Phase Inversion Of Cellular Circadian Rhythms By Spatiotemporal Illuminations In Transgenic Lettuce Leaves

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Abstract

Plant factory in OPU is a facility which can control multiple environmental conditions. This has several advantages for plant growth. However, the running cost, charges for the illumination, is serious problems. Hence, the technique for growing plants well is needed. The circadian rhythm is supposed to be related to the plant growth. Control of circadian rhythm plays important role in plant cultivation. In this study, we tried to control the circadian rhythm in the transgenic lettuce using a LCD projector or a laser projector. We investigated spatiotemporal dynamics in leaves generated by artificial initial phase of circadian rhythm conditions in a transgenic lettuce strain AtCCA1::LUC, in which the cellular circadian rhythm was measurable by bioluminescence imaging. Our experiments were carried out using transgenic lettuce. To apply artificial initial conditions to the leaf, illumination with a set of star-shaped patterns, a bright star within a dark rectangle and its inverted image, was applied using a LCD projector or a scanning laser projector. The bright and dark star images were alternately applied. Bioluminescence of detached leaves was monitored with a highly sensitive cooled CCD camera in a temperature-controlled dark box. Leaves of transgenic lettuce showed circadian oscillations of bioluminescence under continuous dark condition. White and black star patterns were observed in the bioluminescence, implying that their phase was almost the inverse of each other. We have investigated artificial spatiotemporal dynamics that was started from a special initial condition by training the circadian clock in lettuce leaves.

INTRODUCTION

The fully-controlled plant factory is a facility which produces plants in enclosed environment under controlled light, temperature, humidity, CO₂ concentration, and some environmental conditions. Therefore, the facilities have many advantages, e. g., food safety, stable supply, and production of functional vegetables in any location. However, the facility cost and running cost, especially the charges for electricity of lighting, are serious problems. The improvement of lighting efficiency is an important subject of technological research and development in the fully-controlled plant factory.

The fluorescent lamp (FL) is used at the plant factory in recent years. However, the FL illumination illuminates even blank space where no plant is cultivated, which is significant dissipation of electricity. The FL light intensity is also not spatially uniform, that is, the FL illumination at the center region is higher than that at the edge. Therefore, the
point light source, e. g., the light emitting diode (LED) and the laser diode (LD), have been a focus recently in the study of the plant cultivation. LED panel can illuminate just the space where the plants are being cultivated by controlling the illumination of LED elements adapted for the plant growth. LD also can illuminate locally, because it can scan the region of the plant accurately. These light sources can illuminate with less gap between the center and the edge of the cultivation area. Moreover, the cost of maintenance of the LD and LED are lower than the FL.

In particular, the laser also has multiple advantages for the plant growth. A laser can emit a pulsed light. The pulsed laser light enhances the growth and photosynthetic rates of a lettuce (Mori, Takatsui, & Yasuoka, 2002). The application of laser to the plant has been studied as follows. Lettuce has been cultivated using LD light alone, and the vitamin C content has been increased (Mori, Takatsui, & Yasuoka, 2003). Simultaneous irradiation with red LD and blue LED, followed by irradiation with UV-A has led to an increase of polyphenols in the red perilla (Iwai, Ohta, Tsuchiya, & Suzuki, 2010). Thus, a laser is one of optimal light source of plant factory.

In this study, we analyzed the activity change of circadian clock in order to evaluate the effect of the local illumination on the plant metabolism. Clock gene expression produces a circadian rhythm with an approximately 24-hour period. Thus, the each of the plant cells act as oscillators by clock gene expression and cell-cell interaction. The location of a pacemaker has not been determined in higher plants (Thain, Hall, & Millar, 2000). The rhythm can be reset strongly by external signals such as light or temperature cycles (Fukuda, Uchida, & Nakamichi, 2008; Harmer, 2009; Pruneda-Paz & Kay, 2010). The circadian rhythm confers to an advantage to plants. The plants with a period matched to the environment, for example, grow faster than plants with a period differing from their environment (Dodd, Salathia, Hall, Kévei, Tóth, Nagy, et al., 2005). Control of growth of the plants requires a detailed understanding of circadian clock.

We tried to control the circadian rhythm locally in the transgenic lettuce, using a LCD (liquid crystal display) projector or a scanning laser projector. We investigated spatiotemporal dynamics in leaves generated by artificial initial conditions in a transgenic lettuce strain AtCCA1::LUC, in which the cellular circadian rhythm was measurable by bioluminescence imaging. The light period of a LCD projector was 24 h and it of a laser projector was 24 h or 26 h.

MATERIALS AND METHODS

Plant materials and growth conditions

Our experiments were carried out using transgenic lettuce (Lactuca sativa L. cv. Greenwave) AtCCA1::LUC, in which an Arabidopsis thaliana CCA1 promoter-luc cassette, pABH-CCA1:: LUC-C, was transformed into Arabidopsis plants via Agrobacterium tumefaciens–mediated transformation. This AtCCA1::LUC lettuce was eliminated the bioluminescence, which was proportional to the expression rate of CCA1. This bioluminescence showed a circadian rhythm even under constant dark condition and the circadian rhythm could be observed in almost all cells of the leaves, as reported in Ukai et al. (2012).

AtCCA1::LUC plants were grown in hydroponic culture (Otsuka-A; Otsuka Co., Ltd., Japan) under light/dark cycles using fluorescent light with about 150 μmol m−2 s−1 (photosynthesis photon flux) for 3~5 weeks. Young leaves in the plants were detached and set on a dish (40 mm in diameter), then about 5 ml of 0.2 mM luciferin solution dissolved in
water was added on the same dish in the experiment using a LCD projector and a laser projector, respectively.

Illumination conditions by LCD and scanning laser projectors and monitoring bioluminescence

To control the cell-level circadian rhythm in leaf, we applied a spatially controlled illumination for the leaves. Illumination with a set of star-shaped patterns, a bright star within a dark rectangle and its inverted image, was applied using a LCD projector (EB-1915, SEIKO-EPSON KK, Japan) or a scanning laser projector (SHOWWX, MicroVision Inc., USA). The spectrum of the super-high pressure mercury vapor lamp, a light source of the LCD projector, shows a broad peak at 435.8 nm, 404.7 nm and 365.4 nm and the spectrum spreads broadly between 350 nm to 500 nm, while that of the scanning laser projector shows three sharp peaks at 442 nm, 532 nm and 642 nm. The frequency of projection was 60 Hz both in the LCD and scanning laser projectors.

In this study, to demonstrate finally the precise entrainment of cellular circadian rhythms by laser projector illuminations we performed two entrainment-protocols: The bright and dark star images were alternately applied with 12 h or 13 h periods. The difference of alternation periods (12 h or 13 h) in the laser projector experiment will provide the different initialized phases, because of the different start time of continuous dark (DD) condition (t = -6 h in the right star, t = 0 h in the left star).

In the experiment using LCD projector, the alternation period of bright and dark star images was 12 h, and the light intensity of the bright and dark regions were 70 and 1 mol m⁻² s⁻¹, respectively (Figure 1a). The illumination in order to entrainment circadian rhythm was started at t = 0. On the other hand, in the experiment using the laser projector, the alternation period was 12 or 13 h in the right or left half, and the bright and dark regions were 3 and 0 mol m⁻² s⁻¹ (70 and 0 W), respectively (Figure 4). In both experiments, the illumination was projected on a detached leaf in the dish (in diameter 40 mm) on the thermo-controller (OKS-C201, OKANO CABLE Co., Ltd., Japan) at 22.0 ± 0.1 °C. Bioluminescence of detached leaves was monitored with a highly sensitive cooled CCD camera (ORCA-AG, Hamamatsu Photonics KK, Japan) every 30 min. The resolution of bioluminescence images were 100 and 200 μm in the experiments using LCD and laser projector, respectively.

RESULTS AND DISCUSSION

Entrainment of cellular circadian rhythm by a LCD projector illumination

Transgenic lettuce AtCCA1::LUC leaves showed circadian oscillations of bioluminescence under DD conditions as reported by Ukai et al. Figure 1b (left and right panel) show bioluminescence images under DD condition at t = 2.5 and 14.5 h after application of LCD illumination, respectively.

We next investigated the phase of circadian rhythm in each pixel of the bioluminescence images. To investigate the phase pattern in the leaf, we introduced the phase of the circadian oscillation in equation (1) (Fukuda, Nakamichi, Hisatsune, Murase, & Mizuno, 2007; Wenden, Toner, Hodge, Grima, & Millar, 2012),

\[
\phi(t) = 2\pi \frac{t - \tau_k}{\tau_{k+1} - \tau_k}, \quad t \in [\tau_k, \tau_{k+1}) \tag{1}
\]
where $\Gamma_k$ is the time of the $k$th peak of the oscillatory time series of bioluminescence in each pixel. To calculate peaks of bioluminescence oscillation, which often showed large noise, the moving average with a window size of 24 (12 h) was applied in each pixel. Figure 2b shows the phase images of the corresponding circadian bioluminescence in Figure 2a. White and black star patterns were observed in the bioluminescence, indicating that their phase was almost the inverse of each other. The star pattern region remained for at least two days, though the intensity of bioluminescence decreased in time (Figure 2a). Figure 3 (right panel) shows the circadian rhythms in the star region (s) and its neighbor region (t) in Figure 3 (left panel), which were extracted the long-term trend of bioluminescence intensity. The initial peak of the region (s) and (t) was 15 h and 23.5 h from switching off the light, respectively. The expression peak of the CCA1 gene was delay for about 2 h from switching on the illumination (14 h from switching on the illumination) when the illumination period was 24 h (Fukuda, Tokuda, Hashimoto, & Hayasaka, 2011). These peaks of the region (s) and (t) were delay 1 h and advanced 2.5 h compared with expected, respectively. The phase in the region (s) was almost reversed to the region (t) with a delay of about 9 h. From the results, we succeed to control the cellular circadian rhythm by a spatially controlled illumination using a laser projector.

**Entainment of cellular circadian rhythm by a Laser projector illumination**

To demonstrate the precise entainment of cellular circadian rhythms by laser projector illuminations we performed two entainment-protocols: The bright and dark star images were alternately applied with 12 h or 13 h periods. The difference of alternation periods (12 h or 13 h) in the laser projector experiment will provide the different initialized phases, because of the different start time of DD condition ($t = -6$ h in the right star, $t = 0$ h in the left star in Figure 4). The center dash-dotted line shows the division between illumination protocol for the right and left region in Figure 4. Bright and dark star pattern illumination on the leaf using a laser projector.

The initial peak in the star region (s) in Figure 6b and region (u) in Figure 6c were at 12 and 15 h after switching off the illumination, respectively. Because the peak of the CCA1 gene expression was no delay from switching on the illumination (12 h from switching on the illumination) when the illumination period was 26 h. On the other hand, the expression peak of the CCA1 gene was delay for about 2 h from switching on the illumination (14 h from switching on the illumination) when the illumination period was 24 h (Fukuda et al., 2011). The phase in the star region (s) was almost reversed to its neighbor region, because the difference between the initial peak of the region (s) and (t) was 9 h. In addition, the phase in the region (u) was reversed to the region (v) with 13 h delay.

Our LCD and laser projector systems applied sufficiently strong (maximally 70 $\mu$mol m$^{-2}$ s$^{-1}$) and weak (3 $\mu$mol m$^{-2}$ s$^{-1}$) illuminations, respectively. The laser illumination is not enough to live the leaf through photosynthesis and has three wavelengths with a very narrow spectrum, but it can entrain the cellular circadian rhythm and can print the reverse phase pattern. From these results, we succeed to control the cellular level circadian rhythm spatially by a spatially controlled illumination using a laser projector.

Illumination of the different period was applied for one leaf. In the right half applied 24 h light period illumination, the bioluminescence was measured for a longer time and the phase difference between the star region and their neighbor region was larger than the other half applied 26 h light period illumination. These results indicate that the bioluminescence in the leaf with a clock period matched to the environment can be measured for a longer term and the circadian rhythms can be controllable with accuracy better than that with the different period from their environment.
CONCLUSIONS
In this study, we aimed to control the circadian rhythm locally. Figures which contained bright and dark region were illuminated to leaves using a laser or LCD projector. The figures emerged in bioluminescence and phase images. These results indicate that the circadian rhythms in the lettuce leaves are controllable by the spatiotemporal illumination using an LCD projector and a laser projector.

Nomenclature
\( \mu \text{mol m}^{-2} \text{s}^{-1} \): photosynthetic photon flux density
\( \text{mM} \): molar concentration, mol m\(^{-3}\)

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Figures

Figure 1. Star-shaped illumination and bioluminescence. (a) Bright and dark star pattern illumination were projected. (b) White and black star pattern bioluminescence images: the star region was at subject dusk (left) and subject dawn (right). (Scale bar, 10mm)

Figure 2. Time series of the bioluminescence and phase images. (The numbers show time in hour. Interval between images, 4 h) (a) Snapshot of the bioluminescence under DD conditions. (b) Phase images of the bioluminescence.

Figure 3. Local bioluminescence of the star region (s) and its neighbor region (t). Small triangle on the curve showed the peaks of bioluminescence respectively.

Figure 4. Bright and dark star pattern illumination on the leaf using a laser projector.
The star patterns were not clearly emerged in the bioluminescence images (Figure 5a) but were emerged in phase images (Figure 5b). The shape of the star region remained for at least one day, because that the intensity of bioluminescence rapidly decreased in time. The regions (s), (t), (u) and (v) in Figure 6b and Figure 6c showed the time series of normalized bioluminescence of these four regions in Figure 6a.

Figure 5. Time series of the bioluminescence and phase images using a laser projector. (The numbers show time in hour. Interval between photographs, 4 h) (a) Photograph of the bioluminescence under DD conditions. (b) Phase images of the bioluminescence.

Figure 6. The time alternation of the bioluminescence of the star region and its neighbor region. Region (s) and (u) showed the area illuminated star-shaped pattern. Region (t) and (v) were next region of star illumination. Small triangle on the curve showed the peaks of bioluminescence respectively.