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

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Abstract

Plant circadian system works autonomously and responds to various environmental information in cellular level. Conventional studies on controlling of 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 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 $t_e = 0$ (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

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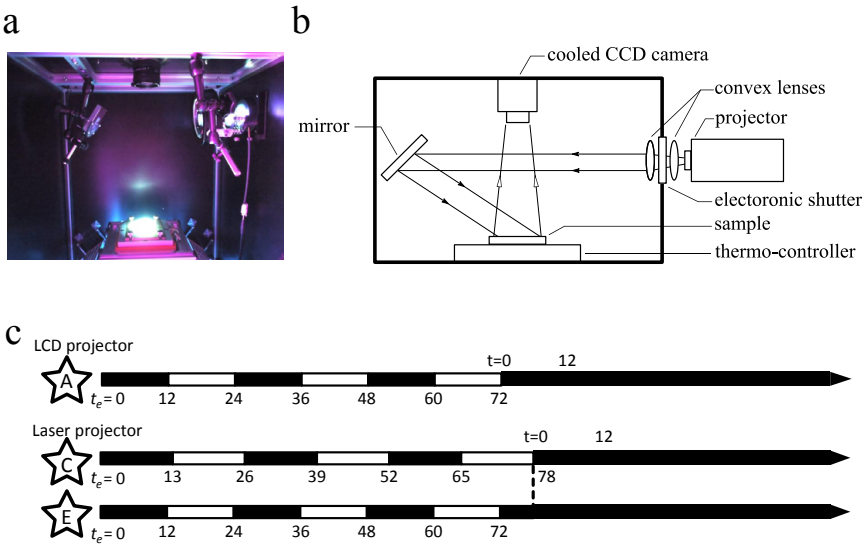

Figure 1: Phase control system of the cellular circadian rhythms. (a) The photograph of the phase control system. (b) The schematic design of the phase control system. Light was reflected in the sputtering mirror through multiple lenses, and it was projected on the sample on the thermo-controller. (c) Illumination protocol of LCD and laser projector. The start time of the entrainment and measurement were at t_e = 0 h and t = 0 h, respectively. The star labelled with A, C and E explained the illumination period of the star-shaped pattern in Figures 2e and 3d. The white and black bar before t = 0 show the bright and dark star-shaped pattern, respectively. Subsequently, black bar shows DD condition.

(G-line), 404.7 (H-line) and 365.4 (I-line) nm [15] and the spectrum spreads 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.

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 = -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 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 monitor with a highly sensitive cooled CCD camera (ORCA-AG, Hamamatsu Photonics KK, Japan) every 30 min at t = 0. The resolution of bioluminescence images was 100 and 200 μm in the experiments using LCD and laser projector, respectively.

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].

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

Where τ_k is the time of the kth 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 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.

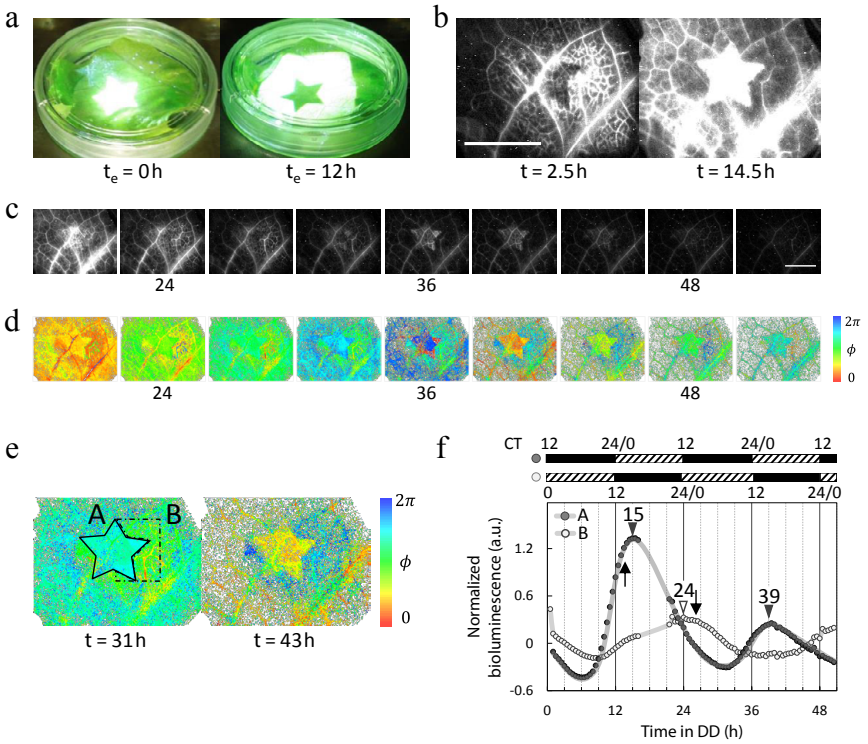

Figure 2: Circadian rhythms with star-shaped initial conditions in an *AtCCA1*::*LUC* lettuce leaf induced by spatiotemporal illumination using an LCD projector. (a) Bright and dark star pattern illumination. (b) Bioluminescence images: the star region was at subject dusk (left) and subject dawn (right). (Scale bar: 10 mm). (c) Snapshots of bioluminescence under DD. (Interval between images = 4 h, scale bar = 10 mm) (d) Phase images obtained from bioluminescence images of the leaf in Figure 2c. (Interval between images = 4 h) (e) Inversion phase images of the leaf of Figure 2c. *t* = 31 (left) and 43 h (right), respectively. (f) Normalized bioluminescence in the star region (black) and the region to its right (white), indicated by the solid line (A) and dashed-dotted line (B) in Figure 2e. There were disturbances in *t* = 16 to 21. Black and white triangles indicated measured peak time of the normalized bioluminescence and arrows indicated expected peak time.Black bars: subjective night; hatched bars: subjective day. The lines were used only to guide the eye.

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 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 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

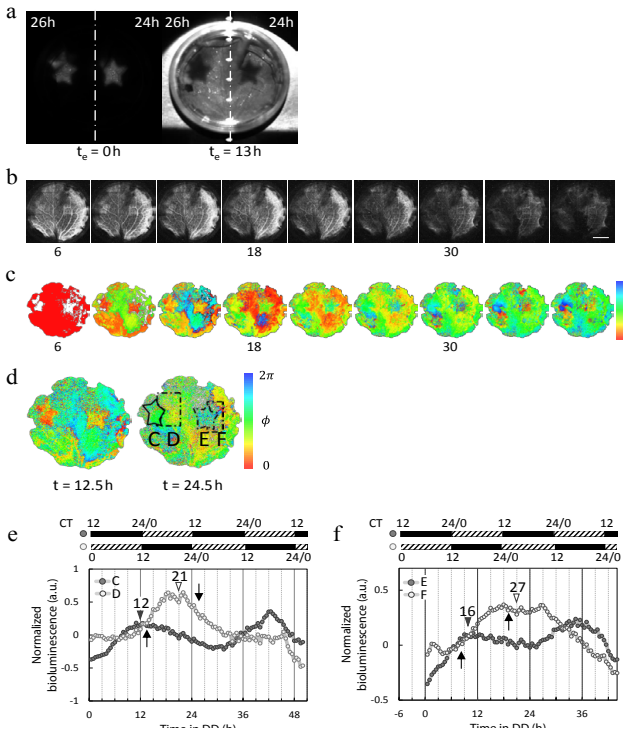

Figure 3: Circadian rhythms with different two star-shaped initial conditions in an *AtCCA1*::*LUC* lettuce leaf induced by spatiotemporal illumination using scanning laser projector. (a) Bright and dark star pattern illumination. Dash dotted line showed the boundary two illumination protocols: LD24 (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 (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.

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 circadian clock accurately, but in our LCD system the light intensity of the blank region was 1 μmol m^{-2} s^{-1}. On the other hand, in our scanning laser projector system, although the laser illumination was significantly weak (maximally 3 μmol m^{-2} s^{-1}) to live through photosynthesis, it entrained successfully the cellular circadian rhythms and printed the reverse phase pattern on the leaves as shown in Figure 3c. From this 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.

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]. Floral 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 inversed 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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