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Resolving plant development in space and time with single-cell genomics

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Abstract

Single-cell genomics technologies are ushering in a new research era. In this review, we summarize the benefits and current challenges of using these technologies to probe the transcriptional regulation of plant development. In addition to profiling cells at a single snapshot in time, researchers have recently produced time-resolved datasets to map cell responses to stimuli. Live-imaging and spatial transcriptomic techniques are rapidly being adopted to link a cell's transcriptional profile with its spatial location within a tissue. Combining these technologies is a powerful spatiotemporal approach to investigate cell plasticity and developmental responses that contribute to plant resilience. Although there are hurdles to overcome, we conclude by discussing how single-cell genomics is poised to address developmental questions in the coming years.

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Introduction

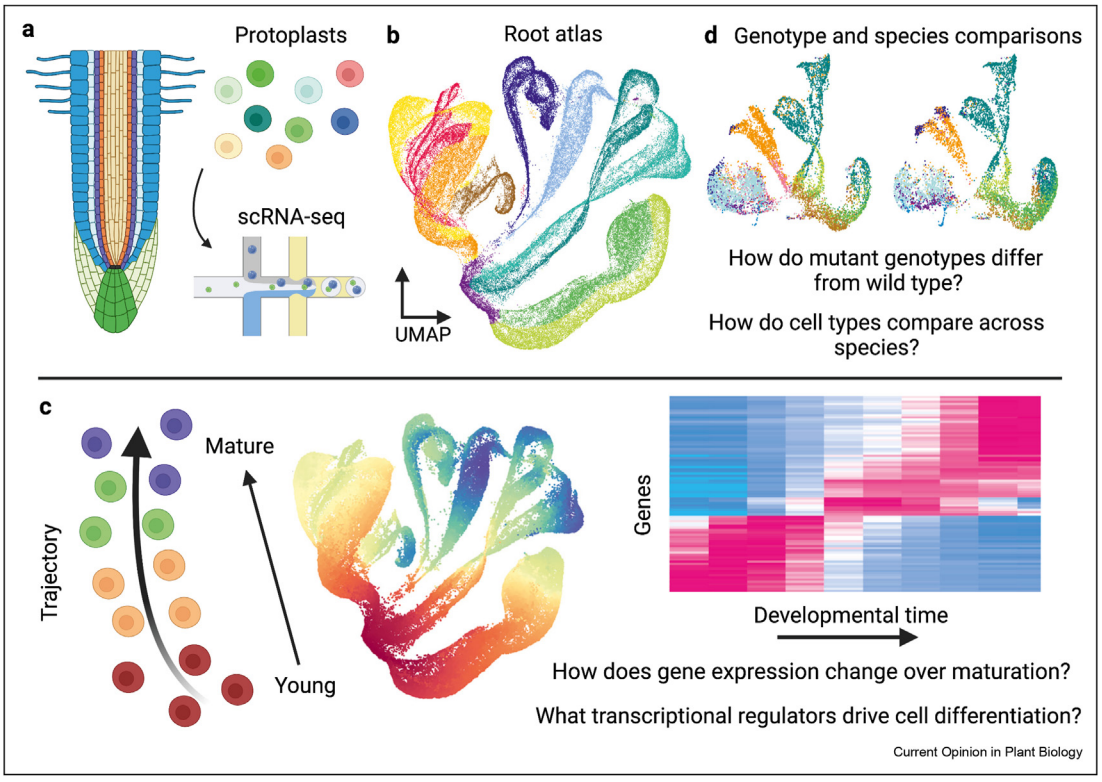
How do cells acquire distinct identities? And how do different types of cells at different stages of maturation contribute to organ form and function? These questions lie at the heart of plant developmental biology. To examine the expression of individual genes with tissue or cell-type resolution, we have long used techniques such as *in situ* hybridization or the fusion of promoters to fluorescent reporters. More recently, single-cell RNA-sequencing (scRNA-seq) makes it possible to profile the expression of hundreds or thousands of genes from thousands of individual plant cells in a single experiment (Figure 1a, b). scRNA-seq thus enables data generation at a scale and resolution that was previously unachievable. However, important technical challenges remain, including the sparsity of the transcripts detected and the loss of a cell's spatial context during the tissue disruption required for sample preparation. These challenges complicate the assignment of each cell's identity and developmental stage, a process that must be done computationally and therefore requires pre-existing knowledge of cell-type markers.

The first droplet-based scRNA-seq experiments in plants were applied to the Arabidopsis root and data analyses were aided by an existing suite of characterized gene expression profiles [1–5]. These studies established that major cell types are successfully identified from scRNA-seq data and present at expected proportions. Additional experiments validated the robustness of Arabidopsis root annotations by confirming *in vivo* expression patterns of new cell-type markers [1,4,6]. scRNA-seq and single-nucleus RNA-seq (snRNA-seq) have since been applied to numerous other plant tissues and species [7–10] and there are ongoing efforts, such as through the Plant Cell Atlas, to establish best practices for data generation, analysis, validation, and publication [11–13]. Here, we review recent applications of single-cell genomics to plant development primarily through the lens of Arabidopsis root studies from the last two years.

A static snapshot captures developmental trajectories

The longitudinal axis of the Arabidopsis root represents a developmental continuum with a full complement of cell types present across developmental stages (Figure 1a–c) [14]. Cell types from each of three morphologically distinct developmental stages, the meristem, elongation, and maturation zones, can therefore be captured from a single snapshot in time. However, finer resolution is required to address a fundamental question: do cells mature via a series of rapid transcriptional switches or does gene expression change gradually? scRNA-seq enables us to address this in a cell-by-cell fashion by 'reconstructing developmental trajectories,' i.e., computationally ordering cells from least to most differentiated. A finely resolved

Figure 1



Single-cell RNA-sequencing reveals tissue composition differences and enables recapitulation of developmental trajectories from a snapshot in time. (**a**) To generate scRNA-seq data, a plant tissue or organ, such as an Arabidopsis root, is first dissociated into protoplasts. All protoplasts can be used for an experiment, as shown in panel b, or a subset of cell types can be isolated with Fluorescence Activated Cell Sorting (FACS). Microfluidic platforms can be used to profile thousands of individual protoplasts in a single experiment. (**b**) After library preparation, sequencing, and pre-processing steps, data are visualized on a Uniform Manifold Approximation and Projection (UMAP) plot. Based on the expression patterns of previously characterized genes, cells are annotated by cell type. Each cell type is indicated on the UMAP plot with a unique color. (**c**) Based on similarities between transcriptional profiles, root cells can be ordered along a developmental progression from least differentiated (warm colors, left) to most differentiated (cool colors, left). Since an actual time component is unknown, these progressions are plotted across developmental 'pseudotime.' After recapitulating a trajectory for a specific cell type, gene expression can be plotted to reveal dynamic patterns over the course of development (right). Developmental trajectories are also used to identify candidate transcription factors that drive cells toward a specific identity [81,82]. (**d**) Using a comparative approach, scRNA-seq is a powerful tool with which to probe differences in cell identity and differentiation as well as tissue composition between developmental mutants or between species. Created with BioRender.com.

trajectory for a specific cell type often requires more cells than can be captured in a single sample generated from whole roots. Enriching a cell type of interest via fluorescence activated cell sorting (FACS) followed by scRNA-seq can therefore be advantageous and a number of publications have applied this approach to Arabidopsis roots.

For instance, two recent studies addressed how stem cell progeny differentiate into protophloem sieve elements, a cell type with accelerated maturation compared to other cell types. Otero et al. (2022) constructed developmental progressions (Figure 1c) for sieve elements as well as three other cell types that, collectively, form the phloem pole. This approach identified shared gene regulatory networks between the cell lineages as well as a new set of transcription factors, the *PINEAPPLE*s, that contribute to the transition to autotrophy in young seedlings [15]. Although the expression of individual genes of interest can be examined *in vivo*, the combination of cells from many different seedlings in a single experiment means that it is not possible to match a location along a computationally derived developmental trajectory with an exact cellular location in a tissue. Therefore, to determine how transcriptional changes relate to morphological changes during sieve element maturation, Roszak and colleagues (2021) combined scRNA-seq data from FACS-sorted cells with cell behavior information captured via live-microscopy lineage tracing [16]. The results reveal that the repression of regulators both early and late in development defines distinct developmental phases along the maturation gradient. This regulation enables a rapid transcriptional change that drives the

differentiation of sieve elements. Taken together, scRNA-seq data generated from FACS-sorted cells can produce a developmental trajectory with sufficient coverage to identify regulatory modules driving distinct phases of cell maturation.

An ultimate goal is to identify the transcriptional programs underlying the acquisition of each cell identity within an organ. Comprehensive coverage of each cell type's developmental timeline is required because, assuming that cells are sequenced to a sufficient depth, discrete groups of cells rather than a continuous progression can be interpreted in two ways: either there are rapid transcriptional changes or there are too few cells to accurately represent all transcriptional states [17]. One way to address this challenge is to integrate multiple datasets generated from whole organs to increase the total number of cells [6,18,19]. Shahan and colleagues integrated sixteen scRNA-seq datasets into an Arabidopsis root atlas (Figure 1b and c) [18]. By maximizing the number of cells, trajectories could be recapitulated with fine-grained resolution, thus enabling the identification of candidate transcriptional regulators that drive differentiation of every root cell type. Using a similar approach, Oliva and colleagues (2022) identified heterogeneity in the transcriptional profiles of cells with the same identity that is due to the differential expression of genes involved in environmental responses. This finding suggests that root cells from the same lineage exist in distinct 'states' despite having the same identity. An intriguing hypothesis is that a range of cell states may underpin the adaptive potential of development [19].

The increased resolution afforded by single-cell studies has also been used to address how distinct cell states and cell identities are influenced by hormone responses. In both roots and shoots, hormones contribute to the balance between cell proliferation, growth, and differentiation that underpins continuous organ development [20–22]. In a recent example, scRNA-seq of Arabidopsis roots revealed non-cell-autonomous functions of xylem-derived cytokinin. Inactivation of cytokinin in the procambium provides feedback to ensure balanced vascular development [23] whereas cytokinin action in the epidermis promotes root hair formation to cope with low phosphorus [6]. Profiling jasmonic acid (JA) responses at single-cell resolution addressed how cell-type-specific outputs of hormone signaling involve the concerted action of activators and repressors in different cell types [24]. Assessing transcriptional responses at the cellular level was also instrumental in characterizing brassinosteroid-mediated root growth. Molecular signatures from tissue-specific rescue lines for the brassinosteroid receptor led to a more nuanced readout compared to morphological analysis alone [25,26]. These studies illustrate that experiments with single-cell resolution can capture opposing responses, cell–cell signaling, and events that occur in only a subset of cell types and/or developmental stages.

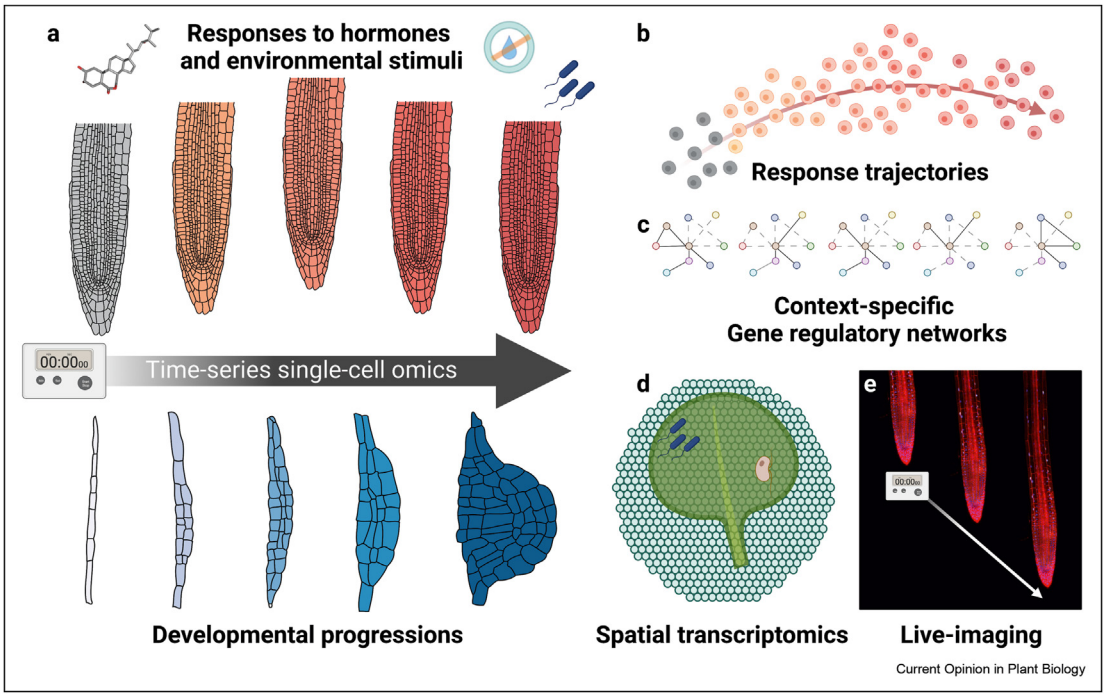
In addition to profiling wild-type plant development, single-cell approaches provide a new level of resolution to probe mutant phenotypes [1,3,18,19,25–27]. For example, scRNA-seq data from a previously characterized developmental mutant yielded a new discovery (Figure 1d). Due to the loss of a formative asymmetric cell division, the root of the *scarecrow* mutant has one layer of ground tissue instead of two. Recapitulating a developmental trajectory from *scarecrow* scRNA-seq data identified a transition from cortex cell identity to endodermis identity in this ground tissue layer. This finding underscores how gene expression as a molecular phenotype offers increased sensitivity compared to morphological analysis alone [18].

Taken together, scRNA-seq data generated from a snapshot in time can facilitate a cell-by-cell dissection of development. Combining data into comprehensive, organ-scale atlases provides a rich resource to investigate transcriptional changes underlying wild-type cell maturation and, via transfer of annotation labels, serves as critical infrastructure to interpret new single-cell datasets [18]. Although these methods are powerful tools for hypothesis generation, researchers should keep in mind that the order of cells in a trajectory is an *in silico* approximation and does not recapitulate the precise spatial arrangement of cells. Hence, additional experimentation *in planta* should be performed to confirm new findings.

Time-series data capture transient transcriptional responses

scRNA-seq data generated from one time point can recapitulate a developmental progression but cannot capture rapid transcriptional dynamics of cell-type-specific responses to stimuli. For example, hormone responses involve perception, signal transduction and complex feedback, and feed-forward networks (Figure 2a–c). To investigate cell type-specific responses to brassinosteroids in the Arabidopsis root, Nolan et al. (2023) generated single-cell transcriptomic data over a time course [25]. The data revealed the elongating cortex as a previously uncharacterized site where brassinosteroids regulate the balance between cell proliferation and differentiation. Leveraging the time course enabled the reconstruction of gene regulatory networks underlying the brassinosteroid response and facilitated the identification of transcription factors that promote cell elongation. This lays a framework for analyzing finely time-resolved single-cell experiments after exogenous treatment and demonstrates the utility of annotation transfer from an organ-scale transcriptomic atlas [18] to inform responses to perturbations.

Figure 2



Transcriptional dynamics captured in time and space using single-cell and spatial omics (**a**) Time-series single-cell omics is a powerful approach to investigate dynamic responses to stimuli such as hormones and environmental factors (top) and developmental progressions (bottom). Color gradients indicate successive times during these processes. (**b**) Single-cell omic profiles taken across time points can be reconstructed into trajectories. (**c**) The increased resolution afforded by single-cell omic approaches allows inference of context-specific gene regulatory networks and identification of associated transcription factors. (**d**) Spatial omic techniques put development and environmental responses in a tissue context. Microbes are shown on different parts of a leaf as an example of how spatial transcriptomics can provide additional information about such interactions. (**e**) Live-imaging techniques complement single-cell and spatial approaches since they allow us to watch development unfold in real-time. Outlining the cells with a plasma membrane marker (red) connects expression changes in the nucleus (yellow and blue) with cellular morphology. Created with BioRender.com with illustrations adapted from the Plant Illustrations repository [83].

Beyond primary root development, time-series scRNA-seq data has also been used to investigate how new root meristems are specified (Figure 2a). Omary et al. (2022) revealed that phloem cells transition to a stem cell-like identity to produce roots from tomato shoot tissue [28]. Gala and colleagues (2021) identified genes involved in early cell fate specification events within lateral root primordia [29]. Serrano-Ron et al. (2021) profiled the assembly of cell lineages within developing lateral root meristems and identified evidence for a hierarchical series of unique cell states [30].

Although time-series experiments can provide a new level of information about dynamic responses, effectively analyzing the data remains a major challenge. For example, root time-series datasets reflect transcriptional signatures underlying distinct cell identities, developmental stages, and responses to external stimuli. We will need to develop tools that effectively parse these multiple dimensions and enable researchers to disentangle the transcriptional responses relevant to their biological question. Overcoming these challenges will allow us to fully exploit single-cell data to address fundamental questions such as how does a cell maintain its identity while changing its state to respond to a given stimulus? And what distinguishes different cell states and are there infinite numbers of possible states?

Comparisons across tissues and species reveal evolutionary innovations

Beyond temporal analysis on the order of hours or days, single-cell analysis has also been used to ask how differences in organ morphologies and cell identities are shaped over evolutionary time scales. In a cross-species approach, Ortiz-Ramirez et al. (2021) created a scRNA-seq map of the maize root and revealed the pathway underlying increased anatomical complexity compared to the Arabidopsis root. This work also revealed the existence of multiple subtypes of cortex cell identity in maize roots, raising the possibility that the different layers may have distinct, but as yet unknown, functions [31]. Guillotin and colleagues (2023) generated a pan-grass single-cell transcriptome encompassing maize, *Setaria*, and sorghum roots [9]. The data revealed that

the transcriptional profiles of some cell types diverged faster than others due, in part, to the recruitment of gene modules from other cell types. This work and others [32–36] highlight how transcriptomics can address an exciting question: what aspects of cell identity and differentiation are conserved in an organ across species and what are evolutionary innovations (Figure 1d)? Answers will shed light on the transcriptional regulation of cell identities that confer key agricultural traits, such as drought resistance [9,37]. These questions are not limited to roots and will also be addressed with single-cell/single-nucleus transcriptomic data from shoot tissues. The last two years have seen numerous datasets profiling leaf and floral tissues from multiple species [38–41].

Moving forward, comparative approaches can address another intriguing question: how do cell identities compare across organs in the same species? In a recent example, Lee et al. (2023) produced a single-nucleus transcriptomic atlas of seed-to-seed development in Arabidopsis to compare cell types and developmental stage progressions across the plant life cycle [42]. Many of the genes unique to both a cell type and an organ have yet to be functionally annotated, suggesting that these signatures could reveal novel biological functions for these specific contexts. These data also provide a framework to compare developmental stage progressions across tissues.

To fully realize the potential of comparative single-cell genomics, we will need to not only generate data from more species and organs but also develop efficient pipelines to integrate the data. A major challenge is comparing datasets when there is not a one-to-one pairwise orthology between species. Machine learning approaches are already being used to address this issue by identifying orthologous marker gene groups [43]. Moving forward, we need to establish transcriptional markers of specific cell identities and cell states to investigate them across species. This effort will be aided by the synthesis and comparison of datasets produced by different labs. To achieve these goals, we will need to maximize the use of available data by adhering to community standards for metadata reporting and developing accessible analysis infrastructure [12].

New technologies preserve spatial context in plant tissues

Across applications, the loss of spatial context is a critical shortcoming of scRNA-seq experiments and necessitates subsequent validation of annotations with other methods such as fluorescent transcriptional reporters [1,6,23,25]. However, creating transcriptional reporters for large numbers of genes is time-consuming and it can be challenging to ensure the inclusion of all cis-regulatory elements. New targeted spatial transcriptomic methods such as PHYTOMap [44], molecular cartography [9], *in situ* sequencing [45], and MERFISH [42,46] now enable high throughput validation of cell type and developmental stage markers identified from plant single-cell data (Figure 2d). These technologies do not require transgenic plants and will simplify the validation of new markers for species that do not yet have comprehensive molecular toolkits [47,48].

Beyond targeting specific genes, recent applications of non-targeted spatial transcriptomic methods with technologies such as Visium from 10× Genomics [49–51], expansion sequencing [52], and Stereo-seq [53] have opened a frontier to generate genome-scale information from individual plant cells while preserving spatial location within a tissue [54]. Untargeted RNA capture, which does not require pre-selection of genes for probe design, is necessary for unbiased experiments. Looking ahead, it will be important to develop methods that leverage the strengths of both approaches, such as updating scRNA-seq annotations based on spatial expression profiles.

Spatial and temporal context informs cell-type responses to biotic interactions

Spatial approaches are proving to be particularly powerful for understanding how plants modulate their development in response to external factors [55]. Biotic interactions, such as microbial infection, may be present in only a subset of tissues and elicit distinct responses from affected cells. Single-cell and spatial approaches have informed plant immune responses [46,56,57] as well the remodeling of plant development that occurs in legume roots during nodule formation in response to bacterial infection [58,59]. Recent studies have deployed sc/snRNA-seq with a combination of either spatial transcriptomics or live imaging. Since plant development is exquisitely plastic and continuously modulated, time-series experiments have also been useful to track responses after infection.

An example combining single-cell transcriptomics and live imaging comes from Zhu et al. (2023), who investigated responses to *Pseudomonas syringae* in the Arabidopsis leaf [56]. Cells within a single time point of scRNA-seq were present at various states of immunity and susceptibility, which enabled the recapitulation of trajectories in pseudotime representing a continuum of immune responses as visualized by live-imaging of reporters. Tang et al. (2023) applied scRNA-seq to two time points after infection with the fungus *Colletotrichum higginsianum*. Complementation of the scRNA-seq data with live imaging was instrumental in identifying cells that directly interacted with fungal hyphae, revealing cell type-specific activation of glucosinolate biosynthesis at the infection site [57].

Nobori et al. (2023) added another layer of information by performing a time-resolved, multi-omic analysis of *P. syringae* infection of the Arabidopsis leaf [46]. Combining transcript and chromatin accessibility readouts from the same nuclei revealed the regulatory logic connecting transcription factors to cis-regulatory elements and downstream target genes. Integration of this multi-omic data with MERFISH spatial transcriptomics datasets placed nuclei in the context of their tissue of origin. Notably, probes targeting bacteria metagenes were also included in the spatial transcriptomics experiments, lending insight to the arrangement of bacterial cells and plant immune responses.

Combining spatial transcriptomics and single-cell omics makes it possible to identify cells that directly interact with pathogens. This increased resolution in space and time will allow researchers to disentangle which responses are cell-autonomous versus non-cell-autonomous, how cell—cell communication is involved in immunity, and ultimately may enable the manipulation of interactions between plants and microbes.

Cell types have distinct responses to abiotic stress

In addition to biotic interactions, single-cell genomics with spatial and temporal context has significant potential to probe how plants remodel their development in response to abiotic stress. A key goal will be to use this information to engineer plants that can withstand volatile conditions caused by climate change, such as drought [60].

An example of developmental adaptations associated with drought stress tolerance comes from snRNA-seq of a strigolactone mutant that revealed changes in the composition of hypocotyl vascular tissue that may enable optimization of water usage during drought [27]. Single-cell approaches also facilitate the characterization of unique cell types such as the exodermis of tomato roots that play an important role in barrier formation during drought [37]. Further defining how abiotic stress signaling networks are rewired in specific developmental contexts should be fruitful. For example, exogenous treatment with abscisic acid (ABA), a hormone that is induced by drought and salinity stressors, causes reduced root meristem size in Arabidopsis and the extremophyte *Schrenkiella parvula*. However, mature root cell length in *S. parvula* nearly doubles upon ABA treatment whereas it is largely unaffected in Arabidopsis [61]. Such sub-tissue level phenotypes are prime candidates for applications of single-cell genomics to capture stress-induced transcriptional responses unique to a specific developmental context.

Understanding how plants recover from drought represents another avenue to improve stress resilience. By combining snRNA-seq with bulk RNA-seq data collected over a finely resolved time series, Illouz-Eliaz et al. (2023) identified over 3000 rehydration-specific genes in Arabidopsis leaves [62]. A rapid increase in immune response upon rehydration suggested a role in preparing plants for microbial challenges accompanying water reappearance after drought. Moderate stress recovery more effectively suppressed bacterial growth compared to severe stress, emphasizing the need for analyzing stress effects at various intensities and integrating abiotic and biotic stress responses in experimental designs.

Unraveling the interplay between development and responses to hormones, microbes, and abiotic stresses requires spatiotemporal information. Single-cell and spatial approaches are poised to probe the stability of cell identities across these conditions as well as the unique cell states that arise during specific responses. Interestingly, responses to some stimuli, such as bacterial infection, produce single-cell expression profiles that appear to cluster primarily by treatment [46,63]. On the other hand, cell identity was the strongest distinguishing feature after brassinosteroid treatment, even though the treatment influences thousands of genes and dramatically alters root growth [25]. Future efforts should address what core aspects of cell identity are maintained across environmental conditions and species as well as how distinct cell states contribute to developmental plasticity in response to stress.

Perspectives

Recent applications indicate that transcriptional information at single-cell resolution is a powerful tool with which to address plant developmental questions. However, there are still significant barriers to entry in terms of experiment cost, protocol development, and computational expertise required for data analysis, especially for biological questions that cannot easily be addressed with off-the-shelf analysis methods. Among the challenges to making plant single-cell data broadly accessible is the dearth of curated reference datasets. Reference atlases for different tissues and organs, such as those produced by the Human Cell Atlas project [64], facilitate the annotation and interpretation of new datasets that push the boundaries of our knowledge. However, comprehensive atlases are available for only a few plant species and they are not yet hosted by a central, easily accessible platform. Further, most atlases are currently limited to the transcriptome. A more complete understanding of cell states and cell identities will require multi-layered regulatory information spanning modalities such as the transcriptome, epigenome [65,66], proteome [67], and metabolome [68,69] gathered over diverse environments and species [70,71]. As the amount of data increases, analytical approaches such as deep learning could facilitate their integration. For

example, a context aware deep learning model called Geneformer was recently trained on 30 million human single-cell transcriptomes [72], enabling the identification of therapeutic targets.

As the plant developmental biology field moves beyond proof of principle studies and atlas generation, our efforts are shifting from simply confirming that single-cell approaches are valid and moving towards deriving biological insights. We will need tools to experimentally test and refine the spatiotemporal models arising from single-cell analysis [13]. For example, single-cell analysis can lead to hypotheses about causal relationships and/or molecular mechanisms that can then be tested by multiple orthogonal approaches. Technologies that can perturb development, such as large-scale CRISPR screening [73,74], will be instrumental in these efforts but have yet to be deployed at the single-cell level in plants.

Given the dynamic nature of development, we anticipate that future experiments will routinely combine single-cell data with spatial information at high temporal resolution. A promising technique is quantitative microscopy to track gene expression dynamics over time in individual cells. These experiments can be used to test hypotheses about gene regulatory network architecture generated *in silico* by network inference techniques from single-cell data. Observations with this level of resolution can reveal unexpected phenomena that are only possible to appreciate by watching development happen in real-time (Figure 2e). For example, the SHR transcription factor was recently shown to initiate asymmetric cell divisions only when present during an early window of the cell cycle [75]. Markers such as the fluorescent plant cell cycle indicator (PlaCCI) will be useful to observe and quantify such cell cycle dynamics [76]. Hormone biosensors [77] can also relate *in situ* expression patterns to the control of spatiotemporal organ development.

3D and 4D microscopy experiments are already being integrated with single-cell omics to connect gene expression to morphological changes *in planta* [78,79]. Future employment of these multifaceted approaches will be critical for understanding how gene regulatory networks operate at specific physical locations and under certain conditions. In particular, unraveling how biotic and abiotic stresses alter growth dynamics will increase our abilities to engineer plant form and function with synthetic biology approaches [80]. This foundational knowledge is essential to produce resilient plants capable of withstanding the climate challenges of the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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