Monitoring Cellular Gene Expression in a Plant Body

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Gene expression is a fundamental cellular process and expression dynamics are of great interest in life science. We succeeded in monitoring cellular gene expression in a duckweed plant, Lemna gibba, using bioluminescent reporters. Using particle bombardment, epidermal and mesophyll cells were transected with the luciferase gene (luc+) under the control of a constitutive [Cauliflower mosaic virus 35S (CaMV35S)] and a rhythmic [Arabidopsis thaliana CIRCADIAN CLOCK ASSOCIATED 1 (AtCCA1)] promoter. Bioluminescence images were captured using an EM-CCD (electron multiply charged couple device) camera. Luminescent spots of the transfected cells in the plant body were quantitatively measured at the single-cell level. Luminescence intensities varied over a 1,000-fold range among CaMV35S::luc+-transfected cells in the same plant body and showed a log-normal-like frequency distribution. We monitored cellular gene expression under light–dark conditions by capturing bioluminescence images every hour. Luminescence traces of ≥50 individual cells in a frond were successfully obtained in each monitoring procedure. Rhythmic and constitutive luminescence behaviors were observed in cells transfected with AtCCA1::luc+ and CaMV35S::luc+, respectively. Diurnal rhythms were observed in every AtCCA1::luc+-introduced cell with traceable luminescence, and slight differences were detected in their rhythmic waveforms. Thus the single-cell bioluminescence monitoring system was useful for the characterization of cellular gene expression in a plant body.

Keywords: Bioluminescence • Gene expression • Lemna gibba • Luciferase • Particle bombardment • Single cells.

Abbreviations: CAB3, Chl a/b-binding protein 3; CaMV35S, Cauliflower mosaic virus 35S; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; EM-CCD, electron multiply charged couple device; GFP, green fluorescent protein; GUS, β-glucuronidase; ROI, region of interest.

Introduction

Changes in gene expression are a fundamental cellular behavior, and information about expression dynamics is critical to analyses for cellular processes. Owing to its non-invasive nature and quantitative performance, bioluminescence monitoring using luciferase reporter genes is a major tool for analyzing the dynamics of gene expression in living organisms. Using transgenic tobacco seedlings, real-time monitoring of gene expression was first developed in plants (Millar et al. 1992). The in vivo bioluminescence system has been efficiently used in research on the circadian clock, which requires observation over at least several days to characterize rhythmic phenomena (Ishiura et al. 1998, Welsh et al. 2005). The circadian clock, a self-sustained oscillator with a period of approximately 24 h, is ubiquitously found in organisms. Circadian systems are synchronized to day–night cycles and control various biological phenomena to adapt cells and organisms to diurnal environmental changes (Dunlap et al. 2005). In studies of plant circadian systems, monitoring luciferase expression driven by circadian-controlled promoters has been successfully applied to plant bodies as well as cultured cells of model plants such as Arabidopsis, tobacco and rice. (Millar et al. 1992, Anderson et al. 1994, Sugiyama et al. 2001, Nakamichi et al. 2004). In addition to being used to generate stable transgenic plants, transient gene expression techniques such as particle bombardment of plant bodies or polyethylene glycol (PEG)-mediated transfection of protoplast cells have been developed for the purpose of monitoring bioluminescence rhythms (Miwa et al. 2006, Serikawa et al. 2008, Kim et al. 2010). These transient expression systems can be applied to any plant species and allow functional analysis of circadian clock-related genes by co-introduction of effectors carrying over-expression or RNA interference constructs.

By employing a high-sensitivity camera to detect bioluminiscence, individual seedlings were monitored and screened for mutants with abnormal circadian rhythms (Millar et al. 1995). Bioluminescence imaging of transgenic plants is capable of spatially distinguishing circadian rhythms in a whole plant body or a specific organ (e.g. a leaf). This technique clearly showed independent antiphase circadian rhythms occurring in two regions in a single leaf (Thain et al. 2000). The plant circadian system can be considered a multicellular system of self-sustained cellular circadian clocks. Spatio-temporal patterns

References


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of circadian bioluminescence in an Arabidopsis leaf suggest that the circadian system is heterogeneous among cellular clocks even in a single leaf (Wenden et al. 2012). In a detached leaf under continuous light conditions, traveling waves of circadian activity were observed (Fukuda et al. 2007, Wenden et al. 2012). These phenomena can be explained by the coupling of cellular clocks, though the interactions are estimated to be weak (Wenden et al. 2012). Recently, a novel stripe pattern of circadian bioluminescence was found in the Arabidopsis root (Fukuda et al. 2012). Simulations suggested that cellular coupling was not necessary for the formation of the stripe pattern, though some observed patterns implied the existence of coupling (Fukuda et al. 2012). Thus, bioluminescence imaging of the plant body and organs led to an understanding of the circadian clock as a multicellular system. These emergent properties are basically the result of the circadian rhythms of individual cellular clocks. However, little is known about the properties of cellular clocks. Recently, a novel method for monitoring circadian rhythms in single cells using a green fluorescent protein (GFP) reporter has been developed in Arabidopsis (Yakir et al. 2011). Using this monitoring system, de-synchronization of cellular circadian rhythms and a difference of period lengths between stomatal guard cells and mesophyll cells were observed. The fluorescence monitoring system requires the illumination of tissues to excite the fluorophore, which restricts its utility for analyzing circadian rhythms which are affected by light conditions. Single-cell luciferase imaging to study the circadian clock system has been developed for cyanobacteria and animals. Microscopic bioluminescence imaging of individual cyanobacterial cells revealed that the circadian rhythm in each cell was stable and that the interactions between cellular clocks in a population were negligible (Mihalcescu et al. 2004). The principal mammalian circadian pacemaker, the suprachiasmatic nucleus, was analyzed for the synchrony of individual neuronal cells by monitoring mouse suprachiasmatic nucleus slice cultures expressing a bioluminescent reporter, and following artificial de-synchronization after tetrodotoxin treatment, autonomous re-synchronization among individual cellular clocks after tetrodotoxin washout was observed (Yamaguchi et al. 2003). Mouse fibroblasts expressing a luciferase reporter were also analyzed for the cellular clock independence, and no communication between cellular clocks was observed (Nagoshi et al. 2004).

Duckweeds are a group of monocotyledonous plants with tiny, floating bodies. *Lemna gibba* has been used for various physiological studies. The tiny, floating plant bodies, rapid growth rates and growth under strictly controllable asptic culture conditions are advantages of this species (Hillman 1961). Physiological rhythms, such as CO2 output and potassium uptake, were initially monitored to analyze duckweed circadian rhythms (Miyata and Yamamoto 1969, Hillman 1970, Kondo and Tsudzuki 1978). Recently, a bioluminescence monitoring system using particle bombardment has been successfully applied to the analysis of circadian rhythms in *Lemna* plants, and several clock-related genes have been isolated from them (Miwa et al. 2006, Serikawa et al. 2008). Gene transfection by particle bombardment dispersely transfects several cells in a tissue. Thus, individual reporter-expressing cells can be separately observed in the tissue (Klein et al. 1988). Using this feature of transfection by particle bombardment and the circadian bioluminescent reporter system, we developed a single-cell bioluminescence imaging system for plants.

## Results and Discussion

### Characterization of cellular luminescence in a single-cell bioluminescence imaging system

Luciferase genes are a common genetic reporter for quantifying promoter activities of transgenes. In vivo imaging of bioluminescence is a good assay to analyze quantitatively the dynamics of gene expression in living cells and tissues. The spatial resolution of luminescence imaging is inferior to that of fluorescence mainly because of the diffusion of luminescence and the low degree of luminescence intensity. In transgenic organisms expressing a luciferase gene, it can be difficult to distinguish luminescence of individual cells in intact tissues. In order to detect bioluminescence signals of individual cells, we used particle bombardment to introduce reporter genes. This method enables disperse transfection into cells on the surface of a plant. Duckweed plants (*L. gibba*) arranged in a single layer on a 35 mm dish were set under the muzzle of a particle bombardment apparatus. Bioluminescent reporter genes were introduced into cells by particle bombardment with reporter-plasmid-coated gold particles. The duckweeds were then transferred to a 35 mm dish with fresh culture medium containing 1 mM luciferin and incubated for at least 12 h to ensure full transgene expression. Luminescent spots were detected using a cooled electron multiply charged couple device (EM-CCD) camera with a bright lens and an extension ring (Fig. 1A). We first introduced a plasmid construct containing firefly luciferase under the control of the *Cauliflower mosaic virus* 35S (*CaMV35S*) promoter (*CaMV35S:Luc*) into duckweed plants. As shown in Fig. 1B, we detected luminescent spots which were scattered across the plant surface. Because luminescence intensities appeared to vary, we investigated the intensity distribution of the luminescent spots and the dependency of the distribution on the amount of DNA used to coat the particles. Aliquot of 0.1, 1 (standard amount) and 10 µg of DNA were used for the analysis. The luminescence intensity of spots on duckweeds with the 0.1 µg DNA dose appeared to be lower than that with 1 and 10 µg DNA doses (Fig. 2A). In order to measure luminescent spots accurately over a broad range of intensities, magnified luminescence images were taken with exposure times of 6, 60 and 600 s for each sample. We also estimated the efficiency of gene transfection by counting all luminescent spots of the five duckweed colonies with the highest spot numbers in each particle bombardment experiment. The numbers of recognized luminescent spots increased as the DNA amounts were increased (Table 1). The 1 µg DNA dose gave rise to ~2 luminescent spots per 1 mm² of frond surface on average. This was
The scale bar represents 1 mm.

![Image](https://example.com/image.png)

**Fig. 1** The single-cell bioluminescence imaging system. (A) Schematic overview of the imaging system (left) and side view (right). A luciferase reporter gene was introduced into duckweeds by particle bombardment. Plants were transferred to culture medium containing 1 mM luciferin in a 35 mm dish, and bioluminescence was captured using an EM-CCD camera with a camera lens (f 0.95/25 mm). A short-pass filter was mounted on the lens to remove the delayed Chl fluorescence. LED white light was guided by optical fiber cables to grow plants. (B) A luminescence image (left) and bright-field image (right). The luciferase reporter CaMV35S::luc + was introduced into *Lemna gibba* plants and bioluminescence from a colony was captured with an exposure time of 600 s. The bright-field image was captured by the same imaging system with a lower sensitivity. The scale bar represents 1 mm.

4- to 5-fold higher than the density of spots following a 0.1 μg dose, and about half of that after a 10 μg dose.

The luminescence intensity of every detectable spot was calculated as photons min⁻¹ in accordance with the manufacturer’s instruction. The frequency distributions of the intensities are shown in **Fig. 2B**. In any DNA dosage condition, luminescence intensities varied over a 1,000-fold range and the distribution was bell shaped. The bell-shaped properties of these distributions suggest that they follow a log-normal distribution, though this was not proven by statistical analyses (**Table 1**; Kolmogorov–Smirnov test and Shapiro–Wilk test for log-normal distribution). It has been theoretically and experimentally demonstrated that the distribution of expression levels of a gene at the single-cell level is ubiquitously log-normal (Bengtsson et al. 2005, Furusawa et al. 2005). Thus, the log-normal-like distribution of cellular luminescence intensities seemed to be reasonable. However, the 1,000-fold range we observed seemed unexpectedly large. We then examined cellular luminescence intensities of a stable transformat.

Leaves of an Arabidopsis transgenic plant carrying *Arabidopsis thaliana* Chl α/β-binding protein 3 (*AtCAB3::luc*) were used for the preparation of mesophyll protoplasts, and the bioluminescence of individual protoplasts was quantified as it was for *Lemna* plants (**Supplementary Fig. S1**). The intensity frequency plot again showed a bell-shaped distribution when plotted on a log scale, and statistical analyses strongly suggested that it was a log-normal distribution. In contrast to a 1,000-fold range of cellular luminescence intensities in *Lemna* plants, transgenic Arabidopsis mesophyll cells had a narrow intensity range (~50-fold), suggesting that transient transfection by particle bombardment was at least partially responsible for the broad range of cellular luminescence intensities. It has been reported that expression of a *CaMV35S-GUS* (β-glucuronidase) construct in tobacco Bright Yellow-2 (BY-2) cells following transfection by particle bombardment was highly dependent on the intracellular end-point of the introduced gold particle (Yamashita et al. 1991). Therefore, variation of the intracellular end-point of introduced gold particles is likely to affect the expression level of the luciferase gene in *Lemna* plants.

We also tested the possibility that the transgene was introduced into different cell types by particle bombardment, and that cell type differences might partially explain the broad range of luminescence intensity observed. It was impossible to distinguish cell types of luminescent spots in our imaging, so we co-transfected a fluorescent reporter (*CaMV35S::GFP-h*) with the bioluminescent reporter by particle bombardment and examined fluorescent cells under a confocal microscope to determine cell types. The GFP-h protein was shown to localize at the endoplasmic reticulum, and was used as a marker for cell shape. We first selected fronds with high transfection efficiency by checking bioluminescence. Highly transfected fronds were scanned for cells with GFP fluorescence, and all of detectable fluorescent cells were examined for their shapes and distribution of adjacent chloroplasts (**Fig. 3A, B**). Epidermal cells of *L. gibba* fronds have a polygonal surface shape and they lack chloroplasts. In contrast, mesophyll cells are round with chloroplasts. Approximately 80% of transfected cells were mesophyll cells and the rest were epidermal cells (**Fig. 3C**). We did not find other types of cells with GFP fluorescence. Any GFP-positive cells were separated by one or more GFP-negative cells. Variation of these cell types might be an additional factor contributing to the broad range of luminescence intensities. However, the frequency distributions for luminescence intensities had a single peak, rather than being a bimodal distribution, suggesting that the broad range of the *CaMV35S::luc +* reporter activity is primarily due to variation of the intracellular end-point of introduced gold particles and a resulting difference of cellular bioluminescence activity.

**Monitoring cellular luminescence**

A bioluminescent reporter system has the advantages of providing quantitative gene expression data and allowing non-invasive measurement. We therefore used this reporter...
system to monitor gene expression in living cells. We made a
time-lapse recording of duckweed cellular bioluminescence
using the single-cell bioluminescence system. *Lemna*
plants were bombarded with gold particles and a colony with an ap-
propriate density of luminescent spots was transferred to a new
dish with culture medium containing 1 mM luciferin. The
colony was anchored on the medium with pins surrounding
it. Pins were stuck and held to a sheet of silicon rubber laid at
the bottom of the dish. Note that this manipulation was done
with care not to stab or damage the plant body. The biolumin-
escence from a frond into which the *CaMV35S::luc* reporter
was introduced was captured every 1 h for 2 d under 12 h dark/
12 h light conditions (Fig. 4). The total luminescence intensity
of traceable luminescent spots (50 spots) peaked at ~5 h in the

![Image](https://academic.oup.com/pcp/article-abstract/54/12/2085/1838896)

**Table 1** Statistical parameters describing single-cell bioluminescence intensities with the three amounts of DNA

<table>
<thead>
<tr>
<th>Amount of DNA (µg)</th>
<th>Experiment number</th>
<th>n*</th>
<th>Density (cells mm⁻²)</th>
<th>Arithmetic mean (photons min⁻¹)</th>
<th>Geometric mean (photons min⁻¹)</th>
<th>Log₁₀ geometric mean (SD)b</th>
<th>Kolmogorov–Smirnov P-valuec</th>
<th>Shapiro–Wilk P-valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Exp. 1</td>
<td>91</td>
<td>0.473</td>
<td>494</td>
<td>144</td>
<td>2.16 (0.58)</td>
<td>0.12</td>
<td>0.00010</td>
</tr>
<tr>
<td>0.1</td>
<td>Exp. 2</td>
<td>70</td>
<td>0.443</td>
<td>305</td>
<td>97.2</td>
<td>1.99 (0.62)</td>
<td>0.57</td>
<td>0.067</td>
</tr>
<tr>
<td>1</td>
<td>Exp. 1</td>
<td>413</td>
<td>1.86</td>
<td>582</td>
<td>285</td>
<td>2.45 (0.51)</td>
<td>0.29</td>
<td>0.011</td>
</tr>
<tr>
<td>1</td>
<td>Exp. 2</td>
<td>384</td>
<td>2.34</td>
<td>528</td>
<td>276</td>
<td>2.44 (0.45)</td>
<td>0.28</td>
<td>0.0038</td>
</tr>
<tr>
<td>10</td>
<td>Exp. 1</td>
<td>751</td>
<td>3.81</td>
<td>828</td>
<td>436</td>
<td>2.64 (0.47)</td>
<td>0.58</td>
<td>0.053</td>
</tr>
<tr>
<td>10</td>
<td>Exp. 2</td>
<td>845</td>
<td>4.38</td>
<td>994</td>
<td>509</td>
<td>2.71 (0.45)</td>
<td>0.13</td>
<td>8.5 x 10⁻⁹</td>
</tr>
</tbody>
</table>

* The number of bioluminescence spots; a total of five colonies.
  b Logarithm of the geometric mean and corresponding SD.
  c For the Kolmogorov–Smirnov test and Shapiro–Wilk test, a high P-value means a good fit. If P > 0.05, log-normality of the data cannot be rejected.

**Fig. 2** Frequency distribution of cellular bioluminescence intensities. *CaMV35S::luc* was introduced into *Lemna* plants on a 35 mm dish. Aliquots of 0.1, 1 and 10 µg of plasmid DNA were used for gene transfection. Plants were cultured under a 12 h dark period followed by a 6 h light period, then whole-dish luminescence images were captured. The five colonies with the highest gene transfection efficiencies were re-captured for single-colony imaging to quantify cellular bioluminescence intensities. Exposures of 6, 60 and 600 s were captured and every luminescent spot was quantified for light intensity under an appropriate exposure condition. (A) Luminescence images of *Lemna* plants in a 35 mm dish of each condition and their bright-field images. Scale bars represent 5 mm. (B) Frequency distribution histograms for cellular bioluminescence intensities. Note that the x-axis is on a logarithmic scale. Experiments were performed twice (Exp. 1 and Exp. 2).
first dark period and then gradually decreased to \(~30\%\) of the peak luminescence at the end of the measurement (Fig. 4B). Responses to dark–light and light–dark shifts were not obvious in the luminescence behavior. However, luminescence behaviors varied among individual cells. Fig 4C and D shows examples of four individual cells in a small region (575 × 675 μm). Their maximum luminescence intensities ranged from 561 to 3,457 photons min⁻¹, and peak times ranged from 5 to 12 h after the beginning of measurement. After peak time, Cell 3 maintained \(~40\%\) of its peak luminescence until the end of measurement, while Cells 2 and 4 dampened severely by the end. Such detected differences among closely located cells suggested that bioluminescence of individual cells should not interfere with time-lapse measurement of multiple cells simultaneously, at least at this distance (\(~150\mu m\)).

We next investigated the luminescence behavior of cells into which a luciferase reporter driven by the circadian Arabidopsis CIRCADIAN CLOCK ASSOCIATED 1 (AtCCA1) promoter (AtCCA1::luc+) was introduced (Nakamichi et al. 2004). This reporter gene was shown to work in two Lema species as a circadian marker with a peak around dawn (Miwa et al. 2006). Bioluminescence of a frond into which the AtCCA1::luc+ reporter was introduced was captured every 1 h for 2 d under 12 h dark/12 h light conditions (Fig. 5). The efficiency of gene transfection into cells was comparable with that of CaMV35S::luc+ (Fig. 5A). The total luminescence intensity of traceable luminescent spots (60 spots) peaked at \(1\)–\(2\) h after the beginning of the first light period (Fig. 5B). The luminescence intensity of the second peak was reduced to \(~60\%\) of that of the first peak. Every single spot showed a diurnal rhythm that synchronized with environmental light–dark cycles. Luminescence intensities of cells ranged over \(~30\) times when their maximum values in the 48 h time course were compared. We calculated the amplitude of each cellular rhythm as half of the difference of luminescence intensities between the peak and the following trough. Then we defined the relative amplitude as the amplitude divided by the mean of those at the peak and the trough, which has a theoretical maximum value of 1. The average of relative amplitudes was 0.88 (SD = 0.056). This high amplitude and the small SD suggested that luminescence waveforms were similar irrespective of luminescence intensities. Almost all spots peaked at \(1\)–\(2\) h after the beginning of the light period. The averages of the first and second peak times were 13.4 h (SD = 1.09) and 38.1 h (SD = 0.88), respectively. The SD for the second peak was smaller than that for the first one, suggesting that the rhythms became more synchronized to the environmental light–dark cycle (Fig. 5B). Fig. 5C and D shows examples for four individual luminescent spots in a small region (575 × 675 μm). The bioluminescence intensities of the first peaks ranged from 80 to 224 photons min⁻¹. The decreases in their luminescence intensities between the first peak and the second varied in a range of 45–75%, and the waveforms of the cells were slightly different from each other. This indicated that diurnal bioluminescence rhythms among closely located cells were observable in the monitoring system, at least at this distance (\(~150\mu m\)).

Using the single-cell bioluminescence imaging system, we succeeded in monitoring the gene expression of individual cells in a plant body. In our particle bombardment procedure, \(~80\%\) of transfected cells were mesophyll cells and the rest were epidermal cells. Thus, the bioluminescence traits presented here mainly reflect the characteristics of mesophyll cells. Optimization of particle bombardment procedures to target epidermal cells will clarify cell type-dependent gene expression behavior. A simultaneous measurement system incorporating a cell type-specific GFP marker and a bioluminescent reporter would allow us to assign cellular bioluminescence traits directly to cell types. Analysis of gene expression of individual cells will be most useful when the specific cell type being characterized is known. In Arabidopsis, a single-cell
Fig. 4 Time-lapse recording of the cellular bioluminescence of the constitutively active reporter, CaMV3SS::luc+. CaMV3SS::luc+ was introduced into Lemna plants on a 35 mm dish. After cultivation under a 12 h dark period followed by a 12 h light period, a whole-dish luminescence image was captured to determine the efficiency of gene transfection. The colony with the highest efficiency was selected and luminescent spots from a frond of the colony were monitored by time-lapse single-colony imaging during two 12 h dark/12 h light cycles. The exposure time was 180 s and the interval of exposure was 1 h. (A) A luminescence image of a frond (left) and its bright-field image (right). Each red square of 6 × 6 pixels (~150 × 150 μm) represents the ROI for the quantification of luminescent spots. The scale bar represents 1 mm. (B) Temporal changes of bioluminescence intensities of the 50 individual ROIs (solid lines) in (A), and the total of their bioluminescence intensities (dotted line). The y-axis is on a logarithmic scale. (C) A series of time-lapse images for four neighboring cells in the blue rectangle in (A) at 2 h intervals. (D) Temporal changes of bioluminescence intensities of the four individual ROIs in (C). Cell numbers correspond to the numbering in (C).

Fig. 5 Time-lapse recording of the cellular bioluminescence of the rhythmic reporter, AtCCA1::luc+. AtCCA1::luc+ was introduced into Lemna plants on a 35 mm dish. Procedures and conditions were the same as described in Fig. 4, except that the exposure time was 900 s. (A) A luminescence image of a frond (left) and its bright-field image (right). Each red square of 6 × 6 pixels (~150 × 150 μm) represents the ROI for the quantification of luminescent spots. The scale bar represents 1 mm. (B) Temporal changes of bioluminescence intensities of the 60 individual ROIs (solid lines) in (C), and the total of their bioluminescence intensities (dotted line). Frequency distribution of peak time of cellular bioluminescence rhythms is shown at the top. The y-axis is on a logarithmic scale. Frequency distribution of peak time of cellular bioluminescence rhythms is shown at the top. (C) A series of time-lapse images for four neighboring cells in the blue rectangle in (A) at 2 h intervals. (D) A series of time-lapse images for the four individual ROIs in (C). Cell numbers correspond to the numbering in (C).
analysis using a circadian GFP reporter revealed that the circadian traits of stomatal guard cells were different from those of epidermal and mesophyll cells (Yakir et al. 2012). It is noteworthy that we were unable to find stomatal guard cells showing a GFP signal following our particle bombardment gene transfection procedure (Fig. 3). It was known that the guard cells of L. gibba carry no mature green chloroplasts and show no trace of stomatal movement (Park et al. 1990, Les et al. 1997). Since the activity of guard cells of L. gibba seems low, signals from the introduced reporter gene might be too weak to be detected. Using a camera to image an entire plant body enabled us to monitor single-cell level bioluminescence from cells dispersed across the plant body (organ) in normal growth conditions. Thus, it has become feasible to obtain gene expression time-series data in many cells in specific positions on the organ. Synchronization among cellular circadian rhythms is of great interest and in Arabidopsis leaves it has been shown that vascular structures influenced spatio-temporal patterns of circadian rhythms (Fukuda et al. 2007).

Duckweeds are suitable for entire plant imaging because of the tiny, flat bodies. In addition, duckweed fronds completely terminate growth after they mature, and they become immobile in the vertical direction, making it unnecessary to adjust the focus during long-term monitoring. These features of duckweed allow long-term monitoring of intact tissues and the whole plant body. Furthermore, the particle bombardment transfection method is adaptable to various plants. Single-cell bioluminescence monitoring will become a powerful tool for the analyses of a variety of physiological phenomena, such as stimulus responses in which cellular responses are stochastic processes that may differ from the ensemble behavior of whole tissues.

**Materials and Methods**

**Plant materials and growth conditions**

*Lemna gibba* G3 was maintained in the laboratory for >40 years by vegetative reproduction. *Lemna gibba* plants were kept aseptically in M medium with 1% (w/v) sucrose under continuous light conditions (Hillman 1961). Approximately five colonies were picked from cultures and grown under continuous light conditions (Hillman 1961). Approximately five colonies were laid on a 35 mm polystyrene dish (IWAKI, http://www.iwaki-kk.co.jp/). All fluorescent cells in the second frond (i.e. the first emerging daughter frond of the parental frond) were investigated for GFP fluorescence-positive cells under a confocal microscope (LSM510-META; Carl Zeiss, http://corporate.zeiss.com/). All fluorescent cells in the second frond (i.e. the first emerging daughter frond of the parental frond) were investigated for transfected cell types.

**Luciferase reporter constructs**

The constitutive bioluminescent reporter pUC18-CaMV35S::luc + (CaMV35S::luc+) was derived from pBI221 (Clontech). The GUS gene under the CaMV35S promoter was replaced by the luc + gene (Promega). pUC-AtCCA1::luc + (AtCCA1::luc+) was used as the rhythmic bioluminescent reporter. The firefly luciferase (luc+) gene was fused at a 5‘ site in the fourth exon of the AtCCA1 gene. Approximately 1.8 kbp of AtCCA1 upstream of the fusion site was included in the reporter construct as the promoter. This reporter was a kind gift from Dr. Nakamichi (construct C in Nakamichi et al. 2004).

**Particle bombardment experiment**

Reporter constructs were introduced into plant cells using particle bombardment. An 8 µl aliquot of pre-washed gold particle suspension (1 µm diameter; Bio-Rad, http://www.bio-rad.com/) in 50% glycerol (60 mg ml⁻¹) was mixed with 1 µl of plasmid DNA (1 µg µl⁻¹), 3.3 µl of spermidine (0.1 M) and 8 µl of CaCl₂ (2.5 M) in a tube. After incubating for 15 min at room temperature, the suspension was briefly centrifuged and the supernatant was discarded. The DNA-coated particles were washed once with 80 µl of 80% ethanol and once with 100% ethanol and resuspended in 15 µl of 100% ethanol. The suspension of DNA-coated particles was then spread on a macrocarrier and dried. A helium gun device (PDS-1000/He; Bio-Rad) was used for particle bombardment according to the manufacturer’s instructions [vacuum, 26 mmHg; helium pressure, 650 psi (rupture disc)]. Approximately 10 *Lemna* colonies were laid on a 35 mm polystyrene dish (IWAKI, http://www.iwaki- kk.co.jp/). The dish was set underneath the muzzle of the gun, and DNA-coated particles on the macrocarrier were fired into plants. After bombardment, 3 ml of M medium containing firefly luciferin (1 mM potassium salt; Wako, http://www.wako-chem.co.jp/) was added to the dish. Plants were cultured under 12 h dark/12 h light conditions.

**Fluorescence imaging**

The GFP reporter pB121-35S::GFP-h, expressing an endoplasmic reticulum-localized GFP, was a kind gift from Dr. Nishimura (Nakano et al. 2009). pBI21-35S::GFP-h and pUC18-CaMV35S::luc + were co-introduced into *Lemna* plants by particle bombardment. Plants were incubated in M medium including 1 mM luciferin under a 12 h dark period followed by 12 h light period. The colony with the highest efficiency was determined by bioluminescence imaging, selected and observed for GFP fluorescence-positive cells under a confocal microscope (LSM510-META; Carl Zeiss, http://corporate.zeiss.com/). All fluorescent cells in the second frond (i.e. the first emerging daughter frond of the parental frond) were investigated for transfected cell types.

**Single-cell bioluminescence imaging**

An EM-CCD camera (ImagEM C9100-13; Hamamatsu Photonics, http://www.hamamatsu.com/) with a camera lens (XENON 0.95/25MM C-mount; Schneider optics, http://www.schneideroptics.com/) with the maximum aperture was set in a dark box placed in an incubator (KCLP-1000I-CT; NK system, http://www.nihonika.co.jp/) and the temperature was maintained at 25 ± 1°C. A short-pass filter (SV630; Asahi Spectra, http://www.asahi-spectra.com/) was fitted in the lens...
hood to reduce delayed autofluorescence from chloroplasts. Extension rings 5 and 16.5 mm thick were used for whole-dish imaging and single-colony imaging, respectively. The EM-CCD camera was controlled by HOKAWO imaging software (Hamamatsu photonics). White light from an light-emitting diode (LED) light source (RFB2-20SW; CCS Inc., http://www.ccs-grp.com/) was guided by optical fibers for plant illumination. LED illumination was controlled by HOKAWO. Bioluminescence images (16-bit TIFF format) were captured with an ImagEM camera (cooled at −80°C) at an EM gain of 1,200 after at least 1 min dark treatment for autofluorescence decay. For time-lapse imaging, bioluminescence images were captured every hour with a 20 min dark period for exposure. For single-colony imaging, the second frond of the colony was anchored with several pins (Austerlitz Insect Pins, stainless steel 0.1 mm diameter; Entomoravia, http://entomoravia.eu/).

Quantification of cellular luminescence

Image analysis and post-processing were carried out with ImageJ (http://rsbweb.nih.gov/ij/). The bioluminescence intensity of a luminescent spot was quantified as an integrated density of the region of interest (ROI; size 6 x 6 pixels). In the single time point measurement (Fig. 2), three bioluminescence images with different exposure times (6, 60 and 600 s) were captured and ROIs in the 600 s exposure were automatically selected by ImageJ functions. Those ROIs were applied to the other images (6 and 60 s). The ROI with the highest signal among the set of three images for a spot, excluding ROIs including saturated pixels, was used for further analysis. In the time-lapse imaging (Figs. 4, 5), gaps between images caused by slight frond movements were corrected by manually tracing two bright luminescent spots in the stack of images followed by automatic parallel and rotation movement of each image to make the trace line to a point. After correcting the positions, luminescent spots that were separated from neighboring spots and maintained bioluminescence throughout the measurement (48 h) were selected. ROIs for those spots were defined manually and calculated for luminescence intensities. Luminescence intensities were converted to photons min⁻¹ based on the rated quantum efficiency and the gain value of the camera as described in the manufacturer’s instructions.

Protoplast isolation and quantification of luminescence of single protoplasts

The CAB3::luc transgenic Arabidopsis strain was a gift of Dr. Mochizuki (unpublished). Transgenic seedlings were grown on Murashige and Skoog medium (Sigma Aldrich, http://www.sigmaaldrich.com) supplemented with 3% sucrose grown on Murashige and Skoog medium (Sigma Aldrich, http://www.sigmaaldrich.com) supplemented with 3% sucrose under 12 h dark/12 h light conditions. Protoplasts were isolated from the transgenic Arabidopsis strain with minor modifications. After overnight enzyme treatment, the released protoplasts were filtered through a sterile 100 μm nylon mesh. Protoplasts were harvested by centrifugation at 50 x g for 5 min. After decanting the supernatant, the protoplast pellet was resuspended in 1 ml of WS solution containing 1 mM luciferin. After 1 h incubation, an aliquot of the protoplast solution (300 μl) was spread on a glass slide for imaging. Five distinct regions of the slide were captured by the EM-CCD camera at the same magnification as single-colony imaging, with exposure times of 60 and 180 s. Every luminescent spot was quantified for bioluminescence intensity as described above.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


