The nature of the root clock at single cell resolution: Principles of communication and similarities with plant and animal pulsatile and circadian mechanisms
Pablo Perez-Garcia, Laura Serrano-Ron and Miguel A. Moreno-Risueno

Abstract
Oscillatory mechanisms are present in most life forms and regulate biological processes periodically. In multicellular organisms where more than one oscillatory mechanism is present, they are organized forming a hierarchical coordinated system even at the cellular level. Here, we focus on the Root Clock, an oscillatory mechanism located at the tip of roots that patterns the spacing of lateral organs through oscillating gene expression. We present a series of recent findings and hypotheses about the cellular mechanisms driving the oscillations, how oscillatory information is transmitted within this clock and similarities with other oscillatory systems. Next, we review principles of communication in other pulsatile mechanisms such as circadian rhythms in plants and mammals, and address the possible communication between plant circadian rhythms and the Root Clock. Finally, we advocate for the use of single-cell approaches to address cell communication, synchronization and integration of external outputs into the Root Clock system.

Introduction
Biological oscillators are ubiquitous in living organisms and produce intermittent responses. These mechanisms assure the formation of repetitive structures or the activation of biological responses every certain time. Formation of lateral roots (LRs) and somitogenesis are examples of the generation of repetitive structures [1–5] regulated by developmental clocks (the Root and Segmentation Clocks, respectively), while circadian rhythms synchronize the organisms with the day cycle to anticipate biological responses every 24 h [6,7]. Recent research has deciphered organ and tissue-specific functions regulating the Root Clock, although important remaining questions such as the origin of the cellular pulsatile signal, its propagation as waves of oscillating gene expression and cell communication are not resolved [8–11]. Novel single cell approaches offer the possibility to address these questions. As these techniques relate individual cells to each other based on patterns of gene expression [12–16] they can be used to study the oscillating gene expression responses.

The root clock is a plant developmental clock fed by an unknown pulsatile signal
The repetitive generation of root branching depends on the activity of the Root Clock [1]. This biological clock was first identified using the synthetic 7x-direct-auxin-response-element-DR5 promoter (DR5) [17,18] that was intermittently visualized at the root tip when fused to the Luciferase reporter [1]. DR5 drives expression in the root tip as pulsatile waves propagating through the longitudinal axis. The area of DR5 oscillatory expression was designated as the oscillation zone (OZ), and concomitantly with DR5 several hundreds of genes oscillate in phase or antiphase [1,2]. As the root grows, cells exposed to the in-phase oscillations are imprinted to initiate a new LR. These cells are designated as prebranch sites (PBSs) (Figure 1a), auxin response factor 7 (ARF7) and its inhibitor IAA18/POTENT have been recently identified at the core of the Root Clock oscillator [2]. These regulators form an auxin-regulable oscillatory circuit that sets the frequency and amplitude of the oscillations, functioning, therefore, as the pacemaker of the Root Clock. Simulations of this circuit with cellular resolution show that in-phase oscillations would be initiated at the OZ region known as the basal meristem (BM), being growth and expansion of the...
DR5-expressing cells that would define the propagating waves (Figure 1a). Root Clock frequency can be entrained by auxin pulses produced outside the OZ [2], and which could be triggered by programmed cell death events in the adjacent lateral root-cap [19,20]. The transportation of the auxin pulses into the OZ requires auxin importers and exporters [20], although it is unknown if auxin could be specifically transported into the BM cells. Periodic priming of PBSs also correlates with periodic cell-size variations of vasculature cells, which is predicted to cause an oscillating load of auxin through a reflux-and-growth mechanism [21]. Although this mechanism might explain periodic priming in a Root-Clock-independent manner through the canonical auxin response, a complex auxin oscillatory circuit, with positive (IAA28) and negative feedback loops (IAA18/POTENT-ARF7), underlies the auxin response in cells of the OZ/priming region [2,22]. Intriguingly, changes in root growth did not substantially change periodic priming [1], suggesting that the reflux-and-growth mechanism would not function independently. In agreement with a clock-based mechanism, cell shape morphology is altered during gravistimulation causing additional auxin load in the OZ [23] that increases the oscillation frequency in an ARF7- and IAA18/POTENT-dependent manner [2].

Parallel to auxin signaling, retinal and its oscillating binding partner temperature-induced lipolin (TIL) predict PBSs formation and might initiate the Root Clock activity, while vesicle trafficking and pectin are also important for Root Clock activity [24,25]. Notably, both the in-phase and antiphase oscillations require from ARF7 imperfect oscillatory behavior in antiphase, whereas ARF7 expression is hormone independent [2]. Therefore, an unknown pulsatile signal regulating ARF7 oscillating expression would be critical to initiate and maintain the Root Clock oscillations.

**Is the Root Clock part of a bigger clock system?**

The more extended type of biological clocks in nature are the circadian rhythms [26]. Circadian rhythms maintain 24-h oscillations autonomously, although external signals synchronize the peaks of the morning/evening/night rhythms with the environment. Plants show a complex system of interconnected circadian clocks [27]. Current evidence shows the existence of a
dominant clock in the shoot apical meristem (SAM), while it is proposed that other meristems would act as centers of synchronization showing subsidiary circadian clocks [28,29] (Figure 1b). The Root Clock oscillates approximately every 6 h, thus peaking four times a day, which represents a subset of the circadian oscillatory pattern. This observation raises the question of whether the Root Clock is part of a bigger coordinated clock system. Cross-talk between the Root Clock and the circadian clocks could act to synchronize the production of LRs according to other daily rhythms (Figure 1c). One possibility is that systemic signals from synchronization circadian hubs traveled to reach the OZ, similar to the observed movement of regulators through vascular tissues to exchange circadian information [30,31], or alternatively be coordinated by spatial waves of gene expression [32].

Notably, new Root Clocks appear to be set in the newly formed LRs, as [6,35] demonstrate for circadian clock establishment and recognized in the newly generated organs as it has been observed even at the tissue level, with tissues showing differentiable oscillatory waves of gene expression and variation in the transcript level [8,9,37] such as in the epidermis and vasculature [11,31,38]. A possible mechanism to generate an organ- or tissue-specific clock is through modification of the oscillatory genes or their function in a tissue- or organ-dependent manner [10]. The expression pattern of many Root Clock oscillating genes [1] is tissue-specific, as observed for instance for pericycle and epidermis, while other genes are expressed across multiple tissues. Notably, PBSs are formed in pericycle cells and root waves (an additional output of the Root Clock [11]) would involve the epidermis. Thus, it is possible that oscillating gene expression in different root tissues varies significantly requiring additional components or regulation.

The ability of tissue-specific circadian clocks to maintain a 24-h oscillatory pattern in the absence of entrainment signals varies between organs [32]. These differences could depend on local coupling among neighboring cells as individualized plant cells can be entrained by light to drive cell-autonomous circadian rhythms [39] (Figure 1b). In the case of the Root Clock, DR5 oscillations are observed in certain xylem cells being later extended to adjacent pericycle cells. However, it is unclear if the rest of OZ tissues and cells oscillate synchronically as suggested by transcriptomic analyses [1,2]. The synchronization observed among plant cells in other systems [28,29,32] indicates that synchronization among OZ cells is possible, thus suggesting that the propagating waves of oscillating gene expression likely occur in most OZ tissues.

Comparison of the Root Clock with vertebrate pulsatile mechanisms

During embryonic development, vertebrate organisms periodic segment their body axis by establishing somites, symmetrical masses of epithelium that flank the neural tube on both sides. Somites are formed at regular intervals from head to tail following the growth axis through oscillating waves of gene expression. Mechanistically, somitogenesis and the Root Clock are similar [5]. The somitogenesis oscillator is known as the segmentation clock and it is located in the posterior presomatic mesoderm (Figure 2a). Similarly, the OZ corresponds to growing tissues, and it is in the OZ where the Root Clock oscillator is located [2]. Both the Segmentation and Root Clock control the rhythmicity of a network of genes that, respectively, will travel along the presomitic mesoderm or the OZ to periodically imprint cells that will make up the somite [40] or a PBS. As the formation of somites arises from the interaction between the Segmentation Clock and gradients of cell fate determinants, it is tempting to speculate if analogous to the somitogenesis Wnt-signaling gradient [41] there might be an unknown plant signaling mechanism defining the size of the Root Clock oscillatory field, or whether the end of the OZ represents a determination front.

Is the Root Clock a compartmentalized pulsatile mechanism?

Different circadian clocks within an organism show organ-specific functions [36,37]. Differences can be observed even at the tissue level, with tissues showing differentiable oscillatory waves of gene expression and variation in the transcript level [8,9,37] such as in the epidermis and vasculature [11,31,38]. A possible mechanism to generate an organ- or tissue-specific clock is through modification of the oscillatory genes or their function in a tissue- or organ-dependent manner [10]. The expression pattern of many Root Clock oscillating genes [1] is tissue-specific, as observed for instance for pericycle and epidermis, while other genes are expressed across multiple tissues. Notably, PBSs are formed in pericycle cells and root waves (an additional output of the Root Clock [11]) would involve the epidermis. Thus, it is possible that oscillating gene expression in different root tissues varies significantly requiring additional components or regulation.

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Similar to plants, circadian clocks also form a hierarchical system in mammals. The central pacemaker is in the hypothalamic suprachiasmatic nucleus, being its neurons and astrocytes entrained by daylight [42–44] while the secretion of factors into the bloodstream [43,45] would synchronize the central pacemaker with peripheral clocks (Figure 2b). Daylight might also contribute to coordinate the plant circadian rhythms and the Root Clock, as photoperiod leads the entrainment of the SAM circadian clock and the Root Clock activity requires light perception in the shoot, which, in turn, might release mobile cues such as auxin or other metabolites [24,34]. In addition to photoperiod, entrainment of mammal peripheral clocks requires a series of inputs such as food, fasting or exercise, while the entrainment of the Root Clock also requires local inputs different from light such as gravistimulation [2].

Similar to what is observed in plants, cell physical contact is not a prerequisite for mammalian clock synchronization as proven by sequencing of individual liver cell nuclei, which shows how the disruption of the circadian clock in hepatocytes can influence rhythms of surrounding cells [46], as well as by previous observations [47–49] (Figure 2b). Thus, it is tempting to speculate about the existence of non-cell-autonomous signals synchronizing cells during the Root Clock oscillations or coordinating cells with the root circadian rhythms.

Single-cell studies can help to decipher the mechanistic organization of the Root Clock
Over last years, single-cell technologies have transformed developmental biology, including our understanding of plant development [50]. New cell types have been described in highly heterogeneous and dynamic cellular environments such as meristems, vascular tissues or the LRP [14–16,51–53]. Notably, the study of the early stages of LRP development identified...
hierarchical root developmental trajectories through the characterization of previously unknown cell populations [51] and the analysis of mutants in root stem cell regulators showed new insights in cell fate specification [52]. In mammals, single-cell studies have permitted to organize cellular identities with taxonomic precision [54] or the discovery of scarce cell populations previously unnoticed [55]. Similarly, single-cell approaches can help unravel the mechanistic organizing principles of the Root Clock oscillations.

As previously mentioned, whether all cells from multiple tissues in the OZ oscillate synchronically is unknown. Single cell approaches would be useful to clarify this question, as this technique can decipher cell heterogeneity even in small and dynamic structures or cell populations [13]. For example, in mammals, the study of highly dynamic developmental processes such as tumorigenesis has been benefited from single cell technologies to determine developmental trajectories during the growth of these structures [12].

One possibility to investigate study tissue synchronization during the Root Clock oscillations is to take advantage of the already available root single-cell data [52,56–60]. These data can be re-clustered based on the expression of the Root Clock oscillating genes (e.g., the in phase and antiphase wave biomarkers [1]) and

Figure 3

Possible models for compartmentalization of the Root Clock oscillations. (a) Simultaneous oscillations might occur in all tissues in the oscillation zone. (b) Individual or certain tissues might primarily contribute to the oscillations. (c) The oscillations might start in one tissue (for instance in those with more auxin load) and be hierarchically coupled with other tissues showing a certain degree of phase differences based on an auxin gradient.
compared with the previously described cell-type clusters. The resulting clusters would provide information on the oscillatory phase of the different cell types or tissues, addressing if they undergo through all phases and/or if specific types of waves associate with cell types. Furthermore, the pseudotemporal dimension can be investigated. Computing the pseudotemporal trajectories for the previous clusters would provide information about the differentiation status and potential location in the root longitudinal axis, thus facilitating a more detailed spatial temporal analysis of the oscillations during root development.

Another option is to generate new single-cell temporal data based on the Root Clock oscillatory phase. Thus, different from using the existing root single-cell datasets, the oscillatory phase would not have to be inferred. As roots do not oscillate synchronically, a time-course experiment of the oscillatory phase would require the synchronization of the oscillations in roots. This synchronization could be achieved upon exogenous application of retinal, as retinal has been described to initiate the oscillations [24]. Moreover, the inducible complementation of the Root Clock oscillator mutants [2] could be exploited to synchronize the oscillations upon induction of the corresponding gene. Using these data, it could be addressed if the Root Clock oscillations occur simultaneously in the different root tissues. While simultaneous oscillations are possible in cells from multiple OZ tissues (Figure 3A), a different possibility is that certain tissues are major contributors to the oscillations (Figure 3B), as tissue-specific rhythms have been reported in plants [8] and the DR5 Root Clock oscillatory marker is primarily detected in xylem-pole-pericycle cells of the OZ [4,18].

Finally, the single-cell analyses of Root Clock mutants can be used to decipher the processes of cellular synchronization and wave propagation. Thus, mutants blocked or impaired at particular phases of the oscillations such as iaa18/potent (in the upper phase) or iaa28 (in the lower phase) would identify clusters of cells (or genes within these clusters) mediating the transition to the subsequent oscillatory phase or involved in communication among tissues in some of the possible oscillatory scenarios (Figure 3).

In mammals, the autonomous cellular clock of astrocytes is sufficient to coordinate the circadian system, indicating a strong hierarchy at the cell type level [44]. It would be possible that xylem-pole-pericycle cells instructed the oscillations by becoming sinks for auxin [21], as auxin can entrain the Root Clock oscillator [2] while delivering information to synchronize the pulsatile gene expression among cells [61]. Thus, the Root Clock oscillatory behavior could be hierarchically coupled among OZ tissues (Figure 3C) with a certain degree of phase differences based on an auxin gradient. Furthermore, tissue-specific differences in components of the Root Clock could explain a different oscillating behavior in amplitude or even in the period in each tissue, which could be comparable to differences observed in plant organ-specific circadian clocks [10]. In any described scenario, whether this compartmentalization is occurring at the tissue level or cellular level shall be under inspection. Importantly, all these possibilities could be addressed using single cell techniques that would help to decipher communication and synchronization among cells of the OZ and cells originating the oscillating waves of gene expression. Finally, single-cell approaches can help to understand the outputs regulated by the Root Clock, such as developmental trajectory/ies leading to PBS formation or possible epigenetic marks [56,62] associated with PBS formation or other Root Clock outputs.

Conclusions
The Root Clock is a critical plant developmental mechanism. The DR5 pattern is conserved in some plant species [63], although the degree of conservation of the Root Clock is unknown. Future experiments will address if the Root Clock mechanism is conserved across the plant kingdom and if root branching periodicity is a characteristic of the species. The Root Clock requires non-cell-autonomous regulation maintaining the oscillatory behavior and/or entraining the frequency of oscillations [2,4,24]. However, it is unclear the nature of pulsatile signal initiating the oscillations as propagating waves, whether all cell types oscillate synchronically or equally perceive entrainment signals, or even what all the outputs of the Root Clock are. Furthermore, it is possible that the Root Clock is regulated by the circadian clock or by other external signals in addition to light [34]. Future research should prioritize the use of single cell approaches to address these remaining questions. Findings will permit to establish analogies with other plant and animal pulsatile mechanisms and address if developmental and circadian clocks are interconnected forming bigger and more complex clock systems.

Author contributions

Conflict of interest statement
Nothing declared.

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References

Papers of particular interest, published within the period of review, have been highlighted as:
* of special interest
** of outstanding interest


In this study, the authors describe the key molecular players defining the core of the Root Clock and find that they form an auxin-dependent negative regulatory loop circuit. This work finds the molecular mechanism that explains the oscillations in gene expression in the OZ.


The authors demonstrate tissue-specific circadian behavior using a tissue-specific split luciferase assay to study gene expression. They use this system in Arabidopsis leaves as a proof of concept.


In this work, the authors study the expression of tissue-specific circadian genes in the sugarcane plant. They conclude the existence of important differences in the expression of central circadian genes and in the number of genes subjected to circadian regulation among tissues.


The authors find that variations in cell length promote the cyclical development of elongated and narrow vascular cells, which could be preferentially loaded by auxins to define an oscillatory behavior of lateral root priming.


The authors address the putative role of retinoids in regulating the Root Clock and they found that the combination of retinal and its oscillatory partner, TEMPERATURE INDUCED LIPOCALIN affects the rhythmicity of the root clock, describing their putative role as initiators of role the Root Clock oscillations.


The authors describe the movement of a circadian core clock protein from shoots to roots as a signal to regulate the period of the circadian root clock in a temperature-dependent manner. Therefore, they show that transcription factors could act as long-distance communicators coordinating the plant circadian network.


In this study, the authors observe a molecular connection between light in the upper part of the plant and the Root Clock involving regulation of HY5 in a light-dependent manner to activate the expression of HY5 and its homolog HYH. In turn, HY5/HYH modulates auxin levels causing root branching alterations.


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