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Insights into multilevel spatial regulation within the root stem cell niche

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All differentiated root cells derive from stem cells spatially organized within the stem cell niche (SCN), a microenvironment located within the root tip. Here, we compiled recent advances in the understanding of how the SCN drives the establishment and maintenance of cell types. The quiescent center (QC) is widely recognized as the primary driver of cell fate determination, but it is recently considered a convergence center of multiple signals. Cell identity of the cortex endodermis initials is mainly driven by the regulatory feedback loops between transcription factors (TFs), acting as mobile signals between neighboring cells, including the QC. As exemplified in the vascular initials, the precise spatial expression of these regulatory TFs is connected with a dynamic hormonal interplay. Thus, stem cell maintenance and cell differentiation are regulated by a plethora of signals forming a complex, multilevel regulatory network. Integrating the transcriptional and post-translational regulations, protein–protein interactions, and mobile signals into models will be fundamental for the comprehensive understanding of SCN maintenance and differentiation.

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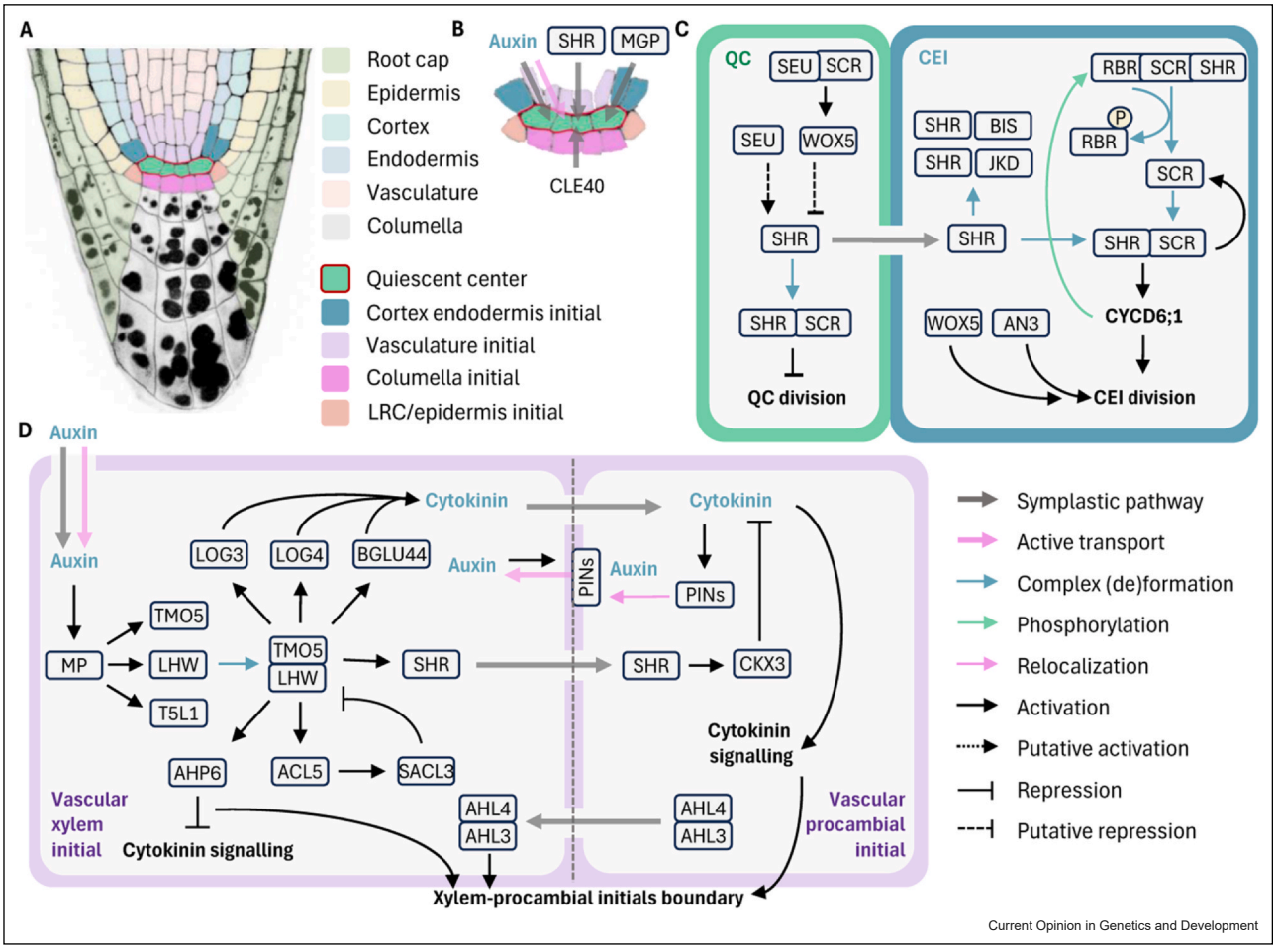
Introduction

Developmental biology focuses on studying how cells, tissues, and entire organs grow and develop. At the foundational level of this developmental process lies the identification of the emergent properties that allow the passage of stem cells to differentiated cells. For plant cells, which are immobile, positional information rather than lineage becomes crucial for determining cell identity and differentiation. The Arabidopsis root apical meristem (RAM) serves as a relatively simply organized, traceable model to study the morphological, molecular, and physiological aspects of stem cell maintenance and differentiation. All root cells originate from the stem cell niche (SCN) in the center of the RAM. The SCN can be separated into two distinct groups of cells: the relatively mitotically inactive quiescent center (QC) and the surrounding initial cells, which have the potential for differentiation. The QC plays a central role in regulating stem cell initial maintenance, which is further discussed in this review. From the SCN shootwards, the root is made up of concentric rings of individual cell files (epidermis, cortex, endodermis, and pericycle) surrounding a central vasculature bundle (phloem and xylem). From the SCN downwards and laterally, we find the columella and lateral root cap (Figure 1a). Thus, along the longitudinal axis of the root, cells divide, transition to expansion, and differentiate into specialized cells. As such, sampling the root into the longitudinal (and/or radial) axis allows dissecting all the developmental stages from stem cells to differentiated cells (in a cell type–specific manner). In this review, we focus on the different levels of regulations, including transcriptional and post-translational regulation, protein–protein interactions, and mobile signals, within the SCN to coordinate cell differentiation and subsequently instruct tissue patterning and overall root development. We specifically focus on the regulatory interactions governing the QC and the initial cells directly adjacent to it, the columella initials (CIs), cortex endodermis initials (CEIs), and vascular initials. The abundance and variety of factors participating in the establishment and maintenance of these cell types and their abundant and tight interconnections make it difficult to decipher system-level rules governing the process. Thus, we finally emphasize how a systems-level perspective, encouraged

Figure 1



Overview of the different levels of regulations within the *Arabidopsis thaliana* root SCN. **(a)** Anatomy of the root tip. **(b)** Anatomy of the SCN with convergence of moving signals in the QC. **(c–d)** Interconnected mechanisms of transcriptional and post-translational regulations, protein–protein interactions, and mobile signals in the QC and CEIs **(c)** and within the vascular initials **(d)**. MGP = MAGPIE; CLE40 = CLAVATA3/EMBRYO SURROUNDING REGION-RELATED 40; BIS = BALDIBIS; AN3 = ANGUSTIFOLIA 3; CYCD6;1 = CYCLIN-D6;1; MP = MONOPTEROS; T5L1 = TMO5-LIKE1; AHP6 = ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6; BGLU44 = B-S GLUCOSIDASE 44; PINs = PIN-FORMED.

and favored by recent technical and methodological advances, could help to unravel the complete picture of root development and, particularly, SCN maintenance/differentiation.

The quiescent center as an organizer center

The QC is responsible for its self-maintenance as well as for generating the initial cells [1,2]. Laser ablation of the QC leads to differentiation of CI into columella cells, suggesting that the QC is also needed to prevent differentiation of its neighbors, working as an organizing center. In line with this, when QC cells are isolated from the surrounding cells by overaccumulation of callose (a cell wall polymer that blocks cell-to-cell communication through plasmodesmata), CIs also differentiate into columella [3]. In addition, it has been extensively demonstrated that QC functions as a damage-resilient source of new stem cells after deterioration of the surrounding initials [4,5].

Many of the molecular factors regulating the balance between stemness and differentiation within the SCN and the particular role of the QC in this process are known, including transcription factors (TFs), microRNAs, hormones, small peptides, and reactive oxygen species (extensive reviews in Refs. [4,6–8]). These many and diverse factors interact into complex regulatory networks, where new players are still being identified. WOX5 (WUSCHEL-RELATED HOMEOBOX 5) is one of the most studied TFs in this process. It is weakly expressed in vasculature and strongly in QC, where it plays a cell-autonomous role in QC maintenance [6–9]. This is achieved, at least in part, by WOX5's transcriptional repression of CYCLIN-D1;1 (CYCD1;1) and CYCD3;3 (by

directly binding to its promoter region [9]), which in turn regulate the activity of the cell-cycle inhibitor RETINOBLASTOMA-RELATED (RBR) [10]. From the QC, WOX5 moves one single cell layer out into the SCN initials, preserving their stemness [11,12]. According to mathematical modeling, the interaction of WOX5 with its target gene, the TF BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO), would restrain the mobility of WOX5, leading, in turn, to self-confinement of *BRAVO* expression to the QC, where it also promotes QC quiescence [13,14]. The sequestration of WOX5 at QC by BRAVO would avoid quiescence out of the SCN while reassuring quiescence of the QC by the expression of both *WOX5* and *BRAVO* [14]. This attenuation of a mobile activator model could be a general mechanism for the self-confinement of TFs in a specific region while maintaining communication with other regions in the vicinity.

In the columella, WOX5 was proposed to function as a QC-derived, non–cell-autonomous factor controlling CI differentiation [11,12]. In the CI, WOX5 represses the differentiation-promoting TF CYCLING DOF FACTOR 4 (CDF4), creating an opposed gradient of WOX5 and CDF4 that would promote differentiation of columella cells (high CDF4) and repress differentiation of CI (low CDF4 due to repression by WOX5) [12]. However, recent results challenge this hypothesis as a non-mobile WOX5 protein fused to two copies of GREEN FLUORESCENT PROTEIN (GFP) is able to complement the CI-related phenotypes of *wox5* mutant, indicating that WOX5 function solely in the QC is sufficient to restore normal CI differentiation [11]. One candidate as a QC-derived mobile signal is the phytohormone auxin. The QC presents a peak accumulation of auxin that fades out toward the vasculature and columella as a consequence of local biosynthesis, polar active transport, and movement through plasmodesmata [15,16]. This local auxin maxima is promoted by WOX5 and participates in the stemness of QC and CI, as well as in the differentiation of columella cells [17]. It has recently been shown that auxin positively regulates transcription and protein stability of the key cell-cycle factor DIMERIZATION PARTNER A (DPa), which in turn promotes CI maintenance [18]. Nonetheless, auxin-induced upregulation of DPa is not CI-specific, so other CI-specific factors could participate in CI maintenance through DPa. Thus, because a plethora of mechanisms converge and regulate the stem cellness in complex networks, new findings come to light that drive hypotheses to be adapted.

Quiescent center as the convergence point of developmental signals

Regeneration experiments after the abscission of the distal RAM (including the QC) show that SCN organization, particularly columella identity, is recovered before QC identity, indicating that the QC might not be the driving force for the establishment of cell types [4,19]. Similarly, during embryonic development or in the emergence of lateral roots, a coherent QC is only detected at late stages, indicating that it does not lead to the formation of new cell types in the new organs [20,21]. In addition, the study of developmental trajectories of individual cells in single-cell RNA sequencing (scRNA-seq) experiments indicates a high degree of freedom for the cells around the QC to become any cell type. When fate probabilities of individual cells are represented in a UMAP (uniform manifold approximation and projection), a clear bias toward specific cell fates is only observed at later pseudotimes [22].

One option is that the QC is just the place of confluence of several signals controlling cell division and differentiation [23] (Figure 1b), such as PLETHORAs (PLTs), SCARECROW (SCR), SHORTROOT (SHR), and other BIRD family TFs, which are all more broadly expressed, but meet and work together in the QC [24–27]. A complex formed by PLTs, SCR, and TCPs (TEOSINTE BRANCHED1/CYCLOIDEA/PCF) activates WOX5 expression particularly in the QC through a system of dosage-dependent combinatorial interactions. PLTs, SCR, and TCPs are all expressed in several meristematic cells, but only in the QC the needed dosage of each of the proteins is achieved [26]. Furthermore, a QC-defining pathway originating from the distal columella has been recently characterized [11]. The CLAVATA3/ESR-RELATED40 peptide (CLE40p) is a differentiation factor synthesized in the columella from where it moves shootward through plasmodesmata. CLE40p differentiation signal needs to be interpreted by distinctly expressed receptor kinases (CLAVATA2 [CLV2] and CORYNE in the vasculature initials and CLV1 and ARABIDOPSIS CRINKLY4 in the QC), ultimately establishing the SCN and QC position and activity [11] (Figure 1b).

Recent scRNA-seq results combined with gene regulatory network inference showed that few genes are QC specific, and a high degree of regulation is originating from more mature cell types toward the meristem. Additionally, QC cells present an over-representation of transcripts related to callose deposition, suggesting that mobility in and out of the QC is tightly regulated, which is a key aspect for QC functioning [28]. All together, we envision the QC as the result of the addition of several (transcriptomic) signatures, some of them regulated even from distant cells, while, at the same time, serving as the source of non–cell-autonomous signals regulating SCN maintenance.

Interconnected feedback loops regulate cortex endodermis initial divisions

The cortex and endodermis, also known as the ground tissue, are formed through the asymmetric division of the

CEIs. This asymmetric division is anticlinal where one daughter cell will continue as CEI, whereas the other daughter cell will undergo a symmetric periclinal division to form the endodermis and the cortex layers. The molecular events, hormonal signaling, and cellular communication underlying the asymmetrical CEI divisions are well-studied and form a complex network, in which new players are still being identified (Figure 1) [29].

Central to this process is the D-type Cyclin CYCD6;1, a component of the cell cycle machinery that is specifically expressed in the CEIs right before their asymmetric division [30]. This highly specific temporal and spatial expression of CYCD6;1 is tightly regulated by the complex of SHR and SCR (Figure 1c) [30–33]. The expression, activity, and complex formation of these two C2H2 zinc-finger TFs, part of the GRAS family, are in turn spatially restricted by hormone levels, mobile signals, protein interactions, and transcriptional regulation. The main mobile signal controlling complex formation is SHR itself. SHR moves from the vasculature to the CEI, where it forms a complex with SCR to transcriptionally regulate CYCD6;1 (Figure 1c) [34]. In turn, complex formation with SCR hinders SHR movement beyond the CEI/endodermis [35]. In addition, other members of the BIRD family, JACKDAW (JKD) and BALDIBIS, will interact with SCR and SHR to limit SHR movement to the CEI/endodermis (Figure 1c) [31]. Moreover, the interaction between SCR and the unphosphorylated RBR also reduces SCR/SHR complex activity within CEI [10]. This interaction is part of a feedback loop where CYD6;1 phosphorylates RBR to restrict its binding with SCR and increase its activity (Figure 1c). A second feedback loop comes into play where the SHR/SCR complex sustains SCR expression (Figure 1c). Finally, SHR and SCR are also under transcriptional regulation within their expression domain, the vasculature and the endodermis, respectively. For example, glutamine-rich SEUSS (SEU) protein and potentially WOX5 transcriptionally activate and repress SHR in the vasculature initials, respectively (Figure 1c) [36]. Moreover, *WOX5* is regulated by SEU (Figure 1c) [37]. The molecular response of this potential feedforward loop between WOX5, SEU, and SHR requires further investigation.

To understand the complex interplay between the hormonal and transcriptional regulatory systems and connect them to CEI divisions, several mathematical models were designed leading to additional insights [36,38,39]. A first model reported that the longitudinal auxin gradient and the radial SHR expression domain precisely indicate the CEI location and its asymmetric division [38]. In addition, the feedback system between CYCD6;1, RBR, and SCR resulted in the proposal of two stable states, one where SHR transport to the CEI is low and asymmetric divisions are absent, and another state where increasing SHR levels suddenly lead to high SCR levels, which are maintained even with lower SHR levels and result in an asymmetric division [38]. Recently, the model that CEI divisions are regulated through a bistable mechanism is challenged [40]. 4D imaging for two days at high resolution showed that SHR can trigger CEI divisions even at low levels, a state that represents the first state of the bistable model where divisions were absent [38]. In addition, SHR and SCR levels followed sigmoid expression dynamics and not a sudden switch [40]. The authors concluded that a sensitivity to SHR and SCR early in the cell cycle leads to a change in the division plane orientation and thus a formative CEI division [40]. A second model showed that differences in complex stoichiometry and transcript abundances might be associated with differences in cell division between the QC and CEI [36]. Specifically, high levels of the SHR and SCR complex promote CEI but repress QC divisions. Recently, a mathematical model was designed that incorporated different cell types, transcriptional regulations, mobile signals, and cell division rules to shed light on the interplay between these factors [39]. In addition to confirming the importance of SHR transport, the model showed an interdependence of QC and CEI division. QC divisions directly affect SHR abundance in the QC and thus subsequently SHR abundance in the CEI [39]. Moreover, new players, including WOX5 and ANGUSTIFOLIA (AN3), were identified and shown to affect CEI divisions [39]. Updating this model with the high-resolution SHR dynamics [40] would be an intriguing avenue for future research.

Hormonal crosstalk maintains two poles of vascular initials

Vascular initials are not clearly defined in the literature. Here, we are focusing on the first cells immediately above the QC, which can be grouped into a central protoxylem axis (xylem initials) and two domains of procambial initial cells that first give place to the procambium and later to all the different cell types of the phloem [41–43]. The establishment and maintenance of these two poles of vascular initials are tightly regulated by the crosstalk between auxin and cytokinin hormones (Figure 1d). The central protoxylem axis is characterized by high auxin response (and low division rates) and the surrounding procambial initial cells are characterized by high cytokinin response (and high division rates) [44–46]. In the xylem initials, auxin is perceived by the TF MONOPTEROS/AUXIN RESPONSE FACTOR 5 (ARF5), activating the transcription of a series of downstream genes, including several basic helix-loop-helix TFs, such as TARGET OF MONOPTEROS 5 (TMO5), TMO5-LIKE1, and LONESOME HIGHWAY (LHW) (Figure 1d) [47–49]. TMO5 and LHW family members hetero-oligomerize promoting

periclinal divisions. The auxin-induced TMO5-LHW heterodimer upregulates the expression of cytokinin biosynthetic genes *LONELY GUY 3* (*LOG3*), *LOG4* [49], and *B-S GLUCOSIDASE 44* (BGLU44)[50], but in parallel, TMO5-LHW also induces the transcription of an inhibitor of cytokinin signaling, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (AHP6 Figure 1d). As such, although xylem precursors present high cytokinin levels, they do not initiate cytokinin responses and, thus, do not enter differentiation [51]. The xylem-synthesized cytokinin moves to the neighboring procambial initial cells where it induces the expression and inner-lateral polar localization of PIN-FORMED auxin efflux carriers, thus, reinforcing the accumulation of auxin in the xylem (Figure 1d) [43–45].

The abovementioned TMO5–LHW complex induces the expression of *CYTOKININ OXIDASE/DEHYDROGENASE 3* (*CKX3*) in the procambial initial cells adjacent to protoxylem, where it degrades cytokinin, sharpening the border between auxin-signaling and cytokinin-signaling domains (Figure 1d). This is achieved through the cell-to-cell mobile SHR, whose expression is induced in the xylem by TMO5–LHW complexes and binds to the promoter of *CKX3* to regulate its expression in the procambium [52]. Furthermore, TMO5–LHW heterodimer upregulates transcription of the thermospermine synthase *ACAULIS 5* (*ACL5*), which in turn induces the accumulation of another basic helix-loop-helix TF, SUPPRESSOR OF ACAULIS5 LIKE 3 (SACL3), which can interact with both TMO5-related and LHW-related TFs, inhibiting the formation of TMO5–LHW complexes (Figure 1d) [53,54]. Thermospermine is a polyamine with signaling functions and has previously been shown to have a role in xylem patterning [55]. It promotes the accumulation of ACL and SACL proteins [56]. Thus, a possible mechanism for the regulation of cell division in the vascular initials is the domain-specific competition of SACLs with TMO5–LHW complexes. In addition, the mobile TFs AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 4 (AHL4) is expressed in the procambium initials, and, together with AHL3, can move into the xylem initials, where they reinforce the xylem-procambium boundary (Figure 1d) [57].

Still, the interplay between vascular initials, other initial cells, and the QC is poorly studied. Mobile TFs as SHR [31,40] or peptides as CLE40 [11] can move between these cells, and understanding the relationships among them would for sure improve our understanding on SCN maintenance/differentiation.

Approaches for a systems-level perspective

The recent surge in single-cell omics technologies, coupled with the increasing computational capabilities for prediction and modeling, holds great potential for uncovering system-level rules and connections in cell development. The improved resolution of generated single-cell atlases has played a significant role in determining cell fates at early developmental stages through pseudotime models within the SCN [22,28,58]. For example, cortex and endodermis fate probabilities within stem cells were determined through single-cell transcriptomics contributing to identifying cell type–specific cell fate regulators, including JKD, MYB36, and SCR [58]. In addition, single-cell omics revealed a gene expression gradient during development rather than a sharp switch on/off of genes, indicating a complex integration of several cues between transition states. The interactions between these cues, including genes, proteins, post-translational modifications, and metabolites, can be unraveled through network inference approaches and should be focused on in future research to comprehensively capture intercellular regulation within the SCN [28,59,60]. These data-driven inference approaches can be supplemented and optimized with additional data types, including *a priori* knowledge on the regulatory proteins, predicted TF binding or docking sites, or (single cell) chromatin accessibility (ATAC-seq) [60–64]. Several approaches have been developed to integrate multiple types of -omics, both mechanistic and deep learning based, although mainly applied in mammalian science and with limited application in plant science [65–67]. A regulatory network model of the root SCN that included transcriptional regulators, miRNAs, and hormones was used to test *in silico* perturbations of individual regulators. The alteration of a few regulators was sufficient to cause a transition in cell identity. For instance, a decrease in the activity of AUX, PLTs, or ARF5 could cause the transition from the QC to the CEI [65]. The integration of different regulatory mechanisms will be fundamental to understand the biology behind SCN differentiation and, in general, developmental decisions.

Concluding remarks

Many diverse and distributed factors, such as transcriptomic and post-transcriptomic regulation, protein–protein interaction, hormonal crosstalk, and mobile metabolites, participate in the establishment and maintenance of SCN and the differentiation of cell types. The need for the joint and coordinated action of all of them makes the system robust, while the possibility of isolating specific regions when needed (i.e. by plasmodesmata closure) would allow rapid and tunable reactions. To achieve a comprehensive understanding of this biological system, our research and methodological strategies should also integrate this diversity and complexity. Modeling approaches offer a great resource to test and predict the outcome of thousands, even millions of *in silico* hypotheses, but the heterogeneous nature of single cell, -omics, and empirical data makes data

integration and systems biology a challenging task. Nevertheless, the endeavor will reap its rewards, as the integration of different regulatory mechanisms and at different levels (molecular, cellular, and organismal), together with high quality, live, three-dimensional imaging of SCN will be fundamental for the future progress of developmental biology.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

None.

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