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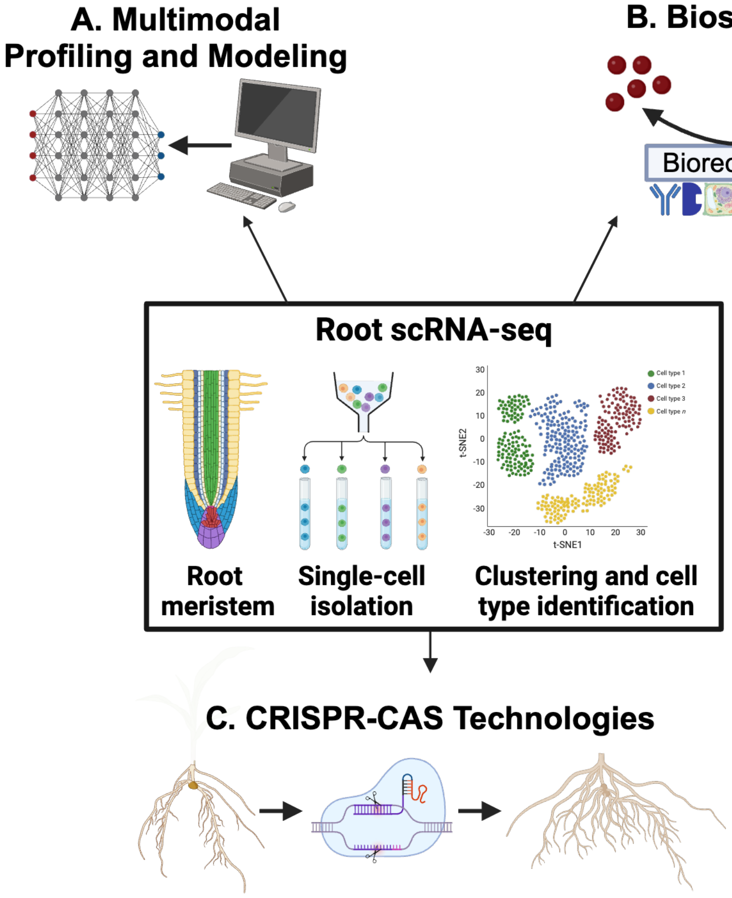
A. Multimodal Profiling and Modeling
B. Biosensors
Bioreceptor
Root scRNA-seq
30
20
10
0
-10
-20
-30
t-SNE2
-30
-20
-10
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30
t-SNE1
Cell type 1
Cell type 2
Cell type 3
Cell type n
Root meristem
Single-cell isolation
Clustering and cell type identification
C. CRISPR-CAS Technologies

Advancing Root Biology: Technologies and Insights from Diverse Examples

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Running Title: ***Advanced Insights into Root Development***

Abstract

Understanding root development is critical for enhancing plant growth and health, and advanced technologies are essential for unraveling the complexities of these processes. This review highlights select technological innovations in the study of root development, with a particular emphasis on the transformative impact of single-cell gene expression analysis. The insights shared here are intended to provide a high-level overview of recent advancements, illustrating how single-cell RNA sequencing (scRNA-seq) has become a pivotal tool in plant biology. Single-cell RNA sequencing has revolutionized root biology by enabling detailed, cell-specific analysis of gene expression. This has allowed researchers to create comprehensive root atlases, predict cell development, and map gene regulatory networks with unprecedented precision. Complementary technologies, such as multimodal profiling and bioinformatics, further enrich our understanding of cellular dynamics and gene interactions. Innovations in imaging and modeling, combined with genetic tools like CRISPR, continue to deepen our knowledge of root formation and function. Moreover, the integration of these technologies with advanced biosensors and microfluidic devices has advanced our ability to study plant-microbe interactions and hormone signaling at high resolutions. These tools collectively contribute to a more comprehensive understanding of root system architecture and its regulation by environmental

factors. As these technologies evolve, they promise to drive further breakthroughs in plant science, with significant implications for agriculture and sustainability.

Advances Box

- **Single-cell RNA technologies (sc/snRNA-seq)**: Enabled detailed, cell-specific gene expression analysis, leading to comprehensive root atlases and precise predictions of cell development.
- **Multimodal Profiling and Bioinformatics**: Integrated analyses of the transcriptome, genome, epigenome, and proteome, enhancing our understanding of cellular dynamics and gene regulatory networks.
- **Innovative Imaging and Modeling**: Advanced imaging and mathematical modeling techniques have provided new insights into cell lineage tracing and non-stereotypic cell division patterns.
- **CRISPR/Cas Applications**: CRISPR technologies have uncovered key genetic elements and regulatory mechanisms in root initiation and formation.
- **Enhanced Plant-Microbe Studies**: Biosensors, microfluidic devices, and sequencing of microbes have advanced our understanding of plant-microbe interactions and root microbiome integration.

Outstanding Questions Box

- How can single-cell RNA technologies be further optimized to capture rare cell types and transient states in root development?
- What are the key regulatory networks and molecular mechanisms governing root-microbe interactions at single-cell resolution?
- How can advanced imaging and modeling techniques be integrated to provide real-time visualization and analysis of root growth and development?
- How can multimodal profiling be expanded to include more comprehensive analyses of the epigenome and proteome in root cells?

These questions aim to guide future research towards a deeper understanding of root development and its broader implications

Review Main Text

Integrating Single-Cell Transcriptomics, Spatial Technologies, Bioinformatics to Uncover Gene Function and Spatial Dynamics in Root Development

Understanding root development is essential for plant growth, as it plays a crucial role in nutrient acquisition, water uptake, and overall plant health (Sparks and Benfey, 2017). Recent technological advancements (Figure 1) have accelerated our comprehension of these complex processes, particularly the interactions between stem cells, developmental and hormone programs, and microbial relationships. Single-cell and single-nucleus RNA sequencing technologies (scRNA-seq and snRNA-seq) have enabled detailed analyses of gene expression at the cellular level, thus providing insights into primary and lateral root development (Minne et al., 2022). By examining cell identities through their molecular profiles, single-cell techniques have facilitated the creation of detailed root atlases and predictions of cell development and gene regulation (He et al., 2024; Shahan et al., 2022). Specifically, scRNA-seq has been effective in defining cell clusters and populations in an unbiased manner, enhancing our understanding of cellular processes. It captures rare cells or cell types, although their low frequency in whole organ atlases may limit statistical power and hinder gene discovery and functional studies. This limitation can be addressed by enriching these rare cell states or tissues, resulting in more specific datasets (Gala et al., 2021). Techniques like Fluorescence-activated cell sorting (FACS) or Fluorescence-activated nuclei sorting (FANS) using fluorescent protein-tagged reporter lines have demonstrated that increased specificity can lead to novel biological insights, as shown in studies profiling the sieve element lineage and early stages of lateral root formation (Roszak et al., 2021; Serrano-Ron et al., 2021b). Alternatively, snRNA-seq is used to access a broader range of cell types across diverse tissues and species, including frozen samples, reducing capture bias (Guillotin et al., 2023). However, snRNA-seq generally captures fewer transcripts and is limited to nuclear RNA, which may not fully represent transcripts with lower nuclear abundance. A combined approach of scRNA-seq and snRNA-seq could provide a more comprehensive analysis, offering both depth and breadth in understanding cell identity in plants. Complementing scRNA-seq, multimodal profiling examines various parameters of the transcriptome, genome, epigenome, or proteome within each cell, offering a deeper understanding of cellular dynamics (Lim et al., 2024).

Single-cell transcriptomics has been proven to be highly valuable in studying root development and initiation. Advances in bioinformatics have enabled the inference of genetic interactions from sequenced transcriptomes and the reconstruction of gene regulatory networks (GRNs), which map the regulatory dependencies among genes (Serrano-Ron et al., 2021a). These advancements, combined with computational models that integrate mechanistic insights with hormonal inputs, have significantly improved our ability to predict root responses in complex scenarios. However, challenges remain in fully utilizing this technology (Kaur et al., 2024). While new protocols using artificial intelligence are beginning to address issues related to data analysis and processing of scRNA-seq raw data (Hsu et al., 2022), other inherent limitations of the technology are still unresolved. One notable limitation of scRNA-seq data is its inherent sparsity. Although scRNA-seq is highly effective for capturing intermediate transcriptional states along developmental trajectories (Seyfferth et al., 2021), it falls short in predicting individual gene expression levels. In contrast, RNA-seq provides a more accurate depiction of gene expression, but it typically reflects specific cell states or a mixture of them. Recent advancements have introduced methods that combine scRNA-seq and RNA-seq datasets, facilitating the identification of novel transcriptional signatures (Li et al., 2024). Despite these advances, comprehensive computational models that integrate scRNA-seq and RNA-seq data to achieve high-resolution, single-cell transcriptomic insights are still lacking. Furthermore, integrating scRNA-seq with other single-cell omics datasets will be crucial for a deeper understanding of cellular identity and function. While strategies to "anchor" different datasets together (Stuart et al., 2019) have been developed, there is a need for new methods that integrate various types of data into a cohesive, multidimensional system. Such innovations could transform our approach to studying root development and advance biotechnological research.

Another limitation of scRNA-seq is its inability to retain the spatial context of cells within tissue samples, which complicates the analysis of spatial organization and biological interactions during root initiation and formation. To address this issue, a new suite of technologies has emerged, offering solutions to the lack of spatial dimensionality inherent in traditional scRNA-seq approaches (Yin et al., 2023). Among these spatial transcriptomics technologies, Slide-seq (Rodriques et al., 2019) and Stereo-seq (Xia et al., 2022) utilize chips embedded with beads of varying diameters. These beads, each carrying molecular identifications and location barcodes, enable reverse transcription and double cDNA synthesis directly on the sample. The tissue is finely dissected, typically through cryo-sectioning, and placed on the chip so that each cell interacts with at least one bead, though ideally, 10-15 beads per cell are preferred. Slide-seq uses beads on a micrometer scale, while Stereo-seq employs beads on a nanometer scale. The choice between these techniques depends largely on cell size, with Stereo-seq offering higher resolution for larger cells by increasing the number of beads and, consequently, the number of reads compared to traditional scRNA-seq.

Additionally, spatial transcriptomics technologies based on high-throughput fluorescent in situ hybridization (Xia et al., 2019; Yin et al., 2023) offer another approach. Although these methods detect a limited number of genes per cell, they are effective for studying multiple genes simultaneously. Multiplexed fluorescence in situ hybridization methods, already applied to root studies (Nobori et al., 2023b), are expected to become more prevalent in addressing root initiation and formation. While single-cell transcriptomics provides detailed insights into gene expression at the cellular level, accurately pinpointing these expressions within the complex architecture of plant tissues remains a challenge. This spatial information is vital for understanding the functional dynamics and interactions of cells within their natural environments. Integrating scRNA-seq data with spatial information (Robles-Remacho et al., 2023) shows promise in revealing RNA functions within specific spatial contexts. Techniques like PHYTOMap (Plant Hybridization-Based Targeted Observation of Gene Expression Map) (Nobori et al., 2023b), and MERFISH (Nobori et al., 2023a) have facilitated single-cell and spatial gene expression analysis in whole-mount plant tissues, including insights into spatial variability in plant immune responses and others.

Notably, scRNA-seq has been combined with spatial transcriptomics to investigate how trees regulate secondary radial growth. In a study in trees (Du et al., 2023), a developmental gradient of meristematic cells from primary to secondary vascular tissues was characterized in stems, identifying two types of cambium meristematic cells that distinctively generate phloem and xylem cells. Combining scRNA-seq with spatial transcriptomics has also been proven to be useful to unravel dynamic molecular maps of cambium differentiation in the primary and secondary growth of trees (Li et al., 2023). Different regulatory networks involved in cell differentiation from cambium to xylem precursors and phloem precursors were identified and connected with auxin accumulation and distribution. scRNA-seq has also been successfully applied to other crops such as pea (Chen et al., 2024b) and medicago (Pereira et al., 2024) to study shoot development and cell-type-specific responses to boron deficiency and cell lineages involved in nodule development, respectively.

Despite these advances, challenges persist in spatially localizing gene expression within non-model plants and more agriculturally relevant conditions such as plants growing in soil. Issues include the need for non-live imaging, tissue sectioning, and limited sensitivity to low-abundance transcripts (Wang et al., 2024). Additionally, the vast amounts of data generated by spatial transcriptomics - often reaching terabytes - pose significant challenges in processing, analysis, and visualization. As technology evolves, the development of advanced data analysis tools and statistical methods (Bressan et al., 2023), along with the potential for 3D and spatial-temporal approaches, will be crucial for offering deeper insights into cellular dynamics over time.

Integrating Spatial Context, Gene Editing, and Advanced Imaging for Enhanced Understanding of Root Development

Integrating single-cell transcriptomics with spatial technologies and advanced bioinformatics represents a significant leap forward in our understanding of root development. The combination of these spatial techniques with CRISPR gene editing allows for precise manipulation of genes identified through single-cell analyses, thereby validating their roles in root development and hormonal responses (Zhang et al., 2021). High-throughput imaging and biosensors further complement these approaches by providing real-time observations of root-environment interactions and molecular events (Rowe and Jones, 2021). Together, these technologies provide a comprehensive understanding of root dynamics, from the cellular and tissue levels to how roots respond to environmental stimuli. This integrative approach enables researchers to gain a more nuanced understanding of root development, especially in unraveling the complexity of how different genetic and environmental factors influence root architecture and function.

The advent of CRISPR-Cas technology has further revolutionized research into root development. By enabling precise and targeted modifications of genes involved in critical processes such as hormonal signaling and the formation of root structures, CRISPR allows us to explore the specific roles of individual genes with unprecedented accuracy (Cardi et al., 2023). This ability to manipulate genetic pathways in root development sheds light on how roots grow, divide, and adapt at the molecular level. As detailed below, CRISPR facilitates both the exploration of gene function and the ability to generate new root phenotypes, allowing for deeper investigations into the regulatory networks controlling root morphology and physiology (Wachsman et al., 2015; Zhang et al., 2021).

Equally transformative are high-throughput imaging techniques, such as confocal microscopy and live-cell imaging, which allow real-time visualization of root growth and interactions with their environment (Sozzani et al., 2014). These technologies provide invaluable insights into how roots dynamically respond to environmental conditions, including changes in nutrient availability, water, and soil structure. Observing these processes in real time has enabled researchers to understand the adaptive strategies of roots, such as how root hairs extend or how lateral roots emerge in response to external signals. In addition to imaging, biosensors have become an essential tool for monitoring molecular events within root cells. These sensors detect and measure key molecules such as hormones and nutrients, revealing how these factors drive developmental changes and signaling pathways within the plant (Beltrán et al., 2022; Madison et al., 2024; Naresh and Lee, 2021). By monitoring the presence and activity of hormones, nutrients, and other key molecules, biosensors will help in understanding of how plants perceive and respond to environmental changes, and how these responses could be modulated by interactions with microbes as briefly described below.

Another key development in understanding root biology is the integration of metagenomics, which characterizes the root microbiome and explores its role in shaping root growth and function (Nwachukwu and Babalola, 2022). The complex communities of microorganisms associated with root systems have been increasingly examined, leading to new understandings of how the microbiome influences root development and nutrient acquisition. These studies have progressively revealed deeper insights into the symbiotic relationships between roots and microbes, underscoring the microbiome's evolving role in promoting root growth, optimizing nutrient uptake, and enhancing plant health across diverse environmental conditions (Das et al., 2023; Edwards et al., 2015). Metagenomic sequencing techniques further provide detailed analyses of these microbial communities, identifying key microbes that support root growth and help plants adapt to environmental stress. Understanding these root-microbe interactions is crucial for gaining a comprehensive picture of root development and function within their natural ecosystems.

These technological advancements are essential for achieving a comprehensive understanding of root development (Figure 1). By integrating stem cell, developmental, and hormonal pathways with microbial interactions, these innovations are set to shape the future of sustainable agriculture, improving crop resilience and productivity. This review briefly highlights key technological advancements, particularly scRNA-seq, which have significantly expanded our knowledge of root development - from studies on the SHOOTROOT and SCARECROW relationship in primary roots (Shahan et al., 2022), to recent breakthroughs in imaging technologies that offer insights into lateral root formation and plant-microbe interactions. Furthermore, the review presents examples of CRISPR/Cas applications in this area.

Revealing Developmental Trajectories Through Single-Cell Transcriptomics

Single-cell root atlas has revealed developmental trajectories in both wild-type and cell identity mutants, with high-resolution characterization of mutant molecular phenotypes achieved using scRNA-seq, particularly in epidermal and ground tissue mutants (Denyer et al., 2019; Ryu et al., 2019; Shahan et al., 2022). These studies have unveiled the steady-state transcriptional status of cells, which contributes to the molecular basis underlying observable phenotypic traits, demonstrating the power of single-cell approaches in molecular phenotype discovery. Additionally, droplet-based scRNA-seq has been effectively applied to Arabidopsis primary roots in multiple studies (Denyer et al., 2019; Jean-Baptiste et al., 2019; Ryu et al., 2019; Shahan et al., 2022; Shulse et al., 2019; Turco et al., 2019; Wendrich et al., 2020; Zhou et al., 2020). These investigations have validated the reliability of scRNA-seq data and confirmed the utility of known cell type markers for annotating root cell populations. Furthermore, plate-based scRNA-seq methods, such as Smart-seq, have been employed to profile specific root cell types, such as phloem cells, revealing developmental trajectories during tissue maturation (Roszak et al., 2021).

Single-cell omics techniques have further shed light into the dynamic responses of *Arabidopsis* roots to environmental cues. These studies investigate how changes in sucrose levels and heat shock stress induce cell type- and developmental stage-specific transcriptional responses (Jean-Baptiste et al., 2019; Shulse et al., 2019). This research underscores the adaptability of roots to environmental fluctuations and provides a molecular understanding of stress responses at a cellular level.

Protoplast-based techniques have demonstrated efficacy in *Arabidopsis* root apical meristem studies, yet they generally necessitate fresh tissues and may induce changes in specific parts of the transcriptome (Denyer et al., 2019). In contrast, nuclei-based methodologies are emerging as viable alternatives, capable of transcriptome profiling through single-nuclei RNA-seq (snRNA-seq) or identification of open chromatin regions via single-cell sequencing using Assay for Transposase Accessible Chromatin (scATAC-seq), applicable to both fresh and frozen tissues. Utilizing scATAC-seq on *Arabidopsis* roots, a few studies have revealed distinct cell identities through accessible chromatin regions, which often differ from those identified by scRNA-seq (Dorrity et al., 2021). This technique has also aided in pinpointing transcription factor-binding sites specific to individual cells or clusters, thereby facilitating the reconstruction of gene regulatory networks (Dorrity et al., 2021; Marand et al., 2021).

Single-cell RNA sequencing has deepened our understanding of the roles of SHR and SCR in Arabidopsis root development, providing insights into how these molecular regulators influence cellular differentiation and identity. Through sophisticated computational analyses, it has been shown that SCR mutant cells span a spectrum between cortex and endodermis identities, indicating a continuum rather than discrete cell types and that SHR mutant cells predominantly maintain cortex-like characteristics throughout developmental stages. These findings challenge previous notions of static cell identities and propose a dynamic model where cells transition between developmental states. This example highlights the power of single-cell gene expression analysis in unraveling complex processes like the trans-differentiation observed in SCR mutant cells, which exhibit characteristics of both cortex and endodermis cell types.

A Combination of New Methods and Traditional Technologies Improves Our Mechanistic Understanding of Root Initiation and Organogenesis

Organ formation typically occurs during embryogenesis. However, plants have the remarkable ability to produce entirely new organs not present in the embryo as part of their normal development. This flexibility allows plants to generate the same organ under different developmental programs, with roots being a prime example. Thus, new roots can be initiated not only during embryogenesis but also through lateral and adventitious (or shoot-borne) root formation, as well as from regenerative programs following wounding. Leveraging novel technologies, in combination with established methods, some of them which have been updated using new molecular technologies, has led to a more comprehensive understanding of the intricate processes underlying the initiation and formation of new roots (Cabrera et al., 2024; Gaudinier et al., 2023; Serrano-Ron et al., 2021a; Zhu et al., 2022).

Lateral root initiation and formation have been extensively studied over the last few decades, with recent approaches being critical in providing insights into both the cellular and molecular contexts. These advances have allowed for a more comprehensive description of developmental mechanisms and the generation of new hypotheses (Torres-Martínez et al., 2022). Seminal studies identified the pericycle in *Arabidopsis* as a reprogrammable tissue from which lateral roots are initiated. These studies also characterized the formative process into seven developmental and/or temporal stages, distinguishing the central and lateral growth domains within the lateral root primordium as intrinsically different in terms of developmental patterns and cell morphologies (Perianez-Rodriguez et al., 2014; Zhang et al., 2022). In the following years, many developmental mechanisms and molecular players were identified, with auxin showing a prevalent role in orchestrating lateral root initiation and formation through different regulatory modules composed of AUXIN RESPONSE FACTORS (ARFs) and their AUX/IAA partners. These factors regulate lateral root initiation, cell division, and growth through specific downstream targets (e.g. LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) factors, GATA TRANSCRIPTION FACTOR 23 (GATA23), etc.), either alone or in combination with various receptor-like protein kinases (e.g. ACR4, TARGET OF LBD SIXTEEN2 (TOLS2), RECEPTOR-LIKE KINASE7 (RLK7), etc.) or peptides (e.g. GOLVEN/ROOT GROWTH FACTORS (GLVs), etc.), while root developmental regulators (PLT factors and SCR) were shown to be involved in tissue patterning and/or stem cell specification (Du and Scheres, 2017; Goh et al., 2016; Torres-Martínez et al., 2022; Zhang et al., 2023b). Additionally, imaging studies combined with mathematical modeling to trace cell lineages and divisions showed that the central and lateral growth domains of the lateral root primordium emerged from non-stereotypic cell division patterns (von Wangenheim et al., 2016). Moreover, the inference of a lateral root formation gene regulatory network identified the central growth domain to be defined by mutual inhibition of ARFs (Lavenus et al., 2015). Despite all this knowledge, it was not until the advancement of the scRNA-seq technology and more sophisticated four dimensional models recapitulating cell behaviors and growth mechanics that a more comprehensive picture of lateral root histogenesis and patterning was obtained.

High-throughput scRNA-seq has emerged as a powerful tool to decipher the processes of lateral root initiation and morphogenesis. Using gravistimulation to synchronize lateral root formation, scRNA-seq was performed in dissected regions of previously gravistimulated roots at various time points (Gala et al., 2021). This study captured the first molecular events associated with lateral root initiation, demonstrating that root initiation could be part of a developmental trajectory involving the stele of the primary root. It also identified an early cell fate switch during lateral root initiation involving novel regulation, such as chromatin remodeling factors. As this dataset also captured primary root tissues, it allowed for the identification of subpopulations of endodermis and pericycle cells responding in coordination during lateral root initiation. Remarkably, this suggests communication between the newly forming organ and the host tissues as part of the organogenesis mechanism. A novel computational model integrating spatial tissue growth mechanics and auxin transport advocates for the necessity of this coordination, proposing that topological cues are interpreted at the molecular level affecting auxin distribution (Ramos et al., 2024).

As lateral root initiation and formation occurs in a small number of cells, a different approach using a lateral-root-specific organogenesis promoter to isolate cells through FACS followed by scRNA-seq was employed (Serrano-Ron et al., 2021b). This research led to a model in which a single group of precursor or founder cells, termed primordial cells, rapidly reprograms to form three developmental trajectories in two sequential steps, with pre-stem cells located in the central region of the new organ, pre-vascular cells on the sides, and flanking cells in the upper and lower parts of the new organ. Intriguingly, as these developmental trajectories are established following three spatial orthogonal axes (a left-right axis to delimit the sides, an upper-lower axis to delimit the flanks and a proximodistal axis to delimit the central part of the primordia) the existence of unknown positional information cues is hypothesized. Additionally, traditional time-course laser ablation of the central and flank regions of the newly forming organ, followed by confocal microscopy, showed a pre-established sequence for tissue initiation that cannot be reversed (Serrano-Ron et al., 2021b). A combination of dynamic Bayesian network inference alongside tree-based methods, based on a time-course dataset of RNA-seq cells isolated through FACS using single and double fluorescent markers across the lateral root stem cell trajectory, demonstrates the existence of a gene regulatory networks structured as a morphogenetic cascade (Cabrera et al., 2024). This network topology could explain why root initiation cannot be easily reversed and requires restarting if the forming organ is damaged.

An implemented version of the classical clonal analyses was used to trace lineages during lateral root initiation through confocal laser microscopy (Torres-Martínez et al., 2020). In this system a heat shock-induced transposition of a DS1 transposon within the construct 35S-DS1-H2B:YFP results, when heat is applied, in the production of a yellow fluorescent protein (YFP) labeling a specific cell lineage. As a result, it was shown that founder cells are progressively recruited by pre-existing ones, which could provide positional information during organogenesis. A different methodological approach proposes to record *in vivo* signaling networks during lateral root initiation using orthogonal serine integrases to induce a switch between fluorescent reporters (the mScarlet and the mTurquoise fluorescent proteins) through site-specific and irreversible DNA recombination (Guiziou et al., 2023). The switch would be driven by the promoter of the gene of interest and can be used to build history-dependent circuits, facilitating the understanding of regulation through developmental trajectories. Light sheet analysis of cell division, combined with information of the cell geometry derived from computational volumetric segmentation was used to generate a four dimensional reconstruction of the early developing organ (Schütz et al., 2021). This study shows different cases of asymmetries in volume partition among daughter cells, which could relate to cell fate specification and be involved in establishing the new developmental axes for the new organ. Future experiments addressing the specific four dimensional reconstruction of the developmental trajectories during root initiation could facilitate a more comprehensive understanding of how tissue growth mechanics and cell fate specification are coupled.

Understanding lateral root formation in the context of plant root systems can be instrumental in modulating and selecting a desired root system architecture. A novel method to quantify the transition through the various lateral root developmental stages was devised (Uemura et al., 2023; Uemura and Tsukagoshi, 2024). In this method, developmental stages are captured using time-lapse imaging of gravistimulated roots followed by deep neural network (DNN) analysis. Time-lapse images of lateral root primordium development through the gravistimulation process serve as input for machine learning, facilitating the construction of the DNN model. The DNN model performs the automated identification of the developmental stages correlating actual time after gravistimulation with development. Next, statistical differences are analyzed for a specific time and associated with a particular developmental stage.

scRNA-seq was also successfully utilized to increase our knowledge of root initiation during shoot-borne root formation (Omary et al., 2022) and from callus (Zhai et al., 2023; Zhai and Xu, 2021). In tomato, shoot-borne roots originate from a particular type of phloem cells, and to isolate these very rare tissues, a combination of microdissection, cell disassociation and FACS upon a doubly fluorescently marked line was used followed by scRNA-seq (Omary et al., 2022). This analysis identified a developmental trajectory from specific phloem cells, which could acquire additional transient identities to initiate a new meristem through the formation of precursor stem cells, termed as transition cells and representing the progenitors of the new root. A critical regulator of transition cell specification was a LBD factor, which was not only required for shoot-borne formation but was also involved in wound-induced and lateral root formation and was part of a conserved superlocus in angiosperms. Roots can also be initiated from callus, and scRNA-seq was also used to profile callus formation and identify regulation specifying root or shoot fates (Zhai et al., 2023; Zhai and Xu, 2021). This study confirms at higher resolution, as initially proposed (Sugimoto et al., 2010), that a callus is organized as a root primordium and that it is the central layer with stem cell properties which can initiate new organs. Intriguingly, fate of the new organ is determined by cytokinin sensibility conferred by levels of WUSCHEL-RELATED HOMEOBOX (WOX) factors, with increased WOX levels leading to shoot fate.

To address the role of the candidate genes identified by scRNA-seq approaches investigating lateral root initiation and formation, CRISPR/Cas9 technology or new methods based on it have been devised. Multiplex CRISPR/Cas9, which can target up to six genes (Bollier et al., 2021), has been used to investigate redundant regulation following scRNA-seq studies of root initiation in tomato (Omary et al., 2022). Remarkably, this approach led to the identification of an ancient angiosperm superlocus formed by class IIIA and IIIB LBD paralogs, with class IIIB specifically regulating shoot-borne root initiation, and both class IIIA and IIIB redundantly regulating wound-induced and lateral root formation. New approaches based on the CRISPR/Cas9 technology leverage promoters with known specific expression during root initiation and formation. One of these methods (Gala et al., 2021) uses an enhancer trap system to drive, under the bacterial UAS promoter, expression of the nuclease-deactivated CRISPR-associated protein (dCas9) fused to the TOPLESS repressor domain (Khakhar et al., 2018). The resulting construct is combined with specific single guide RNAs (sgRNAs) directed to the promoter regions of genes of interest to inhibit their transcription. Specificity is provided by the specific enhancer trap line chosen, in which the bacterial GAL4 transcription factor was already targeted to a specific tissue or group of cells (Laplaze et al., 2005). As a result, the role of novel regulation during root initiation has been investigated with unprecedented spatial and/ or temporal resolution confirming novel hypotheses such as lateral inhibition influencing organ spacing. In a different approach, a tissue-specific knockout system, termed CRISPR-TSKO, was devised (Decaestecker et al., 2019). Specific promoters driving expression of Cas9 during lateral root formation was combined, through Golden Gate assembly, with one of two sgRNAs targeting the genes of interest. As a proof of concept, targeting ARF factors or cell cycle regulators involved in lateral root formation showed reduced organogenesis.

Collectively, the study of root initiation and organogenesis has been greatly facilitated by the utilization of new technologies, such as scRNA-seq, which identified new cell types and developmental trajectories, new computational models that integrate molecular and mechanical or spatial regulation, and genome editing technologies that enable multiple or tissue-specific inactivation of genes. Research has already concentrated on crops, particularly tomatoes, highlighting the exciting potential of these techniques to deepen our understanding of organogenesis in other agriculturally relevant species.

New strategies and tools to modulate root architecture through its interactions with microbes.

As holobionts, plants harbor a variety of external and internal microbes that greatly contribute to their fitness and survival (Vandenkoornhuyse et al., 2015). The root is no exception to that: plant roots release a vast range of substances into the soil, making their surrounding nutrient rich and thus an attractive niche for microbes. These root-derived materials include root exudates abundant in photosynthetically fixed carbon, and rhizodeposition consisting of a mixture of mucilage, shed cells and cellular debris. In this context, the rhizosphere is defined as the layer of soil surrounding the root and the microorganisms that live therein and are affected by plant inputs (Mohanram and Kumar, 2019). Rhizospheric microbes can be beneficial, harmful or neutral for the plant and complex interactions between them also take place ultimately defining microbial population structures (Mendes et al., 2013). Adding complexity, the interactions between the root and microbes in the rhizosphere is strongly modulated by abiotic factors and also by interactions with other plants and animals in the soil (Fitzpatrick et al., 2018). In addition, along with rhizospheric microorganisms, which live outside the root, microbes residing inside, known as root endophytes, are equally important but in comparison far less studied (Verma et al., 2021). As part of their interaction with the plant, all these microbial communities affect and are affected by root development and architecture. In this regard, a better understanding of root-microbe interactions will be key to rationally engineer plant holobionts with radicular systems better adapted to the environmental challenges.

Root-associated microbes communicate with the plant and surrounding via a vast array of signals and this communication will ultimately shape their interaction modulating root system architecture and consequently plant health (Enagbonma et al., 2023). The secondary metabolites produced by rhizobial microbes that have a direct impact on plant root growth and development include both phytohormones (auxins, cytokinins, ethylene, gibberellins and abscisic acid) and a growing list of non-hormonal chemicals (Mukherjee et al., 2022). Perception of these microbial bioactive metabolites by the root activates signaling pathways that result in alterations in the root system architecture mostly involving the changes in the number, length, density and biomass of basal roots, lateral roots and root hairs (Grover et al., 2021). In this regard, a combination of various metabolomics will be key to define the plant holobiont's metabolome. These include liquid or gas chromatographic methods coupled to mass spectrometry and spectroscopic techniques such as nuclear magnetic resonance or Fourier transform-near-infrared (Pang et al., 2021). On the other hand, mass spectrometry imaging has allowed the determination of the spatial distribution of metabolites in the rhizosphere (Döll et al., 2021; Gomez-Zepeda et al., 2021; Korenblum et al., 2020; Sasse et al., 2020), while single-cell mass spectrometry can help discriminating plant- and microbe-produced metabolites (Masuda et al., 2018; Taylor et al., 2021). However, there is still a huge lack of annotation and functional assignment among mass features, which will need to be addressed in the coming years (Aharoni et al., 2023).

While the effects of rhizosphere microbes on roots have been extensively studied, how root system architecture in turn modulates microbial associations remains poorly understood, particularly in agricultural soils (Galindo-Castañeda et al., 2024). This is important to determine the sites of interaction and composition of specific microbe communities along the root axis and the role of these communities in the holobiont. In this regard, the vertical gradients that exist in natural soils determine availability of nutrients, water and oxygen, influencing microbial diversity and potentially affecting the assembly of communities along the root axes (He et al., 2023). The root hair zone and the sites of lateral root emergence constitute microbial hotspots along the root axis (Rüger et al., 2021). Root hairs play a critical role in the interplay among soil, plants and microorganisms, controlling root exudates and contributing to resource exchange (Molefe et al., 2023; Zhang et al., 2023a). In turn, lateral root emergence generates an exit point for various metabolites and other compounds that attract specific microbial communities (Baudoin et al., 2002; Jaeger et al., 1999; Park et al., 2004; Rüger et al., 2021). The root cap is also an important determinant for the assembly of the rhizosphere microbiome (Rüger et al., 2023). Despite major advances in recent years, mechanistic knowledge about the spatial and temporal variability of microbiome assembly on roots is still very sparse. However, a deep understanding of the highly dynamic process of microbial assembly along the root axis will be extremely important for targeted improvement of microbiome function in agricultural soils.

The field of root-microbe interactions has boomed over the last two decades thanks to the advent of next generation sequencing techniques, which has allowed a deeper understanding of the root and rhizosphere microbial communities (Knief, 2014). However, researchers in this field still face many challenges to go beyond mere descriptions of communities and to be able to move into a more mechanistic understanding of the interactions between roots and their interacting microbes that would allow the use of microbes to engineer root functions and overall plant health.

Artificial intelligence will be key to extract valuable information from the large number and diverse omics datasets obtained from roots and their communities. Metagenomics, metatranscriptomics, proteomics and metabolomics techniques have kept becoming more sensitive, relatively affordable and simpler to perform, providing a wealth of information in terms of taxonomic composition and gene expression patterns of root communities (Bai et al., 2015; Lundberg et al., 2012; Saarenpää et al., 2024). However, integrative analyses of these datasets are lacking. In this regard, artificial intelligence can help consolidate data from heterogeneous sources to facilitate unified interpretation. Another challenge faced by the study of root-microbe interactions is the lack of specificity in sampling. Most of the studies are performed with pooled roots, disregarding the spatio-temporal variation in microbial composition along the root axis, or the functional specialization of different root parts. In this regard, it may be important to adapt methods successfully used to isolate single bacterial cells in other bacterial communities to root-associated communities, such as micromanipulation, laser capture microdissection, fluorescence-activated cell sorting or microfluidics (Balestrini et al., 2007; Gomez and Harrison, 2009; Hohnadel et al., 2018; Ishøy et al., 2006; Raghunathan et al., 2005; Riba et al., 2016). Recently, spatial profiling of a root bacterial community has been achieved through a highly multiplexed and accurate imaging method known as sequential error-robust fluorescence in situ hybridization (Cao et al., 2023). In addition, recent advances in root phenotyping, including traditional and machine learning-based methods, will aid in the study of root-microbe interactions and help refine sampling techniques.

In order to define major root microbial colonization sites it will be essential to develop biosensors coupled with advanced live imaging microscopy. In this regard, the development of microfluidic devices that allow for dynamic imaging of plant-microbe interactions at high spatiotemporal resolution (Massalha et al., 2017). In parallel, recent advances have made possible single cell RNA sequencing of microbial community members, which will help define gene expression patterns in individual cells within a consortium and predict spatial organization (Ma et al., 2019).

The assembly of knowledge-based synthetic microbial communities has been leveraged in agriculture to improve plant health. The advances brought about by the CRISPR/Cas9 system has led to more efficient genetic manipulation of microbes, which will facilitate the study of gene function and the targeted engineering of root microbial communities (Aparicio et al., 2019; Bisht et al., 2019). In parallel, this technology can also be employed to develop crops harboring roots engineered to improve colonization/residency of beneficial microbe communities. Ultimately, these modifications can target different aspects of root function to improve water and nutrient uptake, alter selective uptake of solutes from soil, boost immunity to defend from harmful pathogens in the soil or gain tolerance to abiotic stresses.

Root-microbiome analysis has already had a strong impact in agriculture by improving nitrogen use efficiency and reducing the dependence on fertilizers and pesticides of certain crops, resulting in more sustainable practices. New technologies are helping understand the complex and dynamic interactions between roots and microbes, integrating the spatiotemporal dimension of interactions and integrating multiple.

Hormonal Regulation and Technological Advancements in Root Development

Phytohormones influence every aspect of primary and lateral root growth and development. In particular, the coordinated activities of auxin, cytokinin, gibberellin (GA), and abscisic acid (ABA) guide the cell specification, division, and expansion events at the heart of root development. Auxin is generally considered to be the primary initiator and promoter of lateral root formation whereas cytokinin activity opposes those of auxin (Jing and Strader, 2019). Under stressful conditions, ABA inhibits lateral root production and promotes primary root elongation (De Smet et al., 2006; Ranjan et al., 2022). The intricate balance of these hormones drives the ultimate architecture of the root system. Recent new technologies enabling a spatial understanding of phytohormone action has improved our understanding of this balance.

Advancements in hormone biosensors and imaging technologies have revolutionized our ability to visualize hormone distribution within root tissues (Rowe and Jones, 2021). Biosensors and fluorescent reporters provide real-time, high-resolution monitoring of hormone concentrations and signaling events and continual refinement of these biosensors and reporters allow for a deeper understanding of the dynamic relationships between these hormones and environmental modulators of lateral root formation.

Asymmetric auxin distribution is a critical driver of root gravitropic responses and lateral root setpoint angle (Konstantinova et al., 2021). Auxin reporters, such as DR5 (for auxin response) and R2D2 (for auxin perception) have provided much insight into these processes and also of the developmental events underlying *de novo* meristem formation during lateral root initiation. Biosensors can also unmask plant hormone roles in the root meristem. For example, the GPS1 biosensor unmasked a GA gradient in the primary root elongation zone, which correlated with root cell lengths (Rizza et al., 2017). In this system, GA levels increase from the meristematic zone toward the elongation zone (Rizza et al., 2017). A combination of detailed GA sensor experiments and mathematical modeling has led to a deeper understanding of the GA metabolic processes underlying this gradient (Rizza et al., 2021). Further, a second generation GPS2 biosensor with improved orthogonality and reversibility (Griffiths et al., 2024) will surely allow for deeper insights into the multilevel hormone control of root growth, as it has done for our understanding of hypocotyl growth. Indeed, it has already been used to understand the GA dynamics underlying nodulation in *Medicago truncatula* (Drapek et al., 2024). GA accumulates at the site of nodule primordia and acts as a positive regulator of nodule growth (Drapek et al., 2024). A separate GA sensor, called qmRGA and based on GA-induced degradation of the DELLA repressor protein RGA, provides insight into GA signaling dynamics and spatial responses (Shi et al., 2024). Future work with these sensors, and others developed for additional phytohormones, will help elucidate the roles for their distributions in shaping root architecture.

Single cell sequencing of roots has enabled an understanding of phytohormone effects on root developmental progression. For example, surveying the transcriptome changes of increased cytokinin at single cell resolution unmasked a counteraction by SHR to create induction and repression modules that organize root vascular development (Yang et al., 2021). In another example, single cell transcriptomics revealed a role for brassinosteroids in the cortex to trigger a shift from proliferation to elongation (Nolan et al., 2023). Leveraging the power of single cell technologies, combined with the plethora of root development and hormone signaling mutants, will enable a dissection of the coordinated activities of multiple pathways in regulating root system architecture.

In addition to visualizing isomeric auxin derivatives, along with various hormones and their metabolites in roots using ion mobility mass spectrometry imaging (Chen et al., 2024a; Zhang et al., 2023a), coupling single-cell gene expression with biosensors represents a technological advance needed to unravel the complexities of hormone interactions at the cellular level. Despite remarkable progress and the continuing development of new tools, challenges persist in deciphering the hormone interplay regulating lateral root development. In particular, examining the effects of multiple hormones simultaneously against the backdrop of normal developmental programming and stress response will be the next frontier in understanding how root system architecture is dynamically shaped.

Concluding Remarks

The application of single-cell transcriptome technologies, such as scRNA-seq and spatial transcriptomics, as demonstrated in the above examples, underscores that no single method is universally optimal for all research inquiries. Integrating these approaches with biosensors and imaging enables precise identification of cellular spatial organization, significantly enhancing our understanding of whole-plant tissue architecture and intercellular communication. This combination is especially valuable for resolving cell subpopulations in crop plants that lack specific markers or identity genes. Similarly, proteomics is poised for substantial progress, with innovations in single-molecule mass spectrometry, DNA nanotechnologies, and protein fingerprinting driving advances in single-cell protein analysis. In line with this, a recent study, successfully assessed and demonstrated the feasibility of multiplexed single-cell proteomics by analyzing single cells isolated from the cortex and endodermis of *Arabidopsis* roots (Montes et al., 2024). This work highlights the ability of single-cell proteomics to differentiate the proteomes of these distinct cell types.

Such advancements will improve the accuracy of cell-type annotation and facilitate the discovery of new cell types and mechanisms. As single-cell technologies evolve, integrating them with CRISPR-based tools offers transformative potential. CRISPR/Cas knockouts and gene expression modulation can be used to validate key trait genes and improve traits such as stress tolerance, disease resistance, and nutritional quality. High-throughput, genotype-independent transformation techniques will expand genome editing applications to elite breeding materials, accelerating the development of edited plants for agriculture. However, rigorous evaluation of off-target modifications and unintended transgenic insertions remains crucial.

The convergence of single-cell transcriptomics, proteomics, multi-omics, biosensors, imaging, and genome editing technologies (see "Outstanding Questions") is set to revolutionize plant science and agriculture, deepening our understanding of plant biology, advancing plant improvement, and promoting sustainable agricultural practices.

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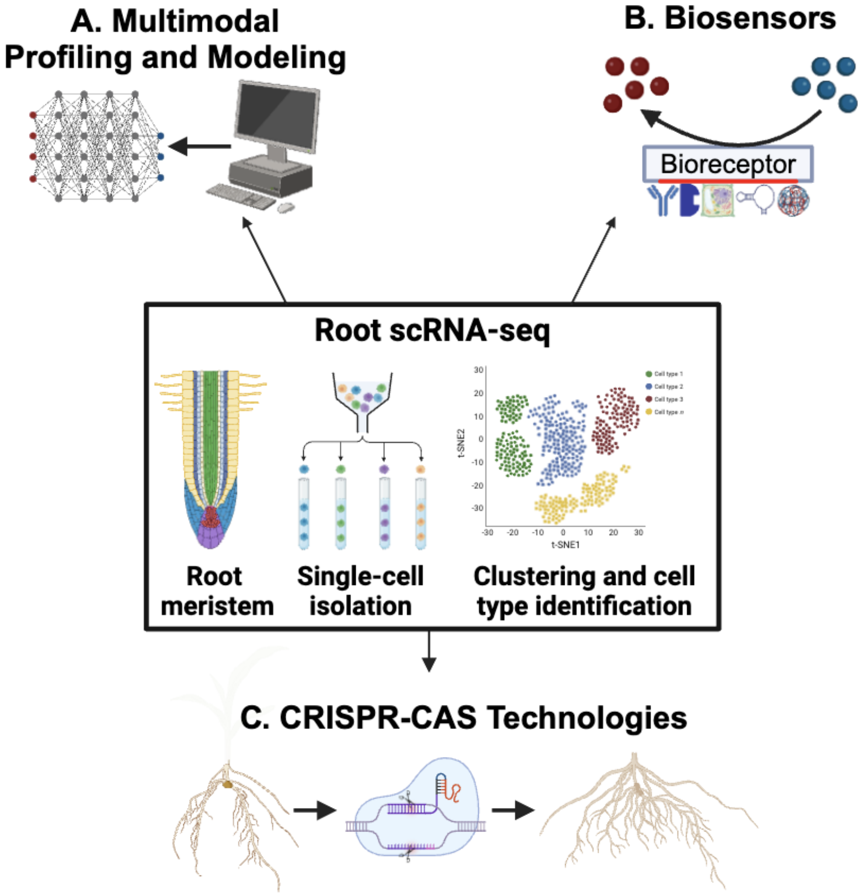

Figure 1. The application of single-cell transcriptome technologies highlights the need for combining methods to optimize investigations. The integration of advanced technologies, such as scRNA-seq and spatial transcriptomes with biosensors and imaging offers a "cloud" of possibilities for precise identification of the spatial organization of cells. This approach enhances our understanding of whole-plant tissue architecture and intercellular communication, paving the way for more comprehensive insights into plant development and function.

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