Spatiotemporal Analysis of Localized Circadian Arrhythmias in Plant Roots

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The circadian system in plants is characterized by substantial cellular oscillations over an approximately 24-h period. Interactions between cellular oscillators trigger phase resets near the root meristem, resulting in localized regions of arrhythmic expression of the clock gene CCA1. The arrhythmicity of the circadian rhythm significantly impacts physiologic processes and growth; however, the exact nature of these arrhythmic regions remains unknown. In this study, we analyzed spatiotemporal patterns in CCA1 expression in arrhythmic regions using a nondestructive imaging technique. We found that formation of root arrhythmic regions involves the emergence of small spiral waves. Near the small spiral waves, the synchrony of cellular oscillations was low. Our findings provide experimental evidence that the arrhythmias are based on desynchronization of cellular oscillators and enhance our understanding of the role of circadian phenomena in root growth.

Keywords : Arabidopsis thaliana, desynchronization, Luciferase bioluminescence assay, phase-reset, spiral wave, stripe wave

INTRODUCTION

Circadian clocks that exhibit rhythmicity over an approximately 24-h cycle are present in almost all living organisms. In higher plants, circadian clocks play crucial roles in regulating numerous biological processes, including gene expression, photosynthesis, water use, and flowering (Harmer, 2000; Nohales and Kay, 2016). An important characteristic of circadian clocks is their entrainment to environmental time cues (zeitgebers) such as changes in external light and temperature cycles (Nakamichi et al., 2004; Higashi et al., 2014). Plant cells act as self-sustained oscillators, with their own circadian clocks that regulate interactions with surrounding cells. Phase waves in leaves and roots (Fukuda et al., 2007; Wenden et al., 2012; Ukai et al., 2012; Fukuda et al., 2012), differences in inherent periods between tissues (Endo et al., 2014; Takahashi et al., 2015; Bordage et al., 2016), and spatiotemporal analytical data (Fukuda et al., 2007; James et al., 2008; Wenden et al., 2012; Fukuda et al., 2013) suggest that the cellular oscillator network involves nonlinear phenomena. It was also reported that growth and developmental processes in roots exhibit marked spatiotemporal patterns, such as striped waves (Fukuda et al., 2012) resulting from strong phase resetting in the elongation-differentiation (ED) region of the root tip. The ED region and meristem, which are core tissues for development, generate specific circadian oscillator network patterns. Moreover, the circadian clock re-phases during lateral root organ initiation because it serves as an auxin signaling gate during lateral root development to facilitate organ emergence (Voß et al., 2015). Thus, investigations of spatiotemporal patterns can provide important data that would enhance our understanding of the mechanisms of plant growth and development.

The root is an ideal system for studying how cellular circadian oscillators generate specific patterns based on their inherent characteristics. For example, a reporter assay for assessing expression of clock genes using AtCCA1::LUC transgenic plants in which a clock gene, CIRCADIAN CLOCK ASSOCIATED 1 (AtCCA1), from Arabidopsis thaliana is fused to the luciferase (LUC) gene as a reporter revealed that A. thaliana and lettuce roots exhibit a striped wave pattern. That is, the locations of gene expression peaks move from the base to the tip along the root (Fukuda et al., 2012; Ukai et al., 2012). These striped waves often exhibit arrhythmic regions in which no circadian oscillations occur. Although our previous mathematical study suggested that this arrhythmicity is caused by desynchronization of the population of cellular oscillators, no experimental data are yet available to confirm this hypothesis.

In this study, we therefore investigated the arrhythmic regions in roots using a bioluminescence imaging method to assess expression of the CCA1 clock gene. Evidence of desynchronization in arrhythmicity was obtained by spatiotemporal analysis of cellular circadian oscillations.

MATERIALS AND METHODS

Experiments were carried out using transgenic A. thaliana (accession number Columbia-0 (Col-0)) harbor-
ing a CCA1::LUC reporter gene (Nakamichi et al., 2005). Seedlings were grown primarily at 22°C for about 4 weeks on a 40-mm diameter dish with 0.2% gellan gum medium containing Murashige and Skoog plant salt mixture supplemented with 2% (w/v) sucrose. Seedlings were grown under 12-h light/12-h dark conditions with fluorescent lighting (80 µmol m⁻² s⁻¹) prior to bioluminescence imaging. Before imaging, approximately 400 µl of 0.2 mM luciferin dissolved in water was poured into the dish. Root bioluminescence was monitored at 22.0±0.1°C using a highly sensitive ORCA-AG camera (Hamamatsu Photonics KK, Japan) in a dark box. The gellan gum medium was very clear and had no color, permitting observation of root bioluminescence.

For precise analysis of the cellular entrainment of circadian rhythm in roots, we introduced the phase of the circadian oscillations, which is determined by following equation:

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

where \(\tau_k\) represents the time of the \(k\)th peak of the bioluminescence oscillatory time series for each pixel. To calculate bioluminescence oscillation peaks, which often exhibited high noise, a moving average with a window size of 12 h was applied for each pixel.

RESULTS AND DISCUSSION

The circadian rhythm in the roots of transgenic A. thaliana CCA1::LUC seedlings was examined using a bioluminescence imaging method. In these seedlings, which carry the firefly LUC gene under control of the CCA1 clock gene promoter, luciferase activity oscillates with a peak at subjective dawn. The oscillation phase, \(\phi\), is an indicator of internal biological time (e.g., \(\phi = 0\) rad near subjective dawn, and \(\phi = \pi\) rad near subjective dusk) (Fukuda et al., 2012). CCA1::LUC plants exhibit circadian oscillations across the entire root region.

Under continuous dark conditions, the main root exhibited a striped bioluminescence pattern in the acquired images (Fig. 1 (a)). Because the number of bioluminescence bands increased by one each day, this striped pattern can be referred to as diurnal growth bands. The bioluminescence peaks in the striped pattern traveled from the base of the root to the tip, with a velocity similar to that of root elongation. Figure 1 (b) shows a space-time plot of bioluminescence images of the main root shown in Fig. 1 (a). Figure 1 (b) also shows that all regions of the root oscillated with an approximately 24-h periodicity (circadian period). Horizontal bright lines at \(x = 4.6, 5.5, 8.1, 9.5,\) and 12.9 mm in Fig. 1 (b) denote the emergence of lateral roots. The regions for the lateral root showed clear circadian oscillation, despite the root tip showing no circadian oscillation in this root. Figure 1 (c) shows a space-time plot of the phase image associated with Fig. 1 (b). Although the striped phase pattern fluctuated over time, the local bioluminescence retained circadian oscillation. In addition, all areas of the main root (0 mm < \(x < 15\) mm in this case) became synchronized with increasing in time, with the exception of some defect regions. These results indicate that the circadian clock in roots is characterized by cellular autonomy and acts to synchronize the entire root system.

The striped waves exhibited some phase defects (phase slips), as shown in Fig. 1 (c). In the regions denoted by red arrowheads in Fig. 1 (b) and (c), phase slips exhibiting large disturbances were observed. Figure 2 shows snapshot enlargements of the phase images in the slip region (#1), demonstrating a wave that rotated counter-clockwise over a 24-h period. Thus, root circadian oscillations generated small rotation waves (small spiral waves) of approximately one-quarter of a millimeter in diameter. Other slip regions (#2, #3 and #4) exhibited similar spiral waves (supplemental Fig. S1). In region #2, a spiral wave that rotated counter-clockwise appeared at \(t = 216\) h and then disappeared at \(t = 336\) h. In regions #3 and #4, spiral waves that rotated counter-clockwise emerged at \(t = 245-302\) h and \(t = 288-360\) h, respectively. Two other roots also exhibited similar spiral waves (supplemental Fig. S2). These results indicate that the slip regions consist of small spiral waves.

To more completely characterize the rotational motion in the slip regions, we compared slip region #1 with a representative normal region (#0). Figure 3 shows a space-time plot of the phases in horizontal sections of these two regions. The representative normal region exhibited
smooth phase variations (Fig. 3 (a)). In contrast, slip region #1 exhibited a disturbed phase pattern (Fig. 3 (b)), indicating that the small spiral waves significantly affected cellular circadian oscillations. Figure 3 (c) shows the bioluminescence oscillations for each pixel on the horizontal sections in regions #0 and #1. The spiral waves strongly suppressed the circadian oscillations and induced phase fluctuations.

Figure 4 shows the synchronization of the cellular oscillators in the slip regions. The synchronization index, $R$, was very small in slip region #1 at $t = 168$ and $192$ h, in which $R$ is defined as $R = N^{-1} \sum_j e^{i \phi_j}$, where $j$ indicates the $j$th pixel, $N$ indicates the number of target pixels, and $\phi$ denotes the average phase. This is strong evidence in support of our hypothesis; that is, root arrhythmias are also caused by desynchronization of the cellular circadian oscillators.

Roots are capable of continuous expansion as a result of branching for lateral root formation. Phase resetting of the circadian rhythm is critical for this branching. Voß et al. (2015) reported that the phase reset might be necessary to ensure hydraulic isolation of cells in the branching root from other cells. Auxin, which is channeled via the lateral root primordium into overlying cells, represses the expression of genes encoding water channel proteins known as aquaporins, leading to complex spatiotemporal changes in hydraulic properties that are necessary for organ emergence (Péret et al., 2012). In addition, lateral root formation is known to be associated with differences in auxin and cytokinin concentrations (Laplaze et al., 2007), and cytokinin suppresses the expression of the clock gene TOC1 (Salomé et al., 2006). Some Arabidopsis pseudo-response regulators (APRRs) respond to cytokinin (Brandstatter and Kieber, 1998; D’Agostino et al., 2000) and are involved in control of the circadian clock (Nakamichi et al., 2005). The C-terminal motif of APRRs is involved in recognition of certain specific DNA for clock regulation (Nakamichi et al., 2012; Gendron et al., 2012). Moreover, the response regulators (RRs) accumulate near the lateral roots, where they regulate cytokinin signaling (Mason et al., 2004; To et al., 2007; Kushwah et al., 2011). Therefore, it is thought that phase reset of the circadian rhythm alters the mecha-
nisms controlling plant hormone crosstalk to enable initiation of lateral root differentiation. However, the behavior of circadian rhythms in the root is not homogeneous, but is complex accompanying with some arrhythmic regions (singularities), as shown in Fig. 1 (c). More detailed spatiotemporal investigations of lateral root formation under fluctuating circadian rhythm conditions will thus be necessary.

The singularity phenomena, that is, arrhythmias in circadian rhythms, are significant in many organisms. The most common conventional example of singularity can be found in “jet lag,” which can pose a significant problem for human health (Yamaguchi et al., 2013). Some studies have revealed that desynchronization of the cellular circadian clocks in the mammalian brain lead to disruption of normal organ function (Ukai et al., 2007). In plants, singularity can be easily induced by employing artificial lighting (Fukuda et al., 2013) (e.g., only four weak perturbations introduced by 2-h dark pulses can induce arrhythmias in the whole plant’s circadian rhythm). These individual-level singularity phenomena induced by external stimuli are understood as a result from desynchronization of the cellular circadian rhythm.

The phase slip in roots is a self-formed singularity of approximately one-quarter of a millimeter in diameter. The horizontal direction of the root is about 5 pixels but the actual number of cells is over 10. This oscillator network size (about 10×10 cells) is sufficient to form spirals, as shown in a previous paper (Fukuda et al., 2007). The novel finding of this study is that the small spiral waves generate localized arrhythmias in phase-slip regions. Moreover, the spiral waves are generally topologically stable, meaning that a relatively strong stimulus is required to disrupt them. However, the spiral waves observed in our study disappeared readily under a stable environment (continuous dark condition), suggesting that the root circadian clock system involves relatively high internal spatial and/or temporal noise. Our results thus suggest that roots are capable of continuous growth despite being exposed to fluctuations in circadian rhythm, although the underlying mechanism remains to be elucidated.

CONCLUSION

Arrhythmias in the circadian rhythm of A. thaliana roots were investigated using a reporter assay to assess expression of the clock gene CCA1. Roots formed stripe waves associated with root elongation and phase-slip regions generated by the emergence of small spiral waves. The detection of small spiral waves provided clear experimental evidence of desynchronization-based arrhythmias and the existence of cell-to-cell interactions in roots. These novel findings of spatiotemporal patterns in roots enhance the current understanding of the role of circadian phenomena in root growth.

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REFERENCES


Figure S1 Enlarged snapshots of the phases located near phase-slip regions #2, #3 and #4. The black (×) symbols indicate the position of the core of the spiral wave.
Figure S2 Example of striped wave with a phase slip in *Arabidopsis thaliana CCA1::LUC* root.

(a) Space-time plot of bioluminescence. (b) Phase in circadian oscillation extracted from the plot shown in panel (a). Red arrowhead indicates the positions of phase slip (#5). (c) Enlarged snapshots of phases located near phase-slip region #5. Black (×) symbols indicate the position of the core of the spiral wave.