Advancing root developmental research through single-cell technologies

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Abstract

Single-cell RNA-sequencing has greatly increased the spatiotemporal resolution of root transcriptomics data, but we are still only scratching the surface of its full potential. Despite the challenges that remain in the field, the orderly aligned structure of the Arabidopsis root meristem makes it specifically suitable for lineage tracing and trajectory analysis. These methods will become even more potent by increasing resolution and specificity using tissue specific scRNA-seq and spatial transcriptomics. Feeding multiple single-cell omics datasets into single-cell gene regulatory networks will accelerate the discovery of regulators of root development in multiple species. By providing transcriptome atlases for virtually any species, single-cell technologies could tempt many root developmental biologists to move beyond the comfort of the well-known Arabidopsis root meristem.

Highlights

- Plant root meristems are uniquely suitable for lineage tracing and trajectory analysis.
- Spatial transcriptomics will initially assist in validation of scRNA-seq data but might soon become the main tool for transcriptomic profiling in plants.
- Gene regulatory networks obtained at single-cell level in multiple species will be an invaluable tool to identify conserved regulators of root development.

Keywords: Root development, single-cell transcriptomics, single-nucleus transcriptomics, spatial transcriptomics

Abbreviations

CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by Sequencing
CRE	Cis-Regulatory Element
FACS	Fluorescence-Activated Cell Sorting
FANS	Fluorescence-Activated Nuclei Sorting
GRN	Gene Regulatory Network
GWAS	Genome-Wide Association Study
IACS	Intelligent Image-Activated Cell Sorting
ISH	*In Situ* Hybridization
LR	Lateral Root
scATAC-seq	Single-cell Assay for Transposase-Accessible Chromatin sequencing
scRNA-seq	Single-cell RNA-sequencing
SNP	Single-Nucleotide Polymorphism
snRNA-seq	Single-nucleus RNA-sequencing
TF	Transcription factor

Introduction

Despite having joined the single-cell RNA-sequencing (scRNA-seq) party somewhat later compared to mammalian and medical research colleagues, the plant field pioneered in generating tissue specific transcriptomic data sets on Arabidopsis root apical meristems by combining fluorescence-activated cell sorting (FACS) and microarray analysis or bulk RNA-seq as early as 2003. These atlases became increasingly more detailed over the years as technology advanced and even included cell-type level responses to a spectrum of abiotic and biotic stresses [1–7]. Although the importance of these datasets for the entire plant community cannot be stressed enough, the introduction of droplet-based scRNA-seq has undoubtedly provided a massive increase in resolution of transcriptome maps in the Arabidopsis root apical meristem [8–17]; and in other organs [18–24]. As this technology is not based on the availability of tissue specific marker lines, it is quickly becoming a very important technology in other plant species as well [25–35]. Despite being fully embraced by the plant community, scRNA-seq technology is mostly being used to query gene expression in a spatiotemporal way, similar to the FACS based data that has been around for almost 20 years [1–4]. There are however clear examples of how the increase in spatiotemporal resolution has advanced our understanding of root development [10,15,23,27], but scRNA-seq technology and the available datasets have much more potential (Fig. 1). We are thus only scratching the proverbial surface of what is already possible and will become possible in the very near future.

The blessing and the curse of using plants for single-cell analyses

It does not come as a surprise that the Arabidopsis root apical meristem was the first organ to be studied using scRNA-seq, as individual cells can easily be generated by enzymatic digestion of the cell wall in a process called protoplasting [1,36]. The capacity to generate single cells has however been and will continue to be a main bottleneck for the plant community [37–40]. Besides potentially introducing an unwanted transcriptional response while generating protoplasts, commercial systems based on microfluidics technology limit the size of captured cells to about 30-50 µm. This poses a real problem as plant cells range from 10 µm to 100 µm in size [41]. Although larger cells up to 125 µm can be captured using specific assays, this was shown to introduce a bias in the relative abundance of subpopulations of large cells [42,43]. Furthermore, certain cell types might fail to be digested or specific cells might burst during the procedure. Because of these reasons, some studies have resorted to nuclei isolation instead of whole cells [29,31,44–51]. This approach is theoretically applicable to any organ and any plant species, including frozen samples, and might prevent inherent capture biases associated with generating single cells [47]. The main disadvantage is capturing fewer mRNAs compared to whole cells, although single-nucleus RNA-sequencing (snRNA-seq) generally performs equally well for sensitivity and classification of cell types [47,49,52–54].

Despite these and other pitfalls in applying single-cell technologies to plant samples, the plant field also has distinct advantages compared to other fields of research which merit more exploitation. For example, linking cell states across periods of time in mammalian systems is very challenging and is currently approached by tracking cell clones via sequencing of inherited barcode sequences [55]. In contrast, every root meristem contains cells at all differentiation

stages, orderly aligned in cell files and fixed within their tissue context. The Arabidopsis root apical meristem is thus highly suitable for lineage tracing and developmental trajectory analysis, eliminating the need for time-series experiments in whole tissues and the associated batch effects, though care must be taken to avoid possible confounding effects due to positional information [56]. So far, pseudotime trajectories have mostly been used to interpret developmental time in scRNA-seq datasets and affirm known cell lineages [8,15,24]. They can however be used to address more complex developmental processes in root biology such as cell ontogeny and specification events. Indeed, trajectory analysis of protophloem cells revealed a differentiation gradient that mediates cellular specification [17] and analysis of the first stages of lateral root (LR) formation led to the identification of a group of precursor cells that rapidly reprograms and splits into various LR cell fates [23]. Furthermore, trajectory analysis can provide insights into the mode and speed of cell state transitions (gradual or switch-like), reveal bifurcations in ontogeny, and discover new regulators of these processes. As the Arabidopsis root meristem is very well studied, trajectory analysis is most likely to reveal novel insights in less characterized species [27,33]. Although its potential is clear, trajectory analysis and gene discovery require sufficient cells at each step of the pseudotime and high data content per cell. At the moment, achieving such high-resolution data using whole organ datasets presents a major financial burden. Thus, until sequencing technologies becomes more affordable, dedicated tissue specific data sets, which contain much fewer cells but with higher sequence coverage per cell will prove useful in studying root development and might allow re-visiting of the text-book concept of tissue identities by e.g. defining functional units of cells that span different tissue types.

Increasing specificity in single-cell experiments

Although scRNA-seq is capable of capturing rare cells or cell types, their occurrence in whole organ atlases might still be insufficient to infer good statistical power or advance to gene discovery and functional characterization studies. Although this issue can be partially solved by profiling a larger number of cells [14], this comes with an unrealistic financial cost if high data content per cell is required or many samples are involved. As mentioned above, this issue can be resolved by enriching these rare cell states or tissues, resulting in more specific datasets. In mammalian systems, this is achieved by using combinatorial antibody staining in e.g. CITE-seq approaches [57–60]. Although large collections of antibodies and tissue-specific epitopes are not readily available in the plant field, increased specificity can be facilitated by manually removing unwanted tissues [20,21] or specific cell enrichment using FACS/Fluorescence-activated nuclei sorting (FANS) on fluorescent protein tagged reporter lines whose expression represent a spatiotemporal domain within the tissue of interest. The fact that increased specificity leads to novel biological insights was elegantly shown by profiling the sieve element lineage from cell birth to terminal differentiation [17], the Arabidopsis inflorescence [61], and the first four stages of LR formation [23]. The major drawback of this method is obviously its reliance on *a priori* knowledge and the availability of specific marker lines, which are rare or absent for most plant species. Alternatives to purify cell types without resorting to specific antibodies or transgenes have been suggested in the form of intelligent image-activated cell sorting (IACS), which performs real-time high-throughput cell microscopy analysis prior to

sorting based on a range of morphological features [62]. Other computational methods combine single-cell transcriptomics with FACS index sorting to set non intuitive sorting gates to purify cell types based on scRNA-seq data [63]. Successful application of these technologies would allow for smooth integration of other single-cell omics and streamline the identification of molecular morphometric phenotypes. However, these methods require accurate training of deep learning algorithms, so other unbiased methods that also do not require markers or antibodies will be needed.

Seeing is believing

In all cases, predictions derived from scRNA-seq data should be validated experimentally, as conclusions drawn from scRNA-seq data analyses can be skewed by biases generated during sample or library preparation and the downstream computational analysis. This can be achieved by generating reporter lines [8,15,17,20] or by performing *in situ* hybridization (ISH) [24,27,33]. However, constructing reporter lines is limited to species that are amenable to transformation and *in situ* hybridization is labour intensive and can be hindered by the lack of robust probes in many plant species [64]. Fortunately, rapid progress is being made in the development of spatial transcriptomics [65–70]. This technology theoretically enables all genes at low spatial resolution (untargeted) or a subset of genes at high spatial resolution (targeted) to be visualized *in situ*, without the need of marker genes or reporter lines and is applicable to any species and tissue. Nevertheless, its application to the plant field is currently still hampered by technical difficulties (see also [71]).

Combining the complementary advantages of single-cell and spatial transcriptomics will revolutionize both fundamental and applied research. Indeed, linking scRNA-seq data with its natural spatial context enables instant cell identity mapping, which is of particular use in non-model organisms. Moreover, it provides unprecedented resolution to study structure-function relationship, cell-cell interactions, plant pathogen interactions and environmental responses in general. If the resolution and accessibility of untargeted spatial transcriptomics increases further, it can even be envisioned that this technology will largely replace scRNA-seq in plant research. Besides advancing basic root biology research in Arabidopsis, spatial transcriptomics is expected to accelerate the establishment of new species for molecular biology applications. No doubt, this technology will also be rapidly adopted to study crops and species which are difficult to transform.

From off-the-shelf to out-of-the-box

Coordinated growth and development in a changing environment requires interplay among many components in complex gene regulatory networks (GRNs), where transcription factors (TFs) and non-coding functional *cis*-regulatory elements (CREs) cooperatively regulate gene expression and as such determine the final cell differentiation start and phenotypical response. Due to the high spatiotemporal resolution, single-cell data is able to deconstruct tissue heterogeneity, making it highly suited for GRN analysis [72]. For example, environmental GRNs have been constructed for Arabidopsis roots where heat-shock treatment led to drastic transcriptional changes in the three outermost cell layers of the root [9]. In another study,

sucrose induced enrichment of root hairs and gene expression changes were highly tissue-specific [11]. However, precise GRN predictions require CREs or TF-binding site information with matching spatiotemporal resolution. This can be achieved by complementing scRNA-seq data with profiling accessible chromatin regions through scATAC-seq [27,50,73]. As such, our understanding of the regulatory networks governing root growth and development can be achieved by pairing scRNA-seq with scATAC-seq across critical growth and developmental transition stages or under a spectrum of environmental stresses.

Apart from answering fundamental research questions using off-the-shelf single-cell applications, the high spatiotemporal resolution embedded in scRNA-seq data can be used in more surprising ways to modulate complex traits in crop species. For example, scRNA-seq data was linked with genome-wide association (GWAS) data in developing maize ears where significant single-nucleotide polymorphisms (SNPs) were found within scRNA-seq marker genes that are associated with yield-related traits [35]. As such, the high-resolution GRNs constructed through scRNA-seq and scATAC-seq will help to pinpoint key regulators underlying traits of interest together with corresponding CREs. SNPs within these CREs can then potentially serve as targets for genome editing to precisely deliver targeted phenotypic changes. Also, such high-resolution SNPs could facilitate applications like GWAS and marker-assisted selection in plant breeding.

Conclusion

In the few years since they have been adopted by the plant field, single cell applications are revolutionizing the way we study root development. Although they are still mostly increasing our spatiotemporal resolution and identifying specific developmental regulators; soon, they might tempt many root biologists to move beyond the well-studied Arabidopsis root meristems and quickly prepare other species for molecular biology applications by providing fully annotated transcriptome atlases. The applications are perhaps even more promising for studying complex systems such as plant-pathogen interactions or cell-cell interactions, but will for sure also be readily adopted in crop species.

Declaration of competing interests

The authors declare no competing interests

Author contributions

M.M., Y.K., M.S.S. and B.D.R. conceptualised and wrote the manuscript.

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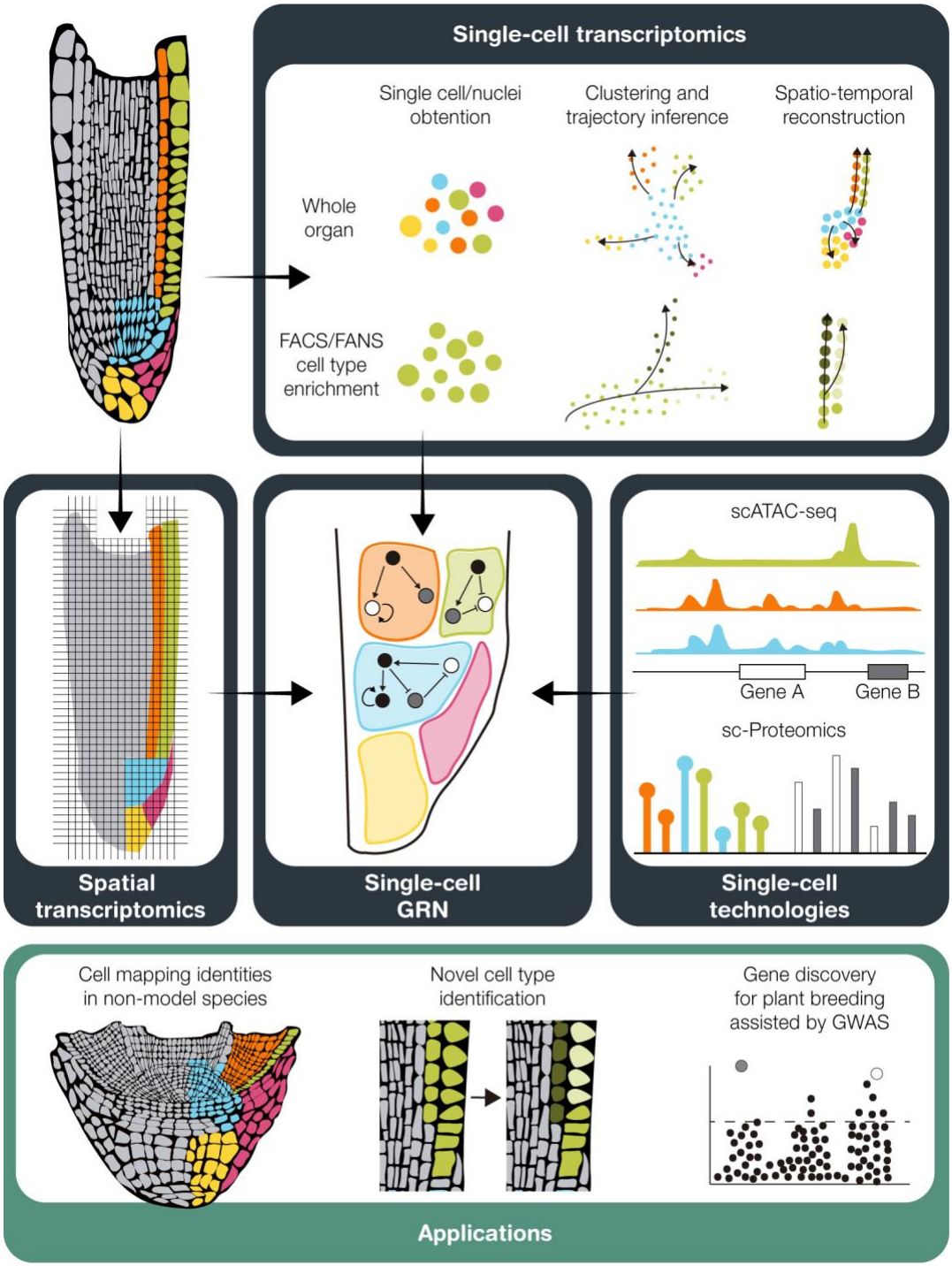

Figure 1. New opportunities for understanding root development using single-cell technologies. The use of RNA-seq applied to individual cells or nuclei allows reconstructing developmental trajectories in root tissues. The resolution can be increased using FACS/FANS to study a specific tissue or cell type of interest. scRNA-seq data will be soon complemented with spatial transcriptomics technology. Combining the transcriptome information with the chromatin accessibility and proteome studies at single cell level could be used to create gene regulatory networks (GRN) with an unprecedent spatiotemporal detail. Root development research will benefit from these technologies to e.g. study non-model plant species, find new functional cell types, or discover target genes for plant breeding.

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In this article, scATAC-seq data and scRNA-seq data are integrated to provide an analytical framework for GRNs.