Entrainment of Cellular Circadian Rhythms in Lactuca sativa L. Leaf by Spatially Controlled Illuminations

Naoki Seki1, Kazuya Ukai1, Takanobu Higashi2 and Hirokazu Fukuda1*

1Department of Mechanical Engineering, Graduate School of Engineering, Osaka Prefecture University, Sakai 599-8531, Japan
2Department of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai 599-8531, Japan

Abstract

Plant circadian system works autonomously and responds to various environmental information in cellular level. Conventional studies on controlling the plant circadian system, however, have not thoroughly considered in cellular level yet. In this study, we investigated spatiotemporal dynamics of cellular circadian rhythms of clock gene CCA1 in leaves that were controlled by the projector lightings in a transgenic lettuce strain AtCCA1::LUC using a bioluminescence imaging. We have succeeded to control the cellular circadian rhythms in the both case of LCD and laser projectors with 24 or 26 h periods of light-dark cycles. Although light intensity of the laser projector was very small to a required light intensity for growth of lettuces, the circadian rhythm was entrained with high sensitivity for illumination. Our results motivate experimental and theoretical studies of circadian control and development for the highly functional lighting technology in plant productions.

Keywords: Circadian rhythm; Clock gene; Oscillators; Phase analysis; Synchronization

Introduction

Circadian clocks that generate approximately 24-h rhythmicity are present in almost all living organisms. In higher plants, circadian clocks play a crucial role in the regulation of a variety of biological processes, including gene expressions, photosynthesis, and flowering [1]. An important characteristic of the circadian clocks is their entrainment to environmental time cues (zeitgebers), such as changes in external light or temperature [2].

Recent studies have revealed that plant cells act as self-sustained oscillators and interact each other [3-5]. The phase sensitivity of circadian rhythm, therefore, possesses essentially in each plant cell to entrain for environmental cycles. In previous studies, however, the individual-level responses in intact plants were ordinarily investigated [6,7]. In addition, the strong pacemaker of circadian system is absent in plant [8]. Therefore, the plant circadian system works as an autonomous distributed system and can show several spatiotemporal dynamics such as spiral wave in Arabidopsis thaliana leaf [4,5,8,9]. In plant circadian system, however, there are few studies of spatiotemporal dynamics in cell population levels, despite many studies in molecular and cellular level [1,10,11].

In this study, we tried to control the cellular circadian rhythm spatially in leaves by spatially and temporally controlled illuminations, which are generated by projectors. Spatiotemporal dynamics of cellular circadian rhythms in leaves in a transgenic lettuce strain AtCCA1::LUC were investigated using a bioluminescence imaging. The period of the LCD projector was 24 h and that of the laser projector was 24 h or 26 h. The bioluminescent images were taken every 30 min using higher sensitive cooled CCD camera in the temperature-controlled dark box. The spatiotemporal dynamics of cellular circadian rhythms were investigated from these bioluminescent images.

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 CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) promoter-luc cassette, pABH-CCA1::LUC-C [10], was transformed into Arabidopsis plants via Agrobacterium tumefaciens - mediated transformation [12]. Luciferase protein was synthesized by activating promoter gene AtCCA1, and then bioluminescence was emitted by the chemical reaction with supplied luciferin. This AtCCA1::LUC lettuce was eliminated the bioluminescence, which was proportional to the expression rate of AtCCA1. This bioluminescence showed a circadian rhythm [13,14] and the circadian rhythm could be observed in almost all cells of the leaves even under constant dark condition, as reported in Ukai et al. [9].

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 poured in the same dish.

Illumination conditions 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 spatiotemporal dynamics of cellular circadian rhythms in leaves in a transgenic lettuce strain AtCCA1::LUC were investigated using a bioluminescence imaging. The period of the LCD projector was 24 h and that of the laser projector was 24 h or 26 h. The bioluminescent images were taken every 30 min using higher sensitive cooled CCD camera in the temperature-controlled dark box. The spatiotemporal dynamics of cellular circadian rhythms were investigated from these bioluminescent images.

*Corresponding author: Hirokazu Fukuda, Department of Mechanical Engineering, Graduate School of Engineering, Osaka Prefecture University, Sakai 599-8531, Japan, Tel: +81-72-254-791; E-mail: fukuda@me.osakafu-u.ac.jp

Received September 11, 2015; Accepted October 17, 2015; Published December 01, 2015


Copyright: © 2015 Seki N, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
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 (Figure 1c). 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 = 0 h). The resolution of projection was 60 Hz both in the LCD and scanning laser projectors.

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 μmol m$^{-2}$ s$^{-1}$, respectively (Figure 2a). 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 μmol m$^{-2}$ s$^{-1}$ (70 and 0 μW), respectively (Figure 3a). 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 started to spread broadly between 350 to 500 nm, while that of the scanning laser projector shows three sharp peaks at 442, 532 and 642 nm. The frequency of projection was 60 Hz both in the LCD and scanning laser projectors.

Figure 2c. Figure 2e shows that enlarged phase images at the phase images of the corresponding circadian bioluminescence in each pixel. To calculate peaks of bioluminescence in each pixel, we introduced the phase of the circadian rhythm in the leaf, we introduced the phase of the circadian oscillation which is determined by following equation [4,8,9].

$$\phi(t) = 2\pi \frac{t - \tau_k}{\tau_{k+1} - \tau_k}, \quad t \in [\tau_k, \tau_{k+1})$$

Where $\tau_k$ 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 h window was applied in each pixel. Figure 2d shows the phase images of the corresponding circadian bioluminescence in Figure 2c. Figure 2e shows that enlarged phase images at 31 and 43 h. The star pattern region emerged very clearly in the bioluminescence and phase images, which means that the phase was very finely initialized with the star form by the LCD illumination (Movie S1). Figure 2f shows the average of circadian rhythms in the star region (A) and its neighbor (B) in Figure 2e, which were extracted the long-term trend (exponential decay in this case) of bioluminescence intensity. The first peak of the region (A) and (B) was 15 h and 24 h from turning off the light, respectively. The peak of the AtCCA1::LUC signal was delay for about 2 h after turning on the illumination, that is, 14 h after turning off the illumination [16,17]. Under above consideration, these peaks of the region (A) and (B) showed, respectively, 1 h delay and 2 h advance compared with the expectation. The phase in the region (A) was almost reversed to the region (B) with a delay of about 9 h. From the results, we succeed to entrain the cellular circadian rhythm by a spatially controlled illumination using an LCD projector.

**Results**

**Entrainment of cellular circadian rhythm by an LCD projector illumination**

Leaves of transgenic lettuce AtCCA1::LUC showed circadian oscillations of bioluminescence under DD as reported by Ukai et al. [9]. Figure 2b (right and left panels) shows bioluminescence images under DD at t = 2.5 h and 14.5 h. 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 rapidly decreased in time (Figure 2c). To investigate precisely the cellular entrainment of the circadian rhythm in the leaf, we introduced the phase of the circadian oscillation which is determined by following equation [4,8,9].
Entrainment of cellular circadian rhythm by a laser projector illumination

To demonstrate 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 (Figure 1c). The simultaneously application of dual periods (12 h and 13 h) for one leaf will provide the different initialized phases by the different start-time of DD condition ($t = 0$ h in the left star in Figure 1c). The center dash-dotted line in Figure 3a shows the boundary line between 12 h and 13 h period regions. Figures 3b and 3c show the bioluminescence and its corresponding phase images. The star patterns were not clearly emerged in the bioluminescence images (Figure 3b) but were emerged in phase images (Figure 3c). The elimination of temporal noise of bioluminescence by the moving average provided successfully the pattern extraction on the phase images. Figure 3d shows that enlarged phase images at $t = 12.5$ and 24.5 h. The shape of the star region remained for at least one day, in spite of the rapid decrease of bioluminescence. Figure 3e shows the time series of normalized bioluminescence of these four regions (C, D, E and F). Because the peak of the AtCCA1::LUC bioluminescence oscillation was observed after 2 h from turning on the illumination, we can estimate the peak-time of the oscillation even under DD, as shown as the arrows in Figures 3e and 3f.

In the experiment for 24 h period illumination, the peak of the oscillation in the regions (E) and (F) was at 16 h and 27 after turning off the illumination, respectively. Although these times were delayed with 2 h and 1 h for the expected peak-time (the arrows in Figure 3f), they were almost consistent with the expectation. The regions (E) and (F) were reversed each other with 11 h time difference. On the other hand, in the experiment for 26 h period illumination, the peak of the oscillation in the regions (C) and (D) was at 12 and 21 after turning of the illumination, respectively. These times were advanced with about 2 h or 5 h for the expected peak-times (the arrows in Figure 3e). The regions (C) and (D) were almost reversed each other with 9 h time difference. The 26 h period entrainment protocol had worse precision than that of 24 h period. This failure might be caused by the mismatch between periods of light-dark cycles (26 h) and circadian clock (approximately 24 h).

Discussion

As shown in bioluminescence and phase images, the circadian oscillation in leaf was not homogeneous [9]. In particular, the vein showed brighter bioluminescence refer to surround one (Figures 2b and 3b), indicating that the constituent surrounding cells of vascular bundles activate the AtCCA1 gene expression. The mature vein cells which have no AtCCA1 genes cannot generate circadian rhythm. Therefore, phase delay in the vein was observed as reported in our previous works [4,9]. Moreover, the circadian oscillation in detached
leaf showed the rapid decay of cellular bioluminescence, which breaks the entrained pattern. Therefore, it was hard to control cellular circadian rhythm with high homogeneity and sustainability in our experiments. The establishment of methodology for homogeneous and sustainability entrainment should be considered in future work.

Our LCD projector system could provide sufficiently strong illumination (maximally 70 µmol m^{-2} s^{-1}) but it has multiple assignments: The power consumption of our LCD projector system was 340 W for photosynthetic photon flux density (PPFD) 70 µmol m^{-2} s^{-1}. The illumination efficiency (light intensity per electric power) of the LCD projector was about 23 and 35 times compared with the FL (15 W) and the LEDs (10 W), that is, the LCD projector system was very high cost. Therefore, the low power consumption LCD projector is required to decrease the lighting-cost. The high contrast between illuminated and blank regions is also demanded in order to control the cellular circadian rhythm by a scanning laser projector.

Moreover, the scanning laser projector has high illumination efficiency and high contrast of illumination of which blank region is completely dark.

There are horticultural meanings in the spatially control of circadian rhythms as follows: The circadian clock gene is upstream of the FLOWERING LOCUS T (FT) involved in floral induction [18,19]. Flora control is expected by regulation of the FT gene expression using local projection such as changing the light quality or period depending on the individual organs. The influence on the plant growth is different relying on the wavelength [20]. Therefore, spatially-controlled illumination with optimal wavelengths and day-lengths for significant organs might increase plant growth and quality.

Conclusions

We showed that the circadian rhythm of lettuce leaf could be controlled spatially by using an LCD or a scanning laser projector. The inverted region of cellular circadian rhythm remained for at least two days, in spite of the rapid decrease of bioluminescence. Although light intensity of the laser projector was very small to a required light intensity for growth of lettuces, the cellular circadian rhythm was successfully controlled. Therefore, the projector illumination is useful to regulate the plant metabolism spatially through the controlling cellular circadian rhythm.

Acknowledgements

This study was partially supported by Grants-in-Aid for Scientific Research (No. 25712029 and 25119721 to H.F.) and the research foundation for Opto-Science and Technology.

References


Figure 3: Circadian rhythms with different two star-shaped initial conditions in an AICCA1::LUC lettuce leaf induced by spatiotemporal illumination using scanning laser projector. (a) Bright and dark star pattern illumination. Dash dotted line showed the boundary two illumination protocols: LD24 (right) and LD26 (left). (b) Snapshots of bioluminescence under DD (Interval between images = 4 h, scale bar = 10 mm). (c) Phase image of bioluminescence in the leaf of Figure 3b (Interval between images = 4 h). (d) Inversion phase images of bioluminescence in the leaf of Figure 3b. t = 12.5 h (left) and 24.5 h (right). respectively. (e,f) Normalized bioluminescence in the star region C and E (gray) and the region to its right (D and F) (white), indicated by the lines in Figure 3d with peak time which was elapsed time since 72 h after applying illumination. Gray and white triangles indicated measured peak time of the normalized bioluminescence and arrows indicated expected peak time. Black bars: subjective day; hatched bars: subjective night.

result, the circadian clock is very sensitive for light cues so that it is able to control the cellular circadian rhythm by a scanning laser projector. Moreover, the scanning laser projector has high illumination efficiency and high contrast of illumination of which blank region is completely dark.


