Title: Model-based analysis on the generation of the circadian rhythm of the bioluminescence reporter activities in duckweed

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Running title: Bioluminescence circadian rhythm of luciferase in duckweed

Abstract

Bioluminescence monitoring techniques are widely used for the study of gene expression dynamics in living plants. Monitoring bioluminescence from a luciferase gene under a circadian promoter is indispensable to examine the plant circadian system. The bioluminescence monitoring technique has been successfully applied to spaciotemporal analysis on circadian rhythms in duckweed plants. It has been reported that the luciferase gene under a constitutive promoter also shows a bioluminescence circadian rhythm in duckweed. The mechanism to generate the rhythm is unknown. Here we performed a model-based analysis to evaluate the generation machinery of the bioluminescence rhythm. Regarding the bioluminescence intensities of luciferase in the cells, we hypothesized the rhythmic factor at three aspects: luminescence efficiency, production rate, degradation rate. Theoretically, if the latter two are involved in the rhythm generation, the difference in luciferase stability affects the amplitude and the phase relation of the bioluminescence rhythm. The luciferase stability is irrelevant to these rhythm properties if the luminescence efficiency is only involved. We first simulated bioluminescence rhythms of two luciferases with different stabilities in association with each of the three rhythmic factors. Luciferase stabilities were set on the basis of the reported values of Emerald-luciferase and Emerald-luciferase-PEST. We then experimentally examined bioluminescence rhythms of those reporters driven under the *CaMV35S* promoter in a duckweed, *Lemna japonica*. Their circadian properties matched those obtained in the simulation for the luminescence efficiency. This supports the idea that cells in duckweed show a circadian rhythm in physiological conditions that are related with the luciferase enzyme reaction.

Key words: bioluminescence, circadian rhythm, duckweed, luciferase, PEST sequence

Physiology of plants is widely regulated by the day/night environmental cycles. The circadian system with a self-sustained clock is critically involved in the diel regulation (Greenham, McClung. 2015). Circadian regulation of gene expression is based on the circadian system. Monitoring of gene expression in living organisms is essential for the study of the circadian system (Millar et al. 1992). To analyze the dynamics of gene expression, a luciferase reporter with a circadian promoter is widely used due to its non-invasive nature and quantitative performance (Muranaka et al. 2013). Spatiotemporal analysis for the plant circadian system at a single cell level was achieved by using a bioluminescence monitoring system in duckweed that transfected with a luciferase reporter (Muranaka et al. 2016). It was reported that bioluminescence of luciferase driven by a constitutive promoter such as *CaMV35S* (the *CAULIFLOWER MOSAIC VIRUS 35S* promoter) showed a circadian rhythm in various duckweeds (Lemnaceae) (Muranaka et al. 2015). The mechanism to generate the rhythm is unknown; it has been suggested that physiological conditions associated with the luciferase reaction in cells mediates the rhythm post-transcriptionally (Watanabe et al. 2021, Watanabe et al. 2023). In this scenario, the bioluminescence rhythm appears even when the luciferase amount is constant in cells. To address how the bioluminescence rhythm is generated, we performed a model-based analysis on circadian rhythms of bioluminescence of two luciferases with different stabilities. Theoretically the stability is associated with circadian properties such as amplitude and phase relation when the production- and/or degradation rates of luciferase are rhythmic (Lück et al., 2014) . We experimentally evaluated the scenarios of rhythm generation by analyzing bioluminescence rhythms of a stable luciferase and an unstable luciferase.

We used *Lemna japonica* 5512 (*Lemna minor* 5512 in previous studies: the Rutgers Duckweed Stock Cooperative (http://www.ruduckweed.org/)) for the experiments. Duckweed plants were maintained in NF medium with 1% sucrose under constant light conditions in a temperature-controlled room (25°C), as previously described (Muranaka et al. 2015). As circadian bioluminescence reporters, we used *pUC18-CaMV35S::ELUC* (*CaMV35S::ELUC*) and *pUC18-CaMV35S::ELUC-PEST* (*CaMV35S::ELUC-PEST*). *ELUC* encodes the Emerald luciferase (ELuc) from the Brazilian click beetle (*Pyrearinus termitilluminans*) (TOYOBO; Nakajima et al. 2010). These reporters are modified versions of *pUC18-CaMV35S::LUC+*, which has been described previously (Muranaka et al. 2013). Transfection of a luciferase reporter by particle bombardment, and bioluminescence monitoring of the transfected duckweed plants were performed as described previously (Muranaka et al. 2015). The luminescence of a sample dish was detected every 20 min using a photomultiplier tube. Single-cell bioluminescence imaging using an EM-CCD camera (ImagEM C9100-13; Hamamatsu Photonics) with a camera lens (VS-50085/C 0.85/50 mm; VS Technology) was carried out as described previously (Muranaka and Oyama 2016). Bioluminescence images (16-bit TIFF format) of plants 3 days after gene transfection were captured with the ImagEM camera (cooled at −80ºC) at an EM gain of 1,200 with 3, 5, 15, 30, 60-s exposure after at least 4 min dark treatment for autofluorescence decay. Time

series analysis was implemented using Python 3.8.8 (NumPy 1.20.1, SciPy 1.6.2). The data analysis codes are available at https://github.com/ukikusa/circadian_analysis v0.9. Further codes and data are available on request from the corresponding authors.

In order to approach the rhythmic factor(s) to generate the bioluminescence rhythm of luciferase driven by a constitutive promoter, we first performed a model-based analysis. Regarding the bioluminescence intensities of cells expressing a luciferase enzyme, we focused on the following three aspects: luminescence efficiency, production rate, degradation rate. The structure of the bioluminescence system is shown in Figure 1A; luminescence emission ($L(t)$) is the output and the luciferase amount ($x(t)$) is the state variable. A circadian rhythm in the luminescence efficiency can confer the rhythmicity of bioluminescence even when the luciferase amount is constant. A circadian rhythm in the production rate or the degradation rate results in a circadian rhythm of the luciferase amounts that consequently generates a bioluminescence rhythm. The stability of the luciferase enzyme can severely affect the phase and amplitude of the bioluminescence rhythm when the luciferase amount is associated with the rhythm (Lück et al., 2014). In order to compare behavior of rhythmic outputs between luciferases with different stabilities (StLUC and UnstLUC), we analyzed three simple cases in the simulation model for the bioluminescence system. We set two luciferases with degradation rates $\gamma_{\mathrm{StLUC}} = 0.07\ \mathrm{h}^{-1}$ and $\gamma_{\mathrm{UnstLUC}} = 0.23\ \mathrm{h}^{-1}$. These values for γ_{StLUC} and $\gamma_{\mathrm{UnstLUC}}$ correspond to those of ELUC (Half-life: 10 h) and ELUC-PEST (Half-life: 3 h), respectively (Yasunaga et al. 2015). ELUC-PEST is a short-life type ELUC containing a 40-aa PEST sequence in the C-terminus. Protein amounts of these two luciferases differ by 3.3 folds in their steady states (Figure 1B; SIM B in Table 1). If the rhythmic factor is simply set in the luminescence efficiency, the amplitudes of bioluminescence rhythms are different, but those of relative values are the same between StLUC and UnstLUC (Figure 1C; SIM C in Table 1). In this case, their phases are the same. If the rhythmic factor is simply set in the production rate, the amplitudes of bioluminescence rhythms are similar between the two luciferases (Figure 1D left; SIM D in Table 1). Regarding the amplitudes of relative values, UnstLUC shows a larger magnitude than StLUC (Figure 1D right; Table 1). A larger phase delay occurs in the rhythm of StLUC (SIM D in Table 1). As shown in Figure 1E and Table 1 (SIM E), the rhythmic factor in the degradation rate results in circadian rhythms for both luciferases similarly to those generated by the rhythmic production rate.

Next, we experimentally compared behavior of bioluminescence rhythms between *CaMV35S::ELUC* (*ELUC*) and *CaMV35S::ELUC-PEST* (*ELUC-PEST*). Bioluminescence intensities of individual cells were measured through the single-cell bioluminescence imaging (Figure 2A). Intensity values of both luciferase reporters show a log-normal distribution. The geometrical mean for *ELUC* was 4.6 folds as large as that for *ELUC-PEST*. This difference was comparable to the result in the simulation (Table 1). Bioluminescence under constant light were monitored for both luciferases (Figure 2B). Bioluminescence intensities of both reporters showed circadian rhythms. While

bioluminescence intensities of *ELUC-PEST* gradually decreased, circadian rhythms of relative values matched those for *ELUC* (Figure 2B and 2C; EXP in Table 1). There was a phase difference between the two reporters when compared at the third trough (Figure 2C; EXP in Table 1). However, the first trough time was almost the same between *ELUC* and *ELUC-PEST,* suggesting that these two luciferase reporters showed a similar phase relation (Figure 2C). In sum, the matching of the bioluminescence rhythms of relative values with a similar phase is the characteristics of the results in SIM C (Figure C; Table 1). Thus it is concluded that the circadian rhythm of the luciferase expressed under the control of CaMV*35S* promoter is generated at the step of luminescence efficiency for the luciferase enzyme reaction. This conclusion does not exclude the possibility that a circadian rhythm in the production rate or the degradation rate may result in the bioluminescence rhythm, but its magnitude should be much smaller than that of luminescence efficiency.

The mechanism to generate the circadian rhythm at the step of luminescence efficiency is unknown, but it is clear that the physiological conditions in the cytoplasm that are associated with the luciferase reaction show a circadian rhythm in duckweed. Then bioluminescence of any luciferase reporter is affected by the circadian changes in the luminescence efficiency. In duckweed, various luciferase reporters driven by a circadian promoter of a specific phase has been used for the studies of the circadian rhythm (Serikawa et al. 2008, Isoda et al. 2022, Watanabe et al. 2023). Bioluminescence rhythms of these reporters include at least two circadian components: the production rate and the luminescence efficiency. Therefore, to analyze the circadian behavior of the promoter activities, it should be necessary to remove the circadian component of luminescence efficiency.

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Figure legends

Figure 1. Simulation of bioluminescence behavior in the three conditions for rhythmic factor

(A) Model. The amount of LUC ($x(t)$) is the state variable and luminescence intensity ($L(t)$) is the output. The production rate coefficient, the degradation rate coefficient, the coefficient of luminescence efficiency are marked in blue, red, green, respectively. k and γ are the mean rate coefficients. Time modulation of $x(t)$ and $L(t)$ is described by cosine-shaped functions with relative amplitudes A_{prod}, A_{deg}, and A_{eff} (values between 0 and 1). (B-E) Simulation of the model. Comon parameter values: $\omega = 2\pi/26$, $\varphi_{\mathrm{prod}} = \varphi_{\mathrm{deg}} = \varphi_{\mathrm{eff}} = 0$, $k = 1\ \mathrm{h}^{-1}$, $\gamma_{\mathrm{StLUC}} = 0.07\ \mathrm{h}^{-1}$ and $\gamma_{\mathrm{UnstLUC}} = 0.23\ \mathrm{h}^{-1}$. A solid blue line and a dashed orange line indicate StLUC and UnstLUC, respectively. Simulation of the luciferase amount without the rhythm in the production rate and degradation rate (SIM B; $A_{\mathrm{prod}} = A_{\mathrm{deg}} = A_{\mathrm{eff}} = 0$) is shown in (B). Simulation of luminescence intensities and relative values with a circadian rhythm in the luminescence efficiency (SIM C; $A_{\mathrm{eff}} = 0.25$, $A_{\mathrm{prod}} = A_{\mathrm{deg}} = 0$) (C), and the production rate (SIM D; $A_{\mathrm{prod}} = 0.25$, $A_{\mathrm{deg}} = A_{\mathrm{eff}} = 0$) (D), and the degradation rate (SIM E; $A_{\mathrm{deg}} = 0.25$, $A_{\mathrm{prod}} = A_{\mathrm{eff}} = 0$) (E). Relative values are the normalized values by the 26-h moving average at each time point. A two-headed arrow for a rhythm represents the twice of the amplitude and the point of the third trough.

Figure 2. Bioluminescence of *CaMV35S::ELUC* and *CaMV35S::ELUC-PEST*

(A) Frequency distributions for cellular bioluminescence intensities of *ELUC* (blue) and *ELUC-PEST* (orange). Geometric means are indicated in the graph. Both histograms show a log-normal distribution. (Kolmogorov–Smirnov test, $P > 0.05$; n = 225 (*ELUC*), n = 69 (*ELUC-PEST*)). Note that the x-axis is on a logarithmic scale. (B) Circadian rhythms of bioluminescence intensities (left) and relative values (right) for *ELUC* (blue lines) and *ELUC-PEST* (orange lines) under constant light conditions. Relative values are the normalized values by the 26-h moving average at each time point. Plants subjected to gene transfection were entrained to two 12-h dark/12-h light cycles and then released into constant light conditions for bioluminescence monitoring by a photomultiplier tube. Time-series data of three replicates in an experiment among three independent experiments are shown. (C) Means of relative values of all 9 samples in the three experiments are shown. A two-headed arrow for a rhythm represents the twice of the amplitude and the point of the third trough.

Table 1. Properties of circadian rhythms in each simulation and the experimental results

Analysis	Mean light intensity			Relative amplitude			Phase property [h]		
	StLUC	UnstLUC	St / Unst	StLUC	UnstLUC	St / Unst	StLUC	UnstLUC	St − Unst
SIM B	14.28	4.35	3.28	NA	NA	NA	NA	NA	NA
SIM C	14.28	4.35	3.28	0.22	0.22	1.00	0	0	0
SIM D	14.28	4.35	3.28	0.06	0.15	0.41	−5.24	−3.27	−1.97
SIM E	14.31	4.41	3.24	0.06	0.15	0.41	7.77	9.54	−1.77
EXP [a]	15.75[b]	3.4[b]	4.64	0.18±0.03	0.22±0.04	0.83	0[c]	0.78[c]	−0.78

[a]Experimental data shown in Figure 2C are summarized for StLUC (ELUC; n = 9) and UnstLUC (ELUC-PEST; n = 9). [b]The unit is 1000 photons min^{-1}. [c]The mean time at the third trough for StLUC is set to 0 h.

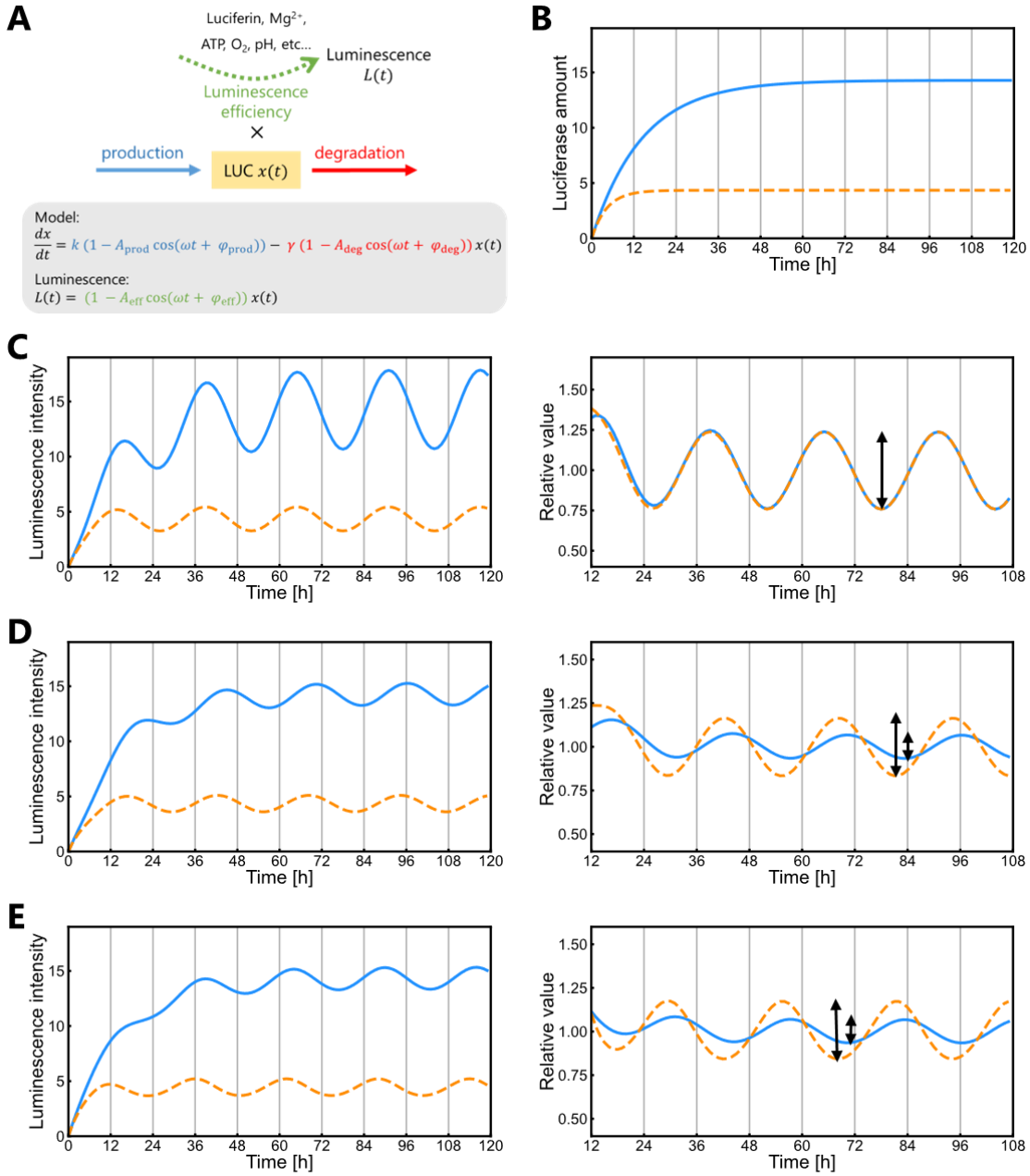
A
Luciferin, Mg2+,
ATP, O2, pH, etc...
Luminescence
L(t)
Luminescence
efficiency
×
production
LUC x(t)
degradation
Model:
dx/dt = k (1 − A_prod cos(ωt + φ_prod)) − γ (1 − A_deg cos(ωt + φ_deg)) x(t)
Luminescence:
L(t) = (1 − A_eff cos(ωt + φ_eff)) x(t)
B
Luciferase amount
Time [h]
C
D
E
Luminescence intensity
Relative value
Time [h]

Figure 1

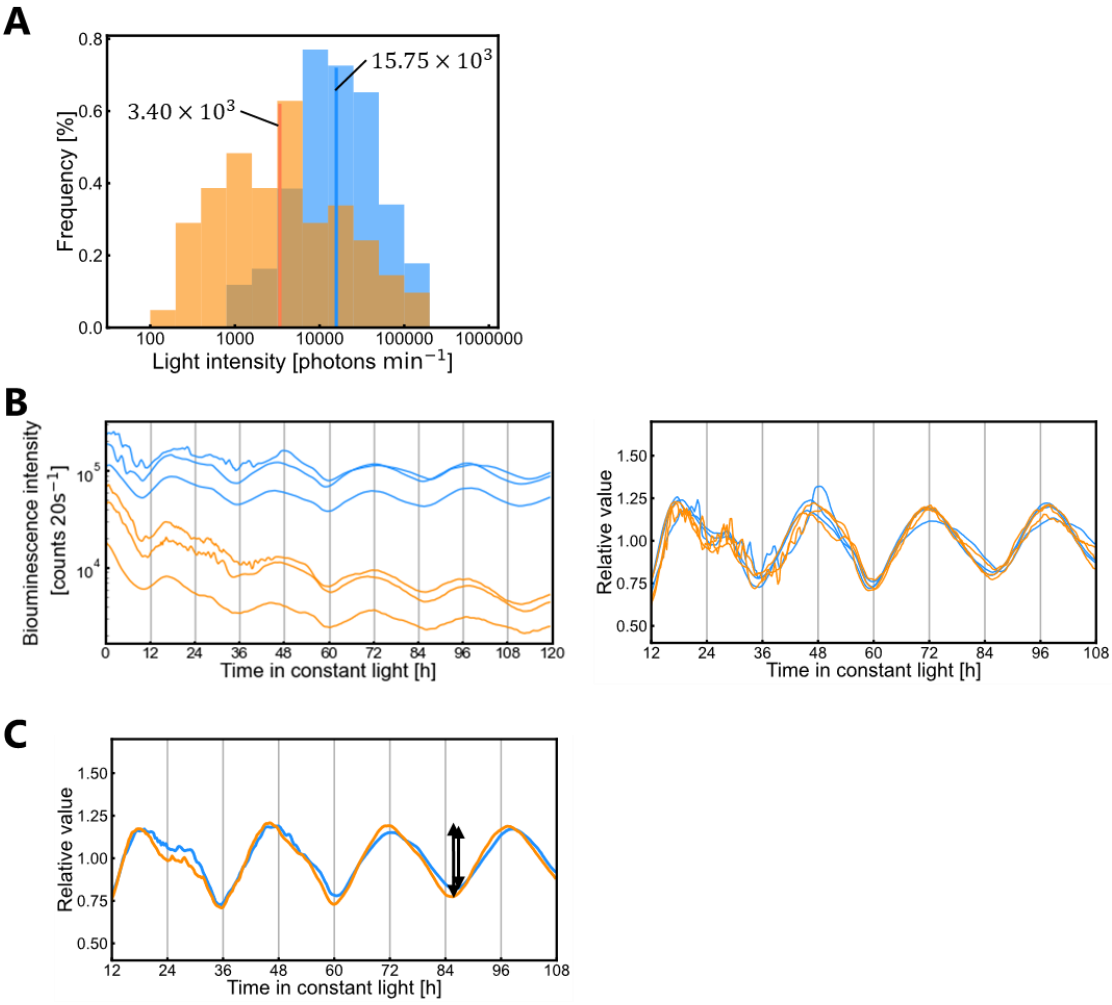
A
0.8
0.6
0.4
0.2
0.0
Frequency [%]
3.40×10^3
15.75×10^3
100
1000
10000
100000
1000000
Light intensity [photons min^{-1}]
B
Biouminescence intensity [counts 20s^{-1}]
10^5
10^4
0
12
24
36
48
60
72
84
96
108
120
Time in constant light [h]
Relative value
1.50
1.25
1.00
0.75
0.50
12
24
36
48
60
72
84
96
108
Time in constant light [h]
C
Relative value
1.50
1.25
1.00
0.75
0.50
12
24
36
48
60
72
84
96
108
Time in constant light [h]

Figure 2