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

Naoki Seki1, Kazuya Ukai1, Takanobu Higashi2 and Hirokazu Fukuda*1

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 AtCCA1 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 only 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 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−2s−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 its inverted image, was applied using a liquid crystal display (LCD) projector (EB-1915, SEIKO-EPSON KK, Japan) or a scanning laser projector (SHOWWX, MicroVision Inc., USA) at t = 0 (Figures 1a and 1b). The spectrum of the super-high pressure mercury vapor lamp, a light source of the LCD projector, shows a sharp peak at 435.8 nm. Our experiments were carried out using transgenic lettuce (Lactuca sativa L. cv. Greenwave) AtCCA1::LUC, in which an

*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

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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].

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

(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 $t = 31$ and 43 $h$. The star pattern 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 illumination, that is, 14 $h$ after turning off the illumination [16,17]. Under above consideration, these peaks 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. 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 2c. Figure 2e shows that enlarged phase images at $t = 31$ and 43 h. The star pattern 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.
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 = -6 h in the right star, 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 bioluminescence by the moving average provided successfully the in phase images (Figure 3c). The elimination of temporal noise of emerged in the bioluminescence images (Figure 3b) but were emerged and its corresponding phase images. The star patterns were not clearly and 13 h period regions. Figures 3b and 3c show the bioluminescence dash-dotted line in Figure 3a shows the boundary line between 12 h 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 h 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 required to decrease the lighting-cost. The high contrast between illuminated and blank regions is also demanded in order to control the 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.

Conclusions

We showed that the circadian rhythm of lettuce leaf could be controlled spatially by using an LCD or a scanning laser projector. The reversed 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.

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Figure 3: Circadian rhythms with different two star-shaped initial conditions in an AINCCAT: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.
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