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Controlling plant circadian clock by pulse perturbation based on phase response curve

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Abstract: The circadian clock orchestrates the rhythms of many physiological events, synchronizing them with ambient rhythms, such as the diurnal light-dark cycle and temperature changes. Precise and ecological control of the circadian rhythm provides a key technology for enhancing plant growth in artificial environments. A phase response curve **,** which describes the phase shift on pulse perturbation, is needed to predict or design the circadian rhythm. In this study, we investigated the phase response of the circadian clock in roots to perturbation by temperature pulse under continuous conditions. Under such conditions, roots included all phases at the same time with forming a striped pattern. We observed this pattern under different temperatures. We also observed that imposing only two pulses on the stripe pattern introduced a synchronization state. These results contribute to development of the control of the plant circadian clock by pulse perturbation.

Keywords: Plant factories, Precision farming, Modeling and control of agriculture.

1. INTRODUCTION

1.1 Controlling circadian clock technology in plant factories

The circadian clock orchestrates many physiological processes of plants, including gene expression, photosynthesis, and growth (Dodd, *et al*. 2005). For healthy growth of plants, it is essential to synchronize activities of the cellular oscillators so as to sustain a stable rhythm. Precise and ecological control of the circadian rhythm provides a key technology for enhancing plant growth in artificial environments, *e.g.*, in a closed cultivation system in which light-dark cycles that differ from the 24-h period can be chosen (a so-called plant factory).

1.2 Molecular mechanism of circadian clock

In *Arabidopsis thaliana*, a model plant, the circadian clock is well studied; the periodic expression of clock genes is caused by transcriptional feedback loops in each cell (Harmer, 2010: Jose, *et al*., 2010). The core feedback loop is comprised of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), transcription factors containing a single MYB domain that repress the expression of their activator, *TIMING OF CAB EXPRESSION 1* (*TOC1*). There are two additional phase-specific feedback loops. One is a morning loop involving two *TOC1* homologs, *PSEUDORESPONSE REGULATOR 7* and *9* (*PRR7* and *PRR9*), which are directly activated by *CCA1* and *LHY*. The other loop is the evening loop, which involves the induction of *TOC1* by an unknown evening-phased clock component or function (Nakamichi, *et al*., 2011: Jose, *et al*., 2010). Phytochrome and cryptochrome photoreceptors provide light input signals to the clock by regulation of the abundance of the clock component transcripts, namely *CCA1*, *LHY*, *PRR9* and *GIGANTIA* (*GI*). Conversely, the photoreceptors are reciprocally regulated by the clock (Jose, *et al*., 2010).

1.3 Controlling circadian rhythm using pulse perturbation

The light-dark signal, as in the natural environment, is the most powerful stimulus to entrain the cellular oscillators. Its effect is so strong that all oscillators are reset to the same phase, resulting in complete synchronization. In contrast, a pulse perturbation such as a short-term injection of a bright pulse induces a phase shift without completely resetting the phase. Whether the pulse injection advances or delays the phase depends upon the timing of the perturbation, which is thoroughly described by a phase response curve (PRC). The effect of the pulse perturbation on the plant rhythm can be precisely predicted and designed using the PRC. The suggested control technique is designed based upon the following properties of the coupled phase oscillator:

$$\frac{d\phi_i}{dt} = \omega_i + \frac{K}{N}\sum_{j=1}^{N}\sin(\phi_j - \phi_i) + (1 - L(t))Z(\phi_i) \quad (1)$$

Here ϕ_k and ω_k are the phase and natural frequency, respectively, of the kth oscillator, K represents the coupling strength, N is the number of oscillators, and $L(t)$ represents light intensity ($L(t) = 0$ for dark pulse; $L(t) = 1$ for lights on). The phase sensitivity function $Z(\phi)$ for the dark pulse is given by $Z(\phi) = -0.093 + 0.327\sin(\phi - 1.64) + 0.079\sin(2\phi - 2.19)$

(Fig. 1a), which is determined from experiments on real plants (Fukuda, et al., 2008). This model has precisely predicted the synchronization dynamics of dark pulse stimulation on the plant circadian clock (Fukuda, *et al*., 2013).

1.4 Temperature stimulation on the stripe pattern in roots

We recently reported that the growing root forms a striped bioluminescence pattern of *CCA1* gene expression, which shows alternating bright (indicating subject morning) and dark (subjective evening) regions along the root (Fukuda, *et al*., 2012). We have succeeded in describing the mechanism of formation of this stripe wave using phase oscillator models. Our model suggests that the stripe wave has all phase in roots at the same time. The circadian clock is responsible for temperature stimulation and *ELF3* is a component that is important for circadian clock temperature response (Thines, *et al*., 2010). However, no research has revealed a tissue-specific response to temperature stimulation. The temperature in the root area is one of the most important parameters for growth. In this study, we investigated the response of the circadian clock in roots to perturbation by temperature pulses.

2. Materials and Methods

2.1 Transgenic Arabidopsis thaliana

The line used was a transgenic *Arabidopsis thaliana* accession Columbia-0 (Col-0) carrying a *CCA1* promoter-luc cassette, pABH-CCA1::LUC-C (Nakamichi, et al., 2004), that had been introduced via *Agrobacterium tumefaciens*-mediated transformation. These seedlings carried the firefly luciferase gene under the control of a clock gene promoter, *CCA1*; the luciferase activity oscillated, with a peak at dawn. The phase θ of the oscillation denotes the internal biological time, e.g. θ = 0 rad at subjective dawn and $\theta = \pi$ rad at subjective dusk.

2.2 Growth and measurement conditions

For measurement of the circadian rhythm in roots, seedlings were grown under 12 h fluorescent light (80 μmol m^{-2} s^{-1})/12 h dark at 22.0 ± 0.5°C for about 1 week on vertical plates (90×120×10 mm) of 0.4% gellan gum medium containing Murashige and Skoog plant salt mixture supplemented with 2% (w/v) sucrose and 0.1 mM luciferin. To investigate the effect of low temperature, seedlings were also grown under continuous fluorescent light (80 μmol m^{-2} s^{-1}) at 17°C for about 1 week on the vertical plates.

Bioluminescence of plants was monitored with a highly sensitive EM-CCD camera (Hamamatsu Photonics KK, Japan) in a dark box. Continuous measurements for time-series data were performed every 30 min under controlled temperature at 22.0 ± 0.1°C using a Peltier device. Because the medium was very clear and colorless, light fully reached the roots and the bioluminescence from the roots could be observed.

2.3 Phase analysis of circadian oscillation

To quantify the spatiotemporal dynamics of the circadian oscillations in the plant roots, we introduced the phase $\theta(t)$ of the bioluminescence oscillations at each pixel.

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

τ_k represents the time of the peak of bioluminescence oscillations. To remove noise in the bioluminescence data, a moving average filter with a 12 h window size was applied. Then, peaks and troughs were respectively identified as local maxima and minima of the smoothed data (Fukuda, *et al*., 2011).

3. Results and Discussion

3.1 Robustness of stripe pattern to temperature changes

It has been reported that roots show a desynchronized pattern under continuous light or dark conditions (Fukuda, *et al*., 2012). Roots under continuous conditions formed a striped

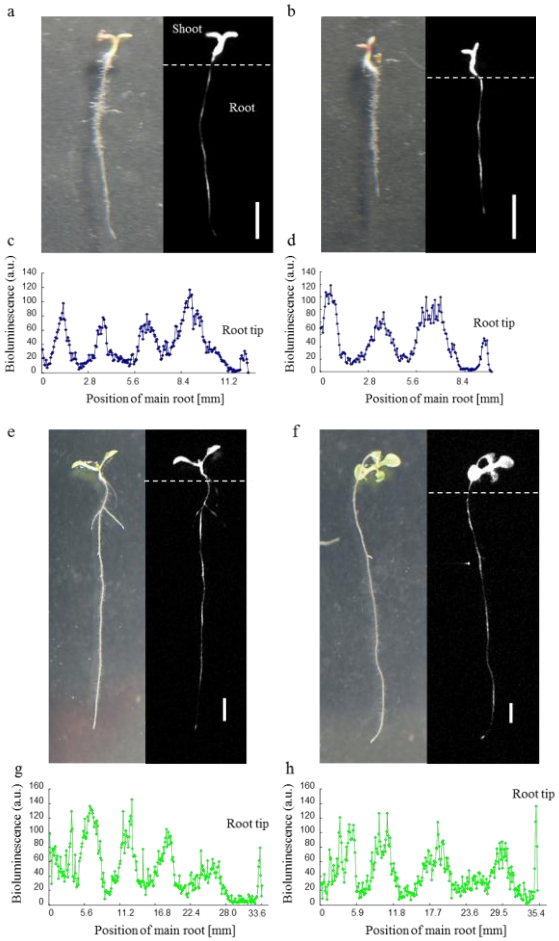

Fig. 1. Bright field images and bioluminescence images of *CCA1::LUC* plant grown under continuous light at 17°C (a,b) or 22°C (e,f). Intensity of bioluminescence along the main root at the junctions of shoot and root in the seedling in Fig. 1(a), (b), (e) and (f) respectively (c, d, g, h). The white scale bars indicate 2.5 mm.

bioluminescence pattern. The number of bioluminescent bands increased by one each day. Bioluminescence peaks travel from the base to the tip along the root with a similar velocity to the growth rate. Phase resetting of circadian oscillation at the root tip plays an important role in pattern formation (Fukuda, *et al*., 2012). To confirm compensation of the stripe pattern at different temperatures, we observed the bioluminescence of *CCA1::LUC* after growth at 17°C or 22°C (Fig. 1). The stripe pattern was observed at both temperatures. In Fig. 1, individual plants sprouted 4 d after germination, except for individual in Fig. 1(b), that sprouted 3d after germination. Control plants (22°C) showed the same number of bioluminescence peaks as number of growth days.(Fig. 1(e, f, g, and h)) Plants grown at low temperature (17°C) also showed the same number of bioluminescence peaks as number of growth days. as (Fig. 1(a, b, c and d)). The results of Fig. 1 indicate that the difference in growth temperature affected only the root elongation rate, not the frequency of circadian oscillation. The stripe pattern was robust for temperature alternation; that is, temperature compensation is one of the most important characteristics of the circadian rhythm. However, the response of the local circadian rhythm to environmental stimuli in roots remains unknown.

3.2 Phase distribution in the stripe pattern

First, we demonstrated that the stripe wave in roots included all phases of circadian rhythm at the same time. Figure 1a shows a snapshot of the stripe wave as a bioluminescence image of plants growing in continuous dark. We calculated the phase at the region of the primary roots and mapped the distribution of the phase with a histogram and phase image (Fig. 2b, c). Primary roots included all phases. Although the

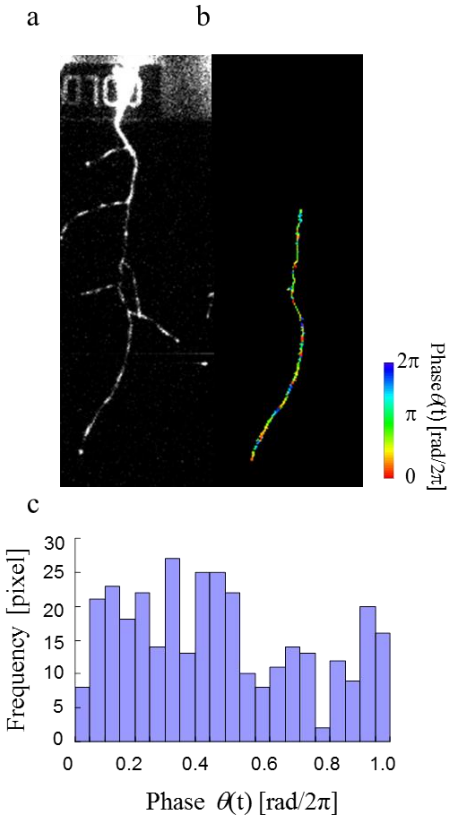

Fig. 2. Bioluminescence image (a) and its corresponding phase θ image (b) of *CCA1::LUC* plant at t = 240 h after the start of measurements when grown under continuous dark. (c) Distribution of phase θ in Fig. 1b.

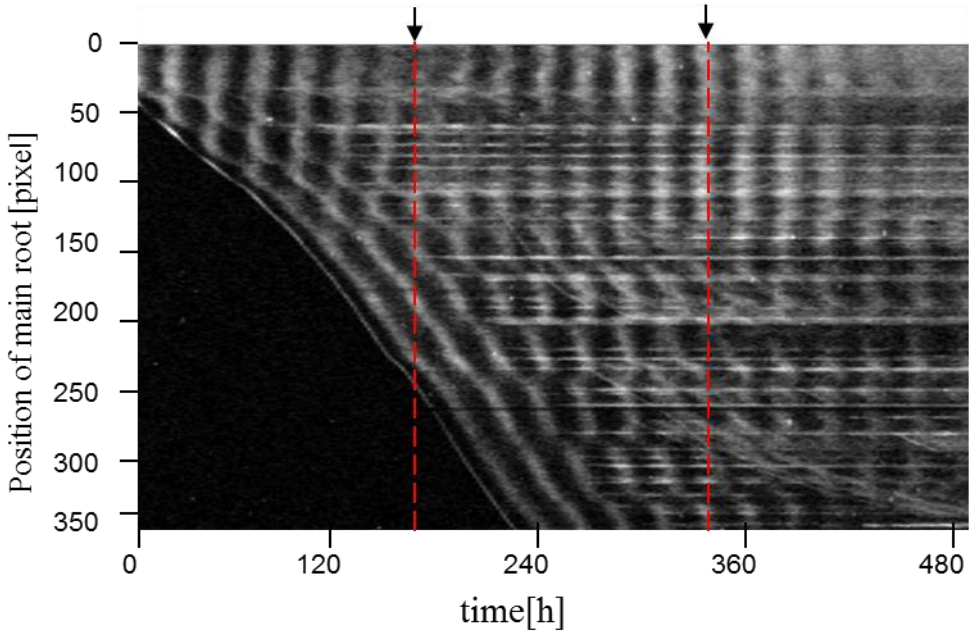

Fig. 3. Space-time plot of bioluminescence of plant grown under continuous dark. Red dotted line indicates the timing of the temperature pulse, which changed from 22°C to 27°C for 30 minutes.

distribution was not flat and the peak of the deviation depended on time, the root stripe pattern contained all phases (0~2 π rad) at one time. stimulation to the stripe wave.

3.3 Pattern perturbation by temperature pulse

To analyze the response of the circadian clock in roots to temperature stimulation, we applied a temperature pulse. We imposed short temperature pulses, alternating from 22°C to 27°C for 30 minutes, on plants at 240 h and 480 h after starting measurements. The upper region in Fig. 3 (root position 0~150 pixels) was synchronized by the first temperature pulse stimulation at 240 h. The lower region in Fig. 3 (root position > 150 pixels) was synchronized by a second pulse. The newly formed area after pulse stimulations formed a stripe pattern. The smooth formation of the new stripe pattern after pulse stimulation indicates that the mechanism of formation of the stripe pattern was not affected by the stimulation. Interestingly, the stripe pattern included a region that had not been fully synchronized by only one pulse stimulation, such as the region of 150 to 160 pixels in Fig. 3.

3.4 Numerical simulation of phase response for pulse perturbation

Controlling the plant circadian clock by pulse perturbation based on PRC is one of the new methodologies for clock control. We could predict the dynamics of the circadian clock to perturbation using a phase oscillator model. First, to analyze the synchronized behavior of individual circadian rhythms by pulse perturbation, we used a periodic pulse perturbation in the phase oscillator model, as in equation (1). $Z(\phi)$ introduced in the model is the PRC for the dark pulse. In the model of individual circadian rhythms, the circadian clock can be entrained by short pulse perturbations. Experimentally, we also observed synchronized behavior for periodic dark pulses. Next we introduced the same PRC into the model of the circadian clock system in roots (Fukuda *et al*., 2012). In a previous study, a three-dimensional coupled oscillator model with a term for phase resetting in the elongation-differentiation (ED) zone was presented as the following:

$$\frac{d\phi_j^{[m,n]}}{dt} = \omega_j^{[m,n]} + \delta_{ik} C \sin(\pi - \phi_j^{[m,n]}) + K \sum_{<l,p,q>} \sin(\phi_j^{[m,n]} - \phi_j^{[m,n]}) + P(t) Z(\phi_j^{[m,n]}) \quad (3)$$

where $\phi_j^{(m,n)}$ and $\omega_j^{(m,n)}$ represent, respectively, the phase and circadian frequency of the cell located at position *(m, n)* of

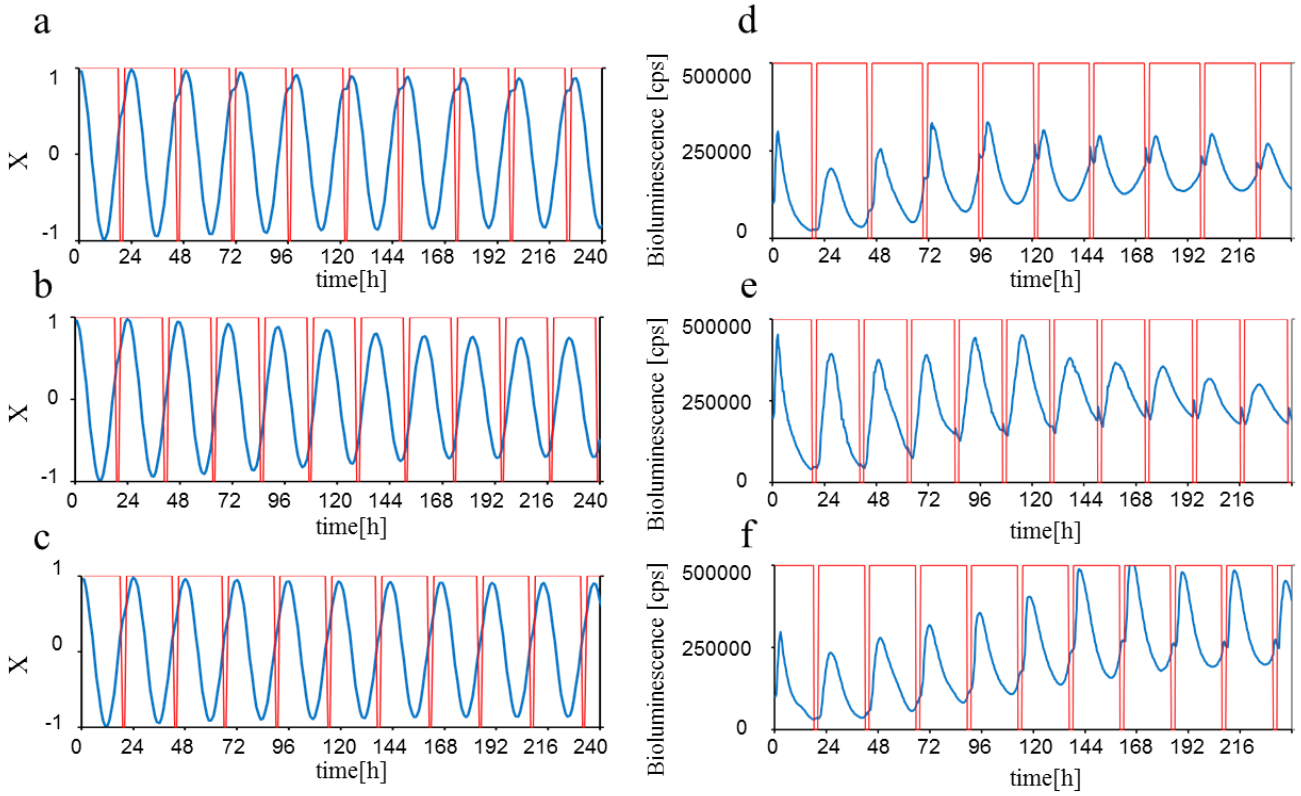

Fig. 4. Response of circadian rhythm to dark pulse perturbation derived from numerical simulation with period of T = 25.6 h (a), 23 h (b), and 22.6 h (c). Bioluminescence signals of the *CCA1::LUC* plant perturbed by 2-h dark pulses with a period of T = 25.6 h (d), 23 h (e), and 22.6 h (f).

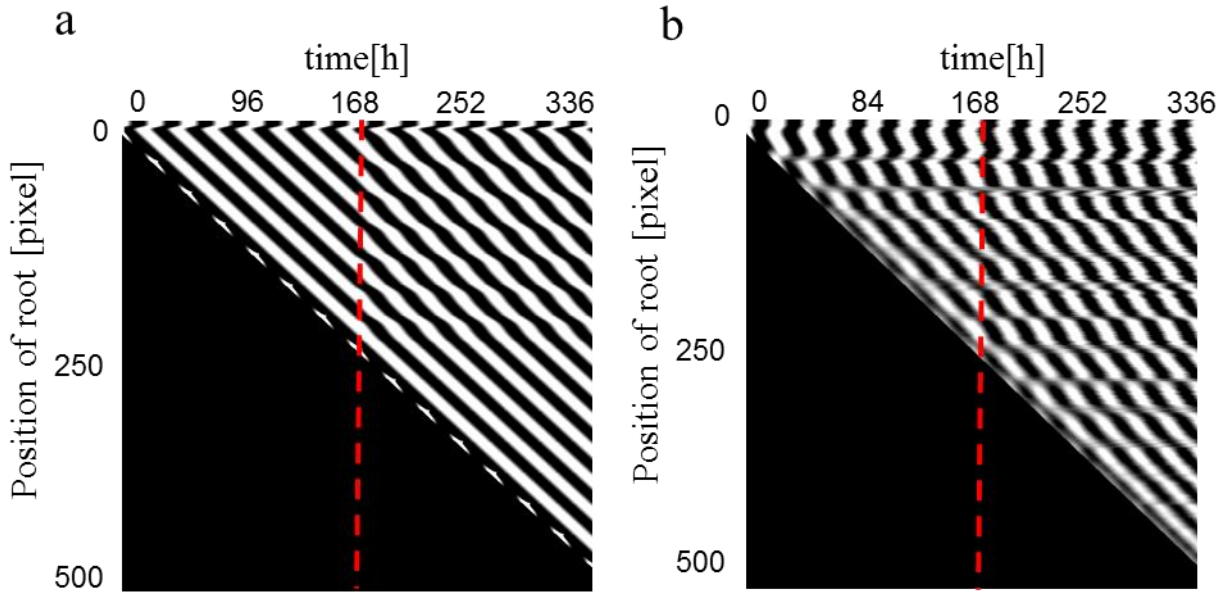

Fig. 5. Numerical simulations of pulse perturbation on the stripe pattern using a model of the circadian clock in roots. In this plot, $K = 0$, $C = 8$, $\sigma_\omega = 0$, $\sigma_\varepsilon = 0$ (a) and $K = 0.02$, $C = 0.8$, $\sigma_\omega = 0.05$ and $\sigma_\varepsilon = 0.05$(b).

section j along the root. We considered a cellular system composed of $N_m \times N_n$ cells (section size) $\times N_j$ cells (root length). The term $\delta_{jk}C\sin(\pi - \phi_j^{(m,n)})$ represents the force of phase resetting, where k indicates the cells in the ED zone, C is the strength of the phase resetting, and δ_{jk} is Kronecker's delta. This force also induces both converse (synchronization) and diverse (desynchronization) effects of the oscillators, depending on $\phi_j^{(m,n)}$. The simulations were performed using equation (3) under a moving boundary, which is denoted $j^* = [(v_R t + x_0)/s]$. Regarding cell proliferation in the meristem, we suppose that daughter cells inherit the phases of their parental cells with stochastic noise $\phi_{j^*}^{(m,n)} = \phi_j^{(m,n)} + \varepsilon$, where ε is a random phase-copying error, which is distributed normally with mean $<\varepsilon> = 0$ and standard deviation σ_ε (Fukuda *et al*., 2012). $P(t)Z(\phi)$ represents the effect of the PRC. In a numerical simulation (Fig. 5), the stripe pattern perturbed by the imposed pulse stimulation was based on the PRC. The area newly formed after the pulse also formed a stripe pattern, although synchronized behavior was not observed. The amount of phase shift could be determined by $Z(\phi)$ and the other parameters. In future work, we will investigate the dependence of the response of the stripe pattern to various parameters using experiments and simulations.

SUMMARY

In this study, we confirmed the responsibility of the circadian clock in roots for perturbation of their rhythm by a short temperature stimulus. We also established the simulation model for circadian clock in roots including a PRC. These results will contribute to develop circadian clock control technology.

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