Tissue-specific circadian clocks in plants

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Abstract

Circadian clocks affect a large proportion of differentially expressed genes in many organisms. Tissue-specific hierarchies in circadian networks in mammals have been contentiously debated, whereas little attention has been devoted to the concept in plants, owing to technical difficulties. Recently, several studies have **demonstrated** tissue-specific circadian clocks and their coupling in plants, suggesting **that** plants **possess** a hierarchical network of circadian clocks. The following review summarizes recent studies describing the tissue-specific functions and properties of **these** circadian clocks and discusses **the** network structure and potential messengers **that might share temporal** information on **such** a network.

Introduction

The rotation and **orbit** of the earth bring daily light/dark rhythms and seasonal rhythms. To anticipate and cope with recurring environmental changes, many organisms have developed a circadian clock, which increases their fitness by regulating behavior and gene expression. In many multicellular organisms, more than a dozen circadian clock genes have been identified, and most of them are expressed universally (**Table 1**), suggesting that circadian clocks generate self-sustaining and cell-autonomous oscillations in each cell [1,2]. However, these cellular circadian rhythms need to be integrated into a tissue or organismal level to achieve coordinated physiological responses. This has been extensively documented in mammals as hierarchical and tissue-specific functions of networked circadian clocks. The circadian clock in the suprachiasmatic nucleus (SCN) in the brain is known as a central oscillator, whereas **clocks** in peripheral tissues, such as liver, are termed peripheral oscillators [3]. In plants, **many studies have clarified the molecular functions of circadian clock genes, such as**

CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), *TIMING OF CAB EXPRESSION 1 (TOC1)*, *GIGANTEA (GI)*, *PSEUDO RESPONSE REGULATOR (PRR)* genes, and *EARLY FLOWERING (ELF)* genes. A circadian clock system comprised of these genes is tightly interlocked and has multi-feedback loops. Despite the important advances at the cellular level, however, only a few studies have investigated the tissue-specific functions of the circadian clock, and it is unclear whether a hierarchical and tissue-specific function of the plant clock exists.

Recently, several reports demonstrated tissue-specific dynamics and hierarchical coupling of circadian clocks in plants (Figure 1). This review focuses on the current state of knowledge regarding the properties of clocks in a specific cell type, and discusses prospective inter-tissue signaling mechanisms that synchronize clock information at the organismal level.

Main text

Tissue-specific regulation of the circadian clock

The first report that hypothesized tissue-specific regulation of the circadian clock came from an observation of stomatal opening, photosynthesis, and leaflet movement in bean plants [4]. The longer periodic timing of leaflet movements relative to the stomatal opening or photosynthesis period under free-running conditions suggested there are multiple circadian clocks in different tissues. Later, cellular observations were performed using transgenic tobacco cells expressing the cytosolic Ca^{2+} -sensitive luminescent protein aequorin and LHCb::LUC [5], and the respective rhythms were shown to free-run with different periods. After that, more direct observations were reported in tobacco. The Ca^{2+} rhythmicity of cells and tissues was shown to oscillate with distinct differences in phase [6]. Similar tissue-specific oscillations of cytosolic Ca^{2+} were also seen in Arabidopsis. The circadian rhythms of *CAB* gene expression are uncoupled from those of cytosolic Ca^{2+} in some situations, suggesting that distinct circadian clocks are located in different cell types [7].

Other clock-regulated genes have also displayed tissue-specific rhythmicities. The *CAB* expression rhythm is distinct from the *CHS* or *PHYB* gene expression rhythms in free-running periods under constant light condition [8,9]. In addition, sensitivity to environmental cues is distinctly regulated by two circadian oscillators regulating CAB2::LUC and CAT3::LUC. The circadian oscillator regulating *CAB2* expression responds preferentially to light/dark cycles rather than temperature cycles, whereas the circadian oscillator regulating *CAT3* expression responds to temperature cycles rather than light/dark

cycles [10]. Since *CAB2* is mainly expressed in the mesophyll, but *CAT3* is expressed both in mesophyll and epidermis, multiple circadian clock systems in plants have been suggested.

Clock gene expression profiles representing the core loop of the circadian clock system in *Arabidopsis* are also distinct in different tissues. *CCA1* oscillations were shown to have a longer period and lower amplitude in guard cells [11], and had a different period in the center of the leaf than in the center of the rosette [12]. The phase of *TOC1* oscillation was also distinct in the vasculature [13, 14]. More interestingly, *CCA1* displayed striped expression patterns in root tissues [15].

Spatial expression patterns of clock genes

Tissue-specific differential expression of clock genes would be the easiest way to explain such tissue-specific oscillations. Although the majority of clock genes are expressed in most cell types [16-19], some clock genes, including *PRR3*, *PRR9*, and *GI*, show vasculature-enriched expression patterns, based on GUS staining assays [16,20] (Table 1). The non-uniform expression of at least several clock genes among different cell types suggests that tissue-specific clock functions can be partially explained by spatial gene expression patterns.

There is continuing debate over whether these genes are exclusively expressed in vasculature; for example, GFP fluorescence from *PRR3::PRR3-GFP* can be detected even in mesophyll cells [21], and another transgenic line, *GI::GI-GUS*, suggests that *GI* is expressed both in mesophyll and vasculature [22]. There is a caveat to the experiments with GUS staining, however. Even if the *GUS* expression levels are almost the same in each tissue, apparent GUS staining levels will be largely dependent on the cell size and cell density. Since mesophyll and epidermal cells are larger, whereas vascular cells are small and tightly aligned, we supposed that the GUS staining level in the vasculature would tend to be higher. To support our view, GUS staining was more intense in the vasculature, even in *35S::GUS* or equivalent transgenic lines [23,24].

To more reliably estimate clock gene expression levels in a specific tissue, a direct tissue-isolation technique with high spatiotemporal resolution was recently developed [13]. The tissue isolation and subsequent microarray analysis showed that only *ELF4* was a particularly vasculature-rich gene (10 fold higher in vasculature), whereas the other clock genes, including *PRR3*, *PRR9* and *GI*, were expressed both in mesophyll and vasculature, and

PRR9 was rather a mesophyll-rich gene [13]. While the *ELF4* expression pattern strongly implies that there are discrete functions of the circadian clock in vasculature, it is also likely that tissue-specific phase, period, and amplitude regulation are crucial; indeed, as evidenced by the phenotype of an *elf4* mutant, not all tissue-specific oscillations can be explained by *ELF4* alone. [25]. Vasculature-specific functions of *cry2*, *COP1*, and *SPA1*, all of which are involved in circadian clock input, might be crucial to determine the tissue specificities of the clock [26-29].

Hierarchical coupling of tissue-specific clocks

To achieve coordinate responses at an **organismal** level, it appears that the circadian clocks in different tissues need to be coupled with each other. In *Arabidopsis* cotyledons, hierarchical coupling of clocks between vasculature and mesophyll was recently reported [13]. Overexpression of *CCA1* driven by a vasculature-specific *SUC2* promoter caused circadian rhythm perturbation not only in vasculature but also in mesophyll cells. On the other hand, *CCA1* driven by a mesophyll-specific promoter, *CAB*, disrupted circadian rhythms only in mesophyll, **while** robust circadian oscillation in vasculature was still observed. Similarly, the short period (18 hour) phenotype of a *light-regulated WD 1* (*lwd1*); *lwd2* double mutant was lengthened (21 hour) by *SUC2*-driven *LWD1*, suggesting **that** partial complementation of *LWD* function in vasculature affects mesophyll circadian rhythms (about 80% of leaf cells are mesophyll) [30]. Furthermore, it has been suggested that a circadian clock in roots consists of limited numbers of components, and the expression of root clock genes is **coupled** to the **hierarchical dominant** shoot clock rhythm [31,32]. These results demonstrate that there is an asymmetric coupling of circadian clocks from vasculature to mesophyll cells and shoot (shoot vasculature?) to root, suggesting plants have a hierarchical circadian clock system.

If there is a hierarchical organization, then do plants have a central oscillator **that governs a centralized network** analogous to the **SCN** in **mammal** brains (Figure 2) [33]? Since food and water anticipatory behaviors are SCN independent [34], **the SCN** is not an absolute center for time measurement in a strict sense; however, in practice, the SCN oscillator governs the greater part of circadian responses. In that sense, animals have a more centralized clock network. Another **type of network is distributed, or “hierarchy-less”**. In a distributed network, a circadian clock in each cell **makes** an equal contribution to the clock system, **and will be minimally affected by the disruption of a hub**. Since we have observed hierarchical coupling in the circadian clock network **and clear phenotypes caused by perturbation of specific tissues**, plants do not appear to have **a** distributed clock network. A third type of network structure is

decentralized. In a decentralized network, tissues operate **with** local circadian rhythms or time measurement to accomplish coordinated responses, and the clocks in each tissue are loosely coupled to each other.

Recently, a circadian clock in shoot apex was reported to display homogenous synchronicity and therefore was proposed as a plant master clock [32]. However, since shoot apical meristem-specific perturbation of clock functions did not affect circadian clock regulated flowering [13], and there are at least two uncoupled clocks that have different sensitivities to temperature [10], it is likely that there are other hubs of circadian clocks, consistent with the decentralized network model.

Candidate inter- and intra-tissue coupling factor

In both the centralized or decentralized models, plant tissues need to share their time information with each other. However these inter-tissue and intra-tissue couplings might not be strongly coupled. Thain et al. entrained each cotyledon of a tobacco seedling with light-dark cycles or inverted dark-light cycles, respectively, and showed that opposite CAB2::LUC or PHYB::LUC oscillation remained in antiphase under continuous light conditions, suggesting that their clocks were not effectively coupled to each other [35]. We suppose that the reason for the lack of global coupling could be explained by the orthostichy of the vasculature. By contrast, two studies have clearly shown that there is weak, local coupling [12,36].

How do clocks in specific tissues transmit time information to other tissues, and what are the coupling factors? Animals, and mammals especially, use neural and humoral signals to share the time information from the SCN. Presumably, in plants an analogous vascular system mediated inter-tissue “messenger” promotes time information integration. One possible candidate is **sugar concentration** oscillation. Sugar from photosynthesis is transported to all plant cells through the vascular system, and endogenous sugar is crucial for maintaining the amplitude of the circadian clock [37]. Consistent with this notion, the simplified root clock is synchronized by a photosynthesis-related signal from the shoot [31]. Micro RNA (miRNA) is another possible candidate for inter- and intra-tissue coupling. For example, in root growth or phosphate homeostasis, short distance and long distance miRNA transport through plasmodesmata and the vascular system were reported [38,39]. The presence of tissue-specifically expressed miRNAs lends support for this hypothesis [40]. At present, there is no iron-clad evidence that miRNA directly affects the plant circadian clock system; but in

mammals or insects, miRNA has a significant role in clock regulation [41].

Concluding remarks

Apart from the extensive characterization of genetic circuits implicated in the ‘averaged’ circadian clock system at a whole-plant level, only a few attempts have so far been made at analyzing tissue-specific clock and inter-/intra-tissue time information sharing. As illustrated above, plants have a hierarchical circadian clock system somewhat like animals. To better understand the coupling mechanisms and the features of clocks in each tissue, one should not only measure gene expression levels, but also focus on post-transcriptional and post-translational regulation in a specific tissue. From a practical perspective, one key objective is the application to crop plants of any forthcoming molecular insights into the tissue-specific circadian clock system. Some clock genes underlie quantitative trait loci that will improve agricultural traits such as flowering, yield, biomass, disease resistance, and so on [42]. Modification of a circadian clock system in a specific tissue might allow us to manipulate specific agricultural traits regulated by the targeted tissue. As plants and flies have tissue-specific *cis* regulatory signatures [13, 43], an initial approach might combine Chip-seq and high-throughput transcription factor screening to identify key molecules that determine distinct circadian clock systems in specific tissues.

Presently, we still have only a limited number of tools for the tissue-specific analysis of circadian clocks, although we have established a few techniques, such as rapid tissue-isolation, Tissue-Specific Luciferase Assay (TSLA), and tissue-specific disruption of clock functions [13]. In addition to the improvement of these techniques, microscopic observations with an unstable variant of EGFP (e.g., d2EGFP), differentiation induction techniques to obtain single cell types, or laser capture micro dissection will create new avenues to investigate circadian clocks at a tissue or cell level.

Acknowledgements

I thank James Alan Hejna of the Kyoto University for his critical reading and contributions to the manuscript. This work was supported by a JST PRESTO 888067, JSPS KAKENHI grants 22770036, 25650097 and 15H05958, a Nakatani Foundation, and a Mitsubishi Foundation.

Table 1. Spatial expression patterns of circadian clock genes

Clock genes	Spatial expression patterns determined by			
	GUS staining	LUC assay	Transcriptome analysis [13]	
<i>TOC1</i>	Whole seedling [16]	Whole seedling [13]	At least in both mesophyll and vasculature	Slightly vasculature rich
<i>CCA1/LHY</i>	Whole seedling [17]	Whole seedling [14]		Slightly mesophyll rich
<i>PRR3</i>	Vasculature rich [16]			
<i>PRR7</i>				Mesophyll rich
<i>PRR9</i>	Vasculature rich [16]			Mesophyll rich
<i>ELF4</i>				Vasculature rich
<i>GI</i>	Whole or vascular rich [20,22]			
The others				

Figure legends**Figure 1**

Tissue-specific circadian clocks and their communications. Red arrows indicate inter- and intra-organ/tissue communications. (a) There is a simplified clock in roots, and the clock is regulated by a shoot clock through photosynthate(s) (inter-organ asymmetric coupling) [35]. (b) A clock in the vasculature regulates the mesophyll clock, whereas the mesophyll clock scarcely affects the vasculature clock (inter-tissue asymmetric coupling) [15]. (c) Mesophyll cells in a leaf are weakly coupled and produce spiral waves (intra-organ coupling) [14, 41]. In contrast to these coupling mechanisms, locally anti-phased circadian rhythms are not synchronized to the clocks in the other leaf (d), or in the other part of the same leaf (dashed arrows) (e) [38]. Furthermore, several clocks in a specific type of cell show distinct circadian rhythms. (f) Circadian rhythms in guard cells have a longer period and lower amplitude [13]. (g) Mesophyll and epidermal clocks might have distinct sensitivities to light and temperature signals [12]. (h) Circadian oscillation of clock gene expression has a striped pattern along with root growth [17].

Figure 2

Schematic models of centralized, decentralized, and distributed networks. Squares indicate a responsive cell/tissue/organ that functions as a hub. Circles indicate targeted cells that operate specific circadian responses such as gene expression, redox regulation, cell elongation, flowering, and so on. In terms of efficiency, a centralized network has a higher performance, but is less robust than a distributed network. A centralized network can be destroyed by perturbation of a small number of hubs. As the mammalian SCN is the center of circadian rhythms in most cases, the mammalian circadian clock is generally assumed to be a

centralized network. **The observed** hierarchical coupling of clocks in plants suggests that the plant circadian clock network should be centralized or decentralized, because a distributed network is known to be a hierarchy-less network.

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Figure 1

Inter- and intra-organ/tissue coupling

(a) Shoot and/or shoot apex $\rightarrow$ Root

(b) Vasculature $\rightleftharpoons$ Mesophyll

(c) Spiral wave in a leaf

No inter- and intra-organ coupling

(d) A leaf to the other side of a leaf

(e) Between tip and bottom of leaf

Distinct circadian clocks

(f) Guard cell vs. Epidermis, Mesophyll

(g) Mesophyll vs. Epidermis (?)

(h) Stripe wave in a root

Figure 2

Centralized network

↑ More centralized network

Mammal

Plant

?

Decentralized network with asymmetric coupling

Distributed network