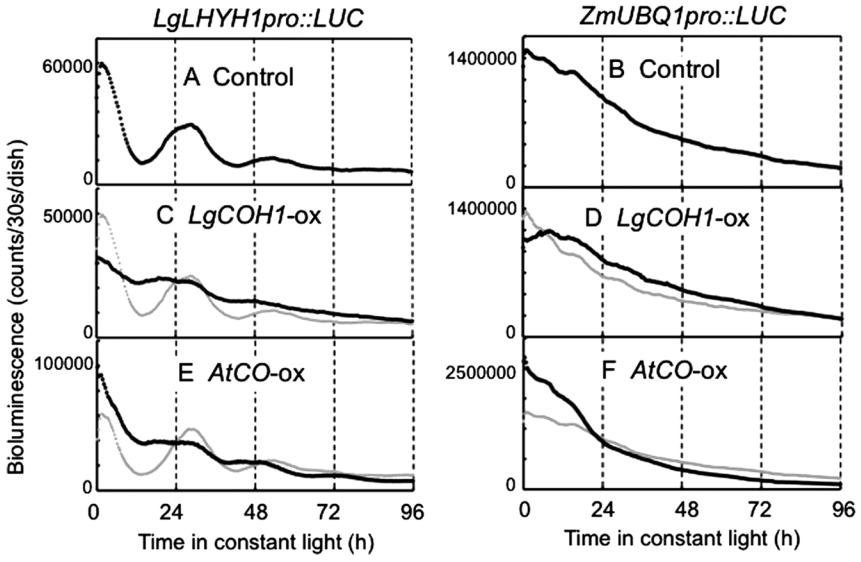

Figure 4. Aberrant circadian rhythms of a bioluminescent reporter caused by overexpression of *LgCOH1* and *AtCO* in *L. gibba*. *LgLHYH1pro::LUC* and *ZmUBQ1pro::LUC* expression patterns under constant light conditions are shown in the left and right panels, respectively. Control vector (*pBI221ΔGUS*; A and B), *LgCOH1-ox* (C and D), or *AtCO-ox* (E and F) were introduced in the plants with each reporter, and the bioluminescence profiles are shown as black dots. The traces for the control vector are also superimposed on C–F (gray dots). *L. gibba* plants cultured under 12 h light/12 h dark conditions were subjected to bombardment, and then entrained by a 12 h light/12 h dark cycle. The bioluminescence of the entrained plants was then measured under constant light conditions. Co-transfection assays were repeated at least three times. Typical bioluminescence traces are shown. Bioluminescent traces of duplicated experiments are shown in Figure S3.

overexpression of *AtCO* as well as *LgCOH1* affected circadian rhythmicity in *Lemna* plants. We introduced an effector construct that expressed *AtCO* under the CaMV 35S promoter with the bioluminescent reporter into *L. gibba*. *AtCO-ox* damped the circadian bioluminescence rhythm, as did *LgCOH1-ox* (Figures

4E, S3). However, there were some differences between the effects of *AtCO-ox* and those of *LgCOH1-ox*. First, in contrast to the complete bioluminescence arrhythmia induced by *LgCOH1-ox*, some traces of the circadian bioluminescence rhythm were observed when the *AtCO-ox* effector was introduced (Figures 4E, S3). Second, bioluminescence levels at Time 0 h (light-on) in *AtCO-ox* plants were high and sharply decreased until 12 h, as in control, implying that *AtCO-ox* left some traces of circadian rhythmicity (Figures 4E, S3). *LgCOH1-ox* showed a flat bioluminescence trace after light-on (Figures 4C, S3). Under 12 h light/12 h dark conditions, the bioluminescence of *AtCO-ox* showed a clear diurnal rhythm while the bioluminescence of *LgCOH1-ox* showed almost no response to light and dark (Figures 3C, E, S2). Therefore, *AtCO-ox* affected the circadian rhythm of *Lemna* plants, though its effects were weaker than those of *LgCOH1-ox*. Bioluminescence traces of *ZmUBQ1pro::LUC* appeared to be unaffected by co-transfection with *AtCO-ox*, suggesting that this overexpression effector was unlikely to affect bioluminescent reporter activity in general, like *LgCOH1-ox* (Figures 3F, 4F, S2, S3). We also confirmed that overexpression of *LgCOH1* and *AtCO* resulted in arrhythmia of circadian rhythmicity of the evening-specific *Arabidopsis PRR1* promoter activity, suggesting that these effectors generally affected the circadian expression of any phases (Figure S5).

Effects of overexpression of truncated LgCOH1 and AtCO on circadian rhythmicity

Plant COL family members have conserved zinc finger B-boxes near the N-terminus and a CCT domain near the C-terminus. In plant transcription factors, B-boxes are thought to be involved in protein–protein interactions (Khanna et al. 2009). It was shown that the CCT domain of the AtCO protein is involved in nuclear localization and DNA binding (Robson et al. 2001; Tiwari et al. 2010). To test which domain gave rise to the disruption of circadian rhythm in the overexpression experiments, we constructed a set of effectors overexpressing truncated protein. *LgCOH1-Zf-ox* and *AtCO-Zf-ox* were designed for overexpression of the N-terminal zinc fingers and middle region (extending from the first Met to a conserved asparagine N-terminal to the CCT domain- Asp286 for LgCOH1 and Asp305 for AtCO). *LgCOH1-CCT-ox* and *AtCO-CCT-ox* were designed for overexpression of the CCT domain and the remaining C-terminal region.

Under constant light conditions, *LgCOH1-Zf-ox* and *LgCOH1-CCT-ox* damped circadian bioluminescence rhythms as did *LgCOH1-ox* (Figures 5A, B, S4). These results indicate that the zinc finger region and the CCT domain both affect circadian rhythm. However, the effect of *LgCOH1-Zf-ox* and *LgCOH1-CCT-ox* appeared to be weaker than that of *LgCOH1-ox*. The sharp decrease of bioluminescence levels were observed in *LgCOH1-Zf-ox* and *LgCOH1-CCT-ox* as well as control (Figures

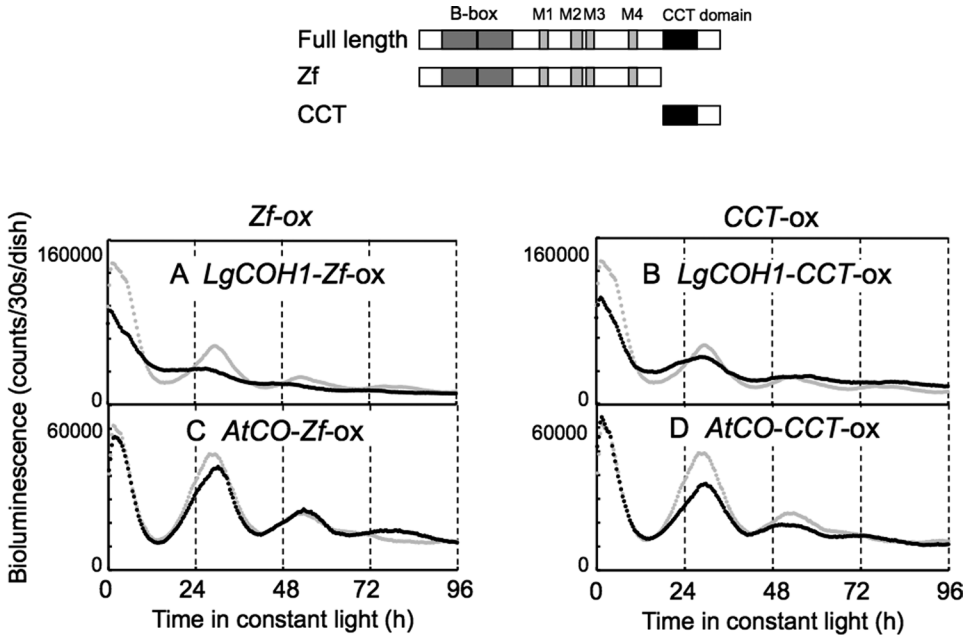

Figure 5. Effects of overexpression of truncated *LgCOH1* and *AtCO* on the circadian rhythmicity of *L. gibba*. *LgLHYH1pro::LUC* expression patterns under constant light conditions are shown. *LgCOH1-Zf-ox* (A), *LgCOH1-CCT-ox* (B), *AtCO-Zf-ox* (C) or *AtCO-CCT-ox* (D) were introduced in the plants with the *LgLHYH1pro::LUC* reporter. Co-transfection assays were the same as those described in Figure 4. Bioluminescence profiles are shown as black dots. The traces for the control vector are also superimposed on A–D (gray dots). Co-transfection assays were repeated at least three times. Typical bioluminescence traces are shown. Schematic diagrams of the full-length, Zf and CCT are shown. Bioluminescent traces of duplicated experiments are shown in Figure S4.

5A, B, S4). These results suggest that both the zinc finger region and the CCT domain are required for the complete damping of circadian bioluminescence rhythms in the overexpression experiments. By contrast, the bioluminescence traces of *LgLHYH1pro::LUC* co-transfected with *AtCO-Zf-ox* and *AtCO-CCT-ox* showed circadian rhythms relatively similar to the control (Figures 5C, D, S4). Bioluminescence levels in *AtCO-Zf-ox* and *AtCO-CCT-ox* cells increased again after attaining trough levels, and circadian bioluminescence rhythms were observed. These results imply that the circadian functions of the zinc finger region and the CCT domain of *AtCO* differ from those of *LgCOH1*.

Discussion

In this report, we demonstrated the properties and functions of *Lemna CO* homologues. Phylogenetic analysis of COL family members using B-box and CCT domain sequences classified LgCOH1 and LaCOH1 into Group I (Figure 1B; Griffiths et al. 2003). The middle region of LgCOH1 has M1, M2, M3 and M4 domains, which are specific to Group Ia members, including AtCO (Figure 1A; Griffiths et al. 2003). Conservation of four small motifs supports the idea that LgCOH1 is a member of Group Ia and similar to AtCO. LaCOH1 lacks M1, but its overall amino acid sequence was 70% identical to that of LgCOH1. Overall similarity of LaCOH1 to LgCOH1 suggests that LaCOH1 is also a member of Group Ia.

The expression of *LgCOH1* was upregulated by light and remained at a high level during daytime (Figure 2). The expression level of *AtCO* is high in late daytime and nighttime, which is important for the measurement of day length (Hayama and Coupland 2003; Suárez-López et al. 2001). The expression pattern of *LgCOH1* was different from that of *AtCO* (Figure 2). Therefore, LgCOH1 is likely to have a different physiological function from AtCO, although its sequence is highly similar to that of *AtCO*. COL family members are also suggested to function in many aspects other than photoperiodic flowering in plants (Datta et al. 2006; Ledger et al. 2001). Overexpression of *COL1* and *COL2* in Arabidopsis has little effect on flowering time, but overexpression of *COL1* shortens the period of circadian leaf movement and *cab2::luc* expression rhythm, suggesting that the *COL1* gene may function in the photoperiodic flowering pathway (Ledger et al. 2001). *COL3* in Arabidopsis functions in red light signaling (Datta et al. 2006). The diurnal expression pattern of *LgCOH1* and the effect of overexpression on the circadian rhythm suggest that it may play a novel role in the circadian oscillator. We also applied an RNAi effector of *LgCOH1* to the co-transfection assay in order to knockdown *LgCOH1*, but no significant differences were observed in circadian bioluminescence traits (data not shown). Genetic redundancy among *COL* family members in the regulation of the circadian clock may explain the results of these LgCOH1 experiments.

Overexpression of *AtCO* as well as *LgCOH1* led to disappearance of circadian *LgLHYH1pro::LUC* bioluminescence rhythm, suggesting that *COL* family members have a role in the plant circadian clock system (Figures 3, 4, S2, S3). It was reported that a *CaMV 35S::AtCO* Arabidopsis line, which expressed *AtCO* at a high level, displayed no defect in the circadian rhythms of leaf movement and *luc*-reported *CAB* expression (Ledger et al. 2001). These phenotypic differences in circadian rhythm might be due to differences in the accumulation of AtCO protein between *Lemna AtCO-ox* cells and the Arabidopsis *CaMV 35S::AtCO* line. AtCO might be produced at much higher levels in the *Lemna* cells that were transfected with a high copy number of the transgene by particle bombardment than in Arabidopsis cells in the stable transgenic plant with one or two copies of the transgene in its genome.

LgCOH1-ox cells showed a more severe phenotype than *AtCO-ox* cells. Bioluminescence of *AtCO-ox* cells showed an acute reduction during the light period under 12 h light/12 h dark conditions, as seen in the control, whereas *LgCOH1-ox* cells showed an almost flat expression level throughout the day-night cycle (Figures 3, S2). The differences in circadian rhythm between *LgCOH1-ox* and *AtCO-ox* might be due to different responses to light. Because the expression of *LgCOH1* is upregulated by light in *Lemna*, LgCOH1 is likely to function in light signaling. On the other hand, *AtCO* is highly expressed in the nighttime (Suárez-López et al. 2001). The difference in expression pattern may reflect the phenotypic difference of circadian rhythms of overexpressing cells. There is a possibility that the protein stabilities of LgCOH1 and AtCO may be different, and that these differences could cause phenotypic differences. It is known that light stabilizes AtCO, and the protein is degraded in darkness (Valverde et al. 2004). In our co-transfection assays using *Lemna* plants, AtCO protein that was produced in transfected cells might be degraded in the 12 h dark period, and the bioluminescence traces were similar to those of control plants (Figures 3, S2). LgCOH1 protein might be more stable than AtCO protein in darkness.

The overexpression of a construct with the zinc finger region including the two B-boxes (*LgCOH1-Zf-ox*) and or a construct with the CCT domain (*LgCOH1-CCT-ox*) damped the circadian bioluminescence rhythms as well as the *LgCOH1-ox* under constant light conditions (Figures 5A, B, S4). Thus, both the regions may be involved in the circadian system independently. This hypothesis is supported by the observation that the effects of *LgCOH1-Zf-ox* and *LgCOH1-CCT-ox* on the damping appeared to be weaker than that of *LgCOH1-*

ox. The zinc finger region of AtCO has been implicated in protein–protein interactions and its CCT domain has been shown to be involved in nuclear localization and DNA binding (Khanna et al. 2009; Robson et al. 2001; Tiwari et al. 2010). Thus, the zinc finger region of LgCOH1 might be involved in the regulatory network of the circadian system through protein–protein interactions, and the CCT domain of LgCOH1 might be involved in modification of clock gene expression. The CCT domain is also conserved in TOC1 and the PRR family, both of which function as key clock components in Arabidopsis. It has been shown that TOC1 binds directly to DNA through its CCT domain, like AtCO, and DNA-binding is necessary for TOC1 function as the clock component (Gendron et al. 2012). Further studies about genes which are directly targets of *LgCOH1* will uncover functional roles of the *COL* family in the circadian rhythm regulatory network.

Acknowledgements

We thank Dr. Ko Shimamoto for the gift of a maize *UBIQUITIN1*-containing vector, and Ms. Midori Sato for technical assistance. Analysis of DNA sequencing was conducted with CREST-Akita Plant Molecular Science Satellite Laboratory in Life Research Support Center of Akita Prefectural University. This research was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (11233203, 15GS0308, and COE 13CE2005 to T.K. and T.O.; 15031215 and 17370088 to T.O.). K. M. was supported by a JSPS fellowship for young scientists (1500948).

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Table S1. Primers for gene isolation and qRT-PCR

Gene	Degenerate primers for gene isolation	Primers for qRT-PCR[a]
LgCOH1	TGYAARGCIGAYGCIGCIGCIYTITGY GCRTAICKDATIGTYTTYTCRAAYTT	TCGTGGTTGATTCCAAACCCTAGC ATCCAGATAACGATCCACCTCGGA CCGGAAGGAAAGGGAGGAGAGTATCTAT (FAM)
LaCOH1	GCIGAYGCIGCITAYYTITGY CKYTTIGCRAAICKICCITT	
LgLHYH1pro		GGCCACACAAGCTTGTGAAAATACCGAAAC ACATGTCTCCATGGGAGCAATTCGCA
LgUBQ		TTCGGACACCATCGACAACGTCAA AGGTGGAGAGTGGATTCCTTCTGA CCAAGATCCAGGATAAGGAGGGAATTCC (FAM)

[a] Third primer sequences are TaqMan probes, and the fluorescent dye (FAM) is shown in parenthesis.

Table S2. Primers for overexpression constructs

Target genes	Target regions	Primer Sequences
LgCOH1	full-length	ATGGGAAGAGAAGAGGCGGGAGAGGGAGTGGAAGG
		TCAAAATGAAGGAACCAGGCCATAGCCAGACGCAG
	Zinc-finger	ATGCAGATCTTATGGGAAGAGAAGAGGCGGGAGAG
		ATGCAGATCTTCAATCCAGCCCCATGGCCGCCTGA
	CCT-motif	ATGCAGATCTTATGCGGGAGGCTCGAGTGATGAGG
		TCAAAATGAAGGAACCAGGCCATAGCCAGACGCAG
AtCO	full-length	ATGCAGATCTATGTTGAAACAAGAGAGTAACGA
		ATGCAGATCTTATCAGAATGAAGGAACAATCCC
	Zinc-finger	ATGCAGATCTTATGTTGAAACAAGAGAGTAACGAC
		ATGCAGATCTTCAGTCCATTGGACTGAGTTGTGTT
	CCT-motif	ATGCAGATCTTATGAGAGAAGCCAGGGTCCTGAGA
		ATGCAGATCTTATCAGAATGAAGGAACAATCCC

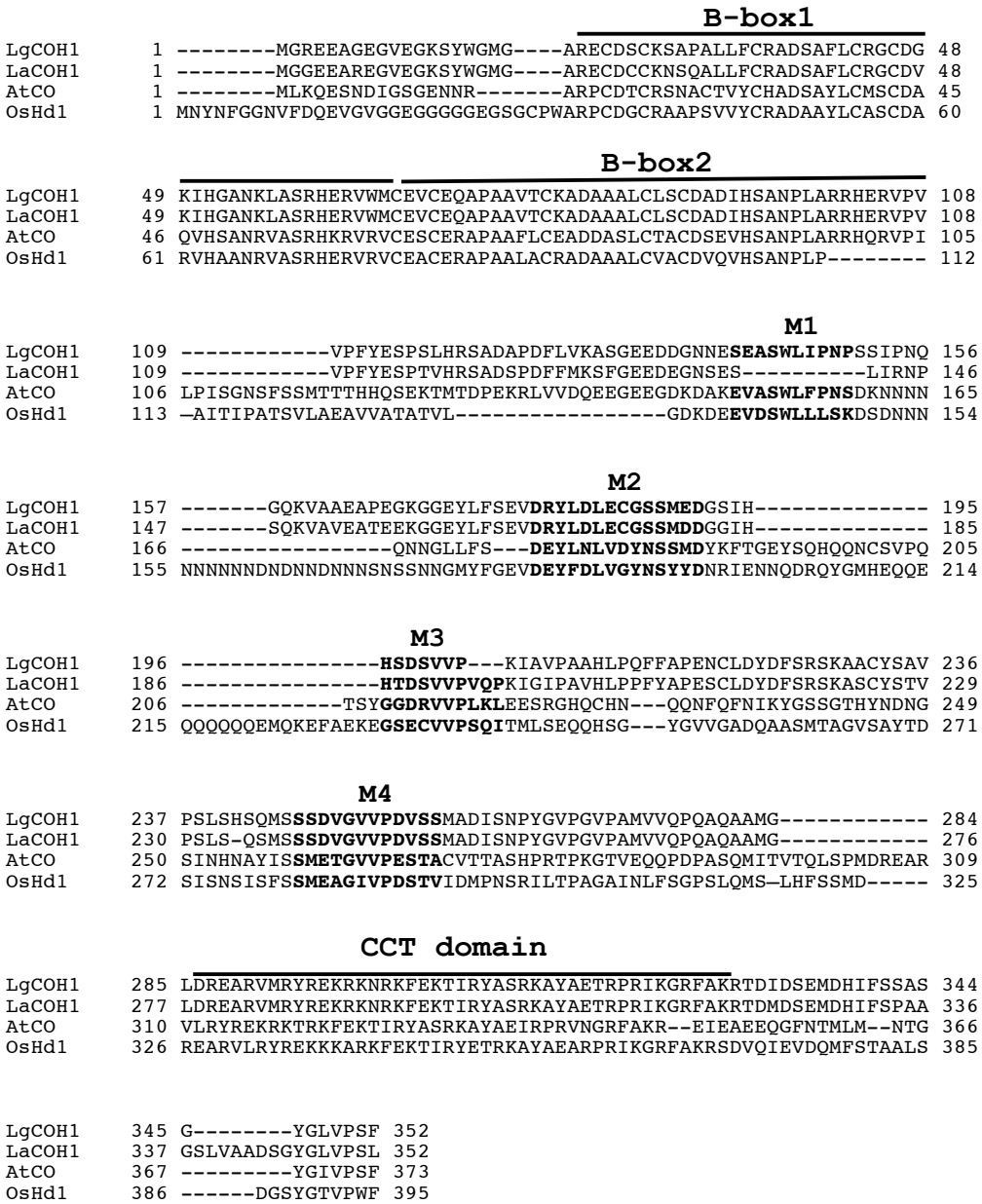

Figure S1. Sequence alignments of LgCOH1, LaCOH1, AtCO and OsHd1. Amino-acid sequences were aligned using ClustalW method, and alignment was modified to match highly homologous regions. Hyphens indicate gaps inserted to maximize the sequence homology. Bold horizontal parts indicate highly homologous regions.

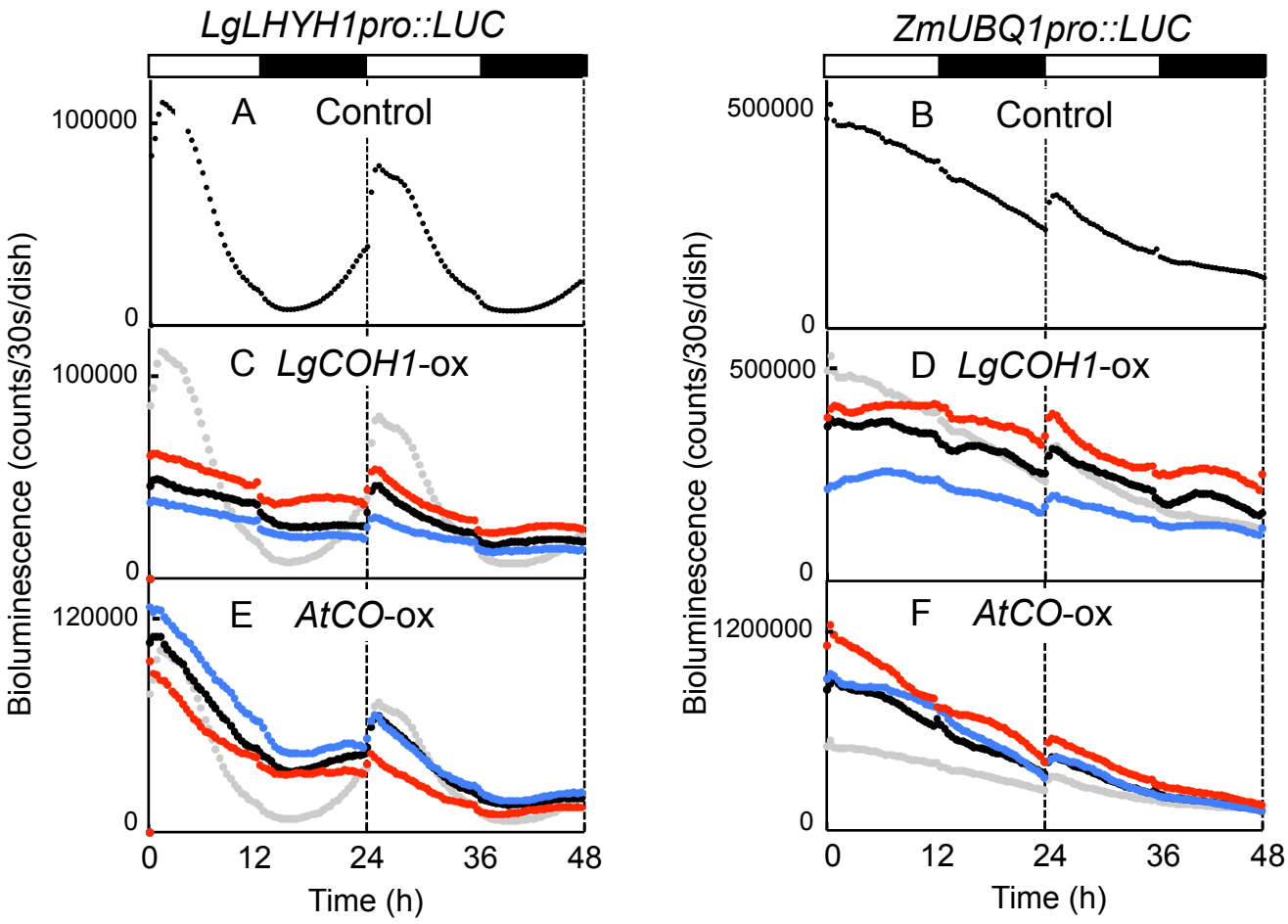

Figure S2. Aberrant bioluminescence rhythms caused by overexpression of *LgCOH1* and *AtCO* in *L. gibba* under 12 h light/12 h dark conditions. Bioluminescence profiles in Figure 3 are shown as black dots and other data of duplicated experiments are shown as red and blue dots. Traces for the control vector are also superimposed on C, D, E and F (gray dots).

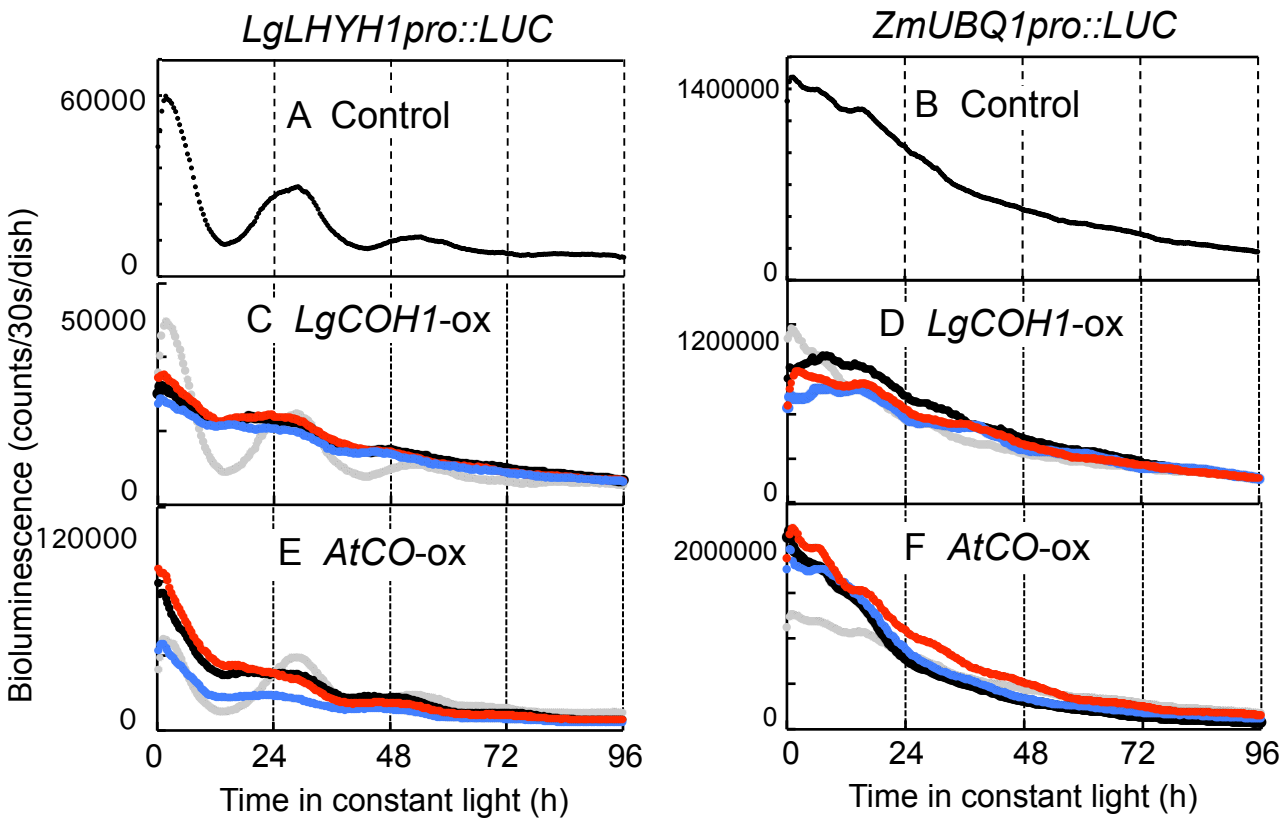

Figure S3. Aberrant circadian rhythms of a bioluminescent reporter caused by overexpression of *LgCOH1* and *AtCO* in *L. gibba*. Bioluminescence profiles in Figure 4 are shown as black dots and other data of duplicated experiments are shown as red and blue dots. Traces for the control vector are also superimposed on C, D, E and F (gray dots).

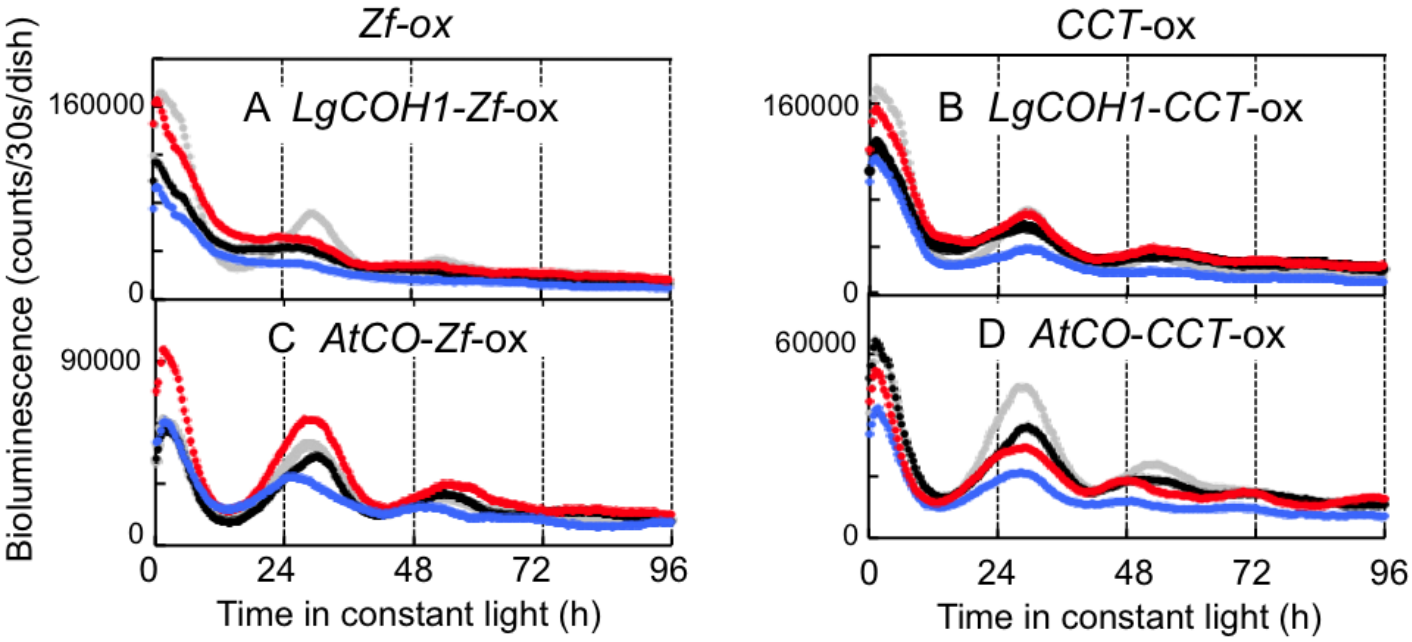

Figure S4. Effects of overexpression of truncated *LgCOH1* and *AtCO* on the circadian rhythmicity of *L. gibba*. Bioluminescence profiles in Figure 5 are shown as black dots and those of duplicated experiments are shown as red and blue dots. Traces for the control vector are also superimposed on A, B, C and D (gray dots).

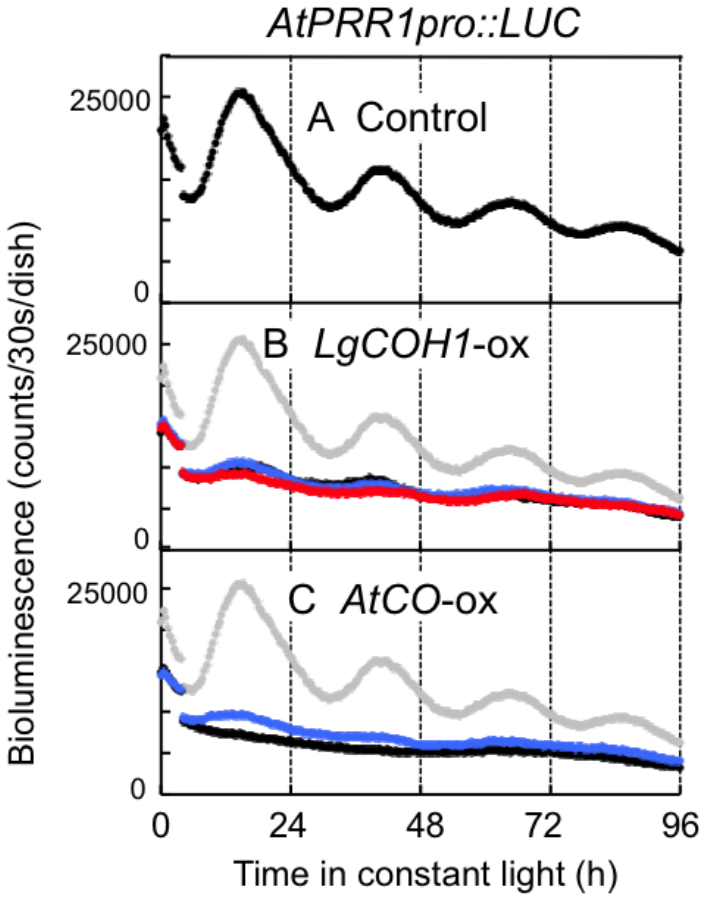

Figure S5. Aberrant circadian rhythms of an evening-specific bioluminescent reporter caused by overexpression of *LgCOH1* and *AtCO* in *L. gibba*. *AtPRR1pro::LUC* expression patterns under constant light conditions are shown. Control vector (*pBI221ΔGUS*; A), *LgCOH1-ox* (B), or *AtCO-ox* (C) were introduced in the plants with each reporter, and the bioluminescence profiles are shown as black, red and blue dots. The traces for the control vector are also superimposed on B and C (gray dots). *L. gibba* plants cultured under 12 h light/12 h dark conditions were subjected to bombardment, and then entrained by a 12 h light/12 h dark cycle. The bioluminescence of the entrained plants was then measured under constant light conditions. Co-transfection assays were repeated at least twice.