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Application of Single-Cell RNA-Seq in Legume Root Development Studies

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Abstract The single-cell RNA sequencing method known as scRNA-seq has revolutionized the study of plant root cellular complexity and developmental processes. The research combines modern scRNA-seq methods to study legume root development by showing how these methods help identify cell types and track cell lineages and detect short-lived cell populations. The research in Arabidopsis model systems produced complete root cell maps which revealed vital elements that regulate cell development and environmental adaptation thus enabling legume research. ScRNA-seq analysis of legumes has enabled researchers to study gene expression patterns in different cell types during root and nodule development which has enhanced our understanding of symbiotic processes and plant stress mechanisms. Single-cell plant research encounters technical barriers because of protoplasting artifacts and cell capture biases but scientists continue to develop their research through the combination of single-nucleus RNA-seq and spatial transcriptomics. The research investigates the necessity of root cell atlases that span multiple species within legumes while exploring the combination of scRNA-seq with other omics methods and their potential to develop new crop improvement approaches based on single-cell research findings. ScRNA-seq technology has the potential to revolutionize our knowledge of legume root biology which will drive major breakthroughs in plant developmental research and sustainable agricultural methods.

Keywords Single-cell RNA sequencing; Legume root development; Cell type identification; Transcriptome atlas; Nitrogen fixation

1 Introduction

The agricultural sector depends on legume crops including soybean and Lotus japonicus because they provide food security and improve soil fertility and support sustainable farming practices. The plants serve as a primary source for food and feed production across the world while their partnership with rhizobia allows them to transform atmospheric nitrogen into soil nutrients which reduces the need for synthetic fertilizers (D'Agostino et al., 2023). The root development of legumes serves as a vital process because their root system with nodules enables successful nutrient absorption and symbiotic relationship preservation (Sun et al., 2022). The study of root development mechanisms at cellular and molecular levels enables researchers to develop improved crops and sustainable agricultural methods.

The field of plant biology has experienced a major transformation through recent transcriptomics breakthroughs because single-cell RNA sequencing (scRNA-seq) has become a revolutionary tool. The single-cell RNA sequencing method scRNA-seq provides researchers with the ability to study gene expression at the individual cell level for identifying unique cell populations and their developmental paths and environmental response patterns (Denyer et al., 2019; Ryu et al., 2019; Shulse et al., 2019; Serrano-Ron et al., 2021). The combination of scRNA-seq and sNucRNA-seq techniques has started to analyze root tissue complexity in legumes which has exposed the regulatory mechanisms that control phytohormone responses and nodulation and plant-microbe interactions (Sun et al., 2022). The new methods for extracting high-quality nuclei from difficult-to-process tissues such as root nodules became possible through technological advancements which enabled single-cell research in legumes (D'Agostino et al. 2023).

The objective of this study is to synthesize current knowledge on the application of scRNA-seq in legume root development studies. The research investigates novel analytical techniques and biological discoveries through the connection between single-cell data and complete transcriptomic and gene regulatory network analyses. The

research presents recent breakthroughs and current obstacles to create a complete guide for scientists who want to use single-cell methods to study legume root biology and its effects on agriculture.

2 Single-Cell RNA Sequencing Technology: Principles, Platforms, and Challenges in Plant Systems

2.1 Principles of scRNA-seq: from tissue dissociation to sequencing

Single-cell RNA sequencing (scRNA-seq) allows researchers to study individual cell transcriptomes which reveals hidden cell diversity and rare cell populations that bulk sequencing methods cannot detect. The process begins with tissue dissociation to generate single-cell suspensions followed by cell capture and lysis and then RNA reverse transcription to cDNA followed by amplification and library preparation and high-throughput sequencing. The application of Unique molecular identifiers (UMIs) and cell barcodes by researchers allows them to detect particular cell-derived transcripts and correct amplification bias for accurate gene expression analysis (Liu and Trapnell, 2016; AlJanahi et al., 2018; Rich-Griffin et al., 2019).

2.2 Technical platforms and their application in plant systems

Multiple scRNA-seq platforms exist for plant research because they provide different benefits for scientific applications. The Drop-seq system uses microfluidics to create droplets that contain single cells and barcoded beads which allows researchers to analyze thousands of cells simultaneously. It is cost-effective but has lower capture efficiency compared to other platforms (AlJanahi et al., 2018). The 10x Genomics Chromium system operates as a commercial droplet-based platform which achieves high capture efficiency and sensitivity levels for working with scarce tissue samples and detecting rare transcripts (Baran-Gale et al., 2017; Rich-Griffin et al., 2019). The SMART-seq/SMART-seq2 method provides a well-based solution to obtain full-length transcripts with high sensitivity which works best for analyzing rare tissues and small cell numbers. It is often combined with fluorescence-activated cell sorting (FACS) (Baran-Gale et al., 2017). Plant systems have proven the effectiveness of these platforms through their successful application to model species Arabidopsis thaliana, maize and rice which generated complete root cell type and developmental pattern maps (Jovic et al., 2022).

2.3 Challenges in applying scRNA-seq to plant tissues compared to animal systems

The application of scRNA-seq to plants requires overcoming specific technical challenges. The process of removing cell walls through enzymatic digestion (protoplasting) for single cell release causes stress responses and cell recovery bias particularly in lignified and suberized tissues (Bawa et al., 2022). The process of cell viability and RNA quality assessment proves more difficult in plants because it affects the final data quality (Rich-Griffin et al., 2019). The complexity of tissue structure together with its cell wall diversity leads to poor dissociation outcomes which results in missing specific cell types (Hazarika et al., 2025). The plant scRNA-seq method operates with lower detection sensitivity and reduced throughput compared to animal systems which need particular optimization protocols (Liu and Trapnell, 2016).

2.4 Data analysis pipelines for plant scRNA-seq

The computational process for plant scRNA-seq data analysis consists of multiple stages which begin with Quality Control. The process of filtering out low-quality cells and doublets and empty droplets relies on gene/UMI counts and mitochondrial RNA content. The normalization process enables researchers to achieve proper cell-to-cell comparison through the adjustment of sequencing depth and technical variation (Bacher and Kendziorski, 2016; Andrews et al., 2020). The process of cell type and state identification involves running PCA t-SNE or UMAP algorithms for dimensionality reduction followed by clustering analysis (Andrews et al., 2020). The analysis of marker genes and developmental lineage reconstruction or stimulus response tracking is described in. Specialized Tools: The development of plant-specific pipelines and adaptations continues to address particular difficulties in plant data analysis which include problems caused by protoplast-induced artifacts and cell-type annotation.

3 Root Development in Legumes: A Biological Framework

3.1 Root architecture and developmental zones

The root system of legumes consists of three main parts which include the primary root and lateral roots and the specialized nodules. The primary root contains three main developmental areas which include the root apical meristem for cell division and the elongation zone for cell growth and the differentiation zone for cell maturation and specialization. The formation of lateral roots happens after embryonic development to create a flexible root system which helps plants adapt to their environment (Bensmihen, 2015). The root system of plants shows strong reactions to environmental factors including nutrient availability and stress conditions like phosphorus deficiency and drought (Ye et al., 2018; Chen et al., 2023).

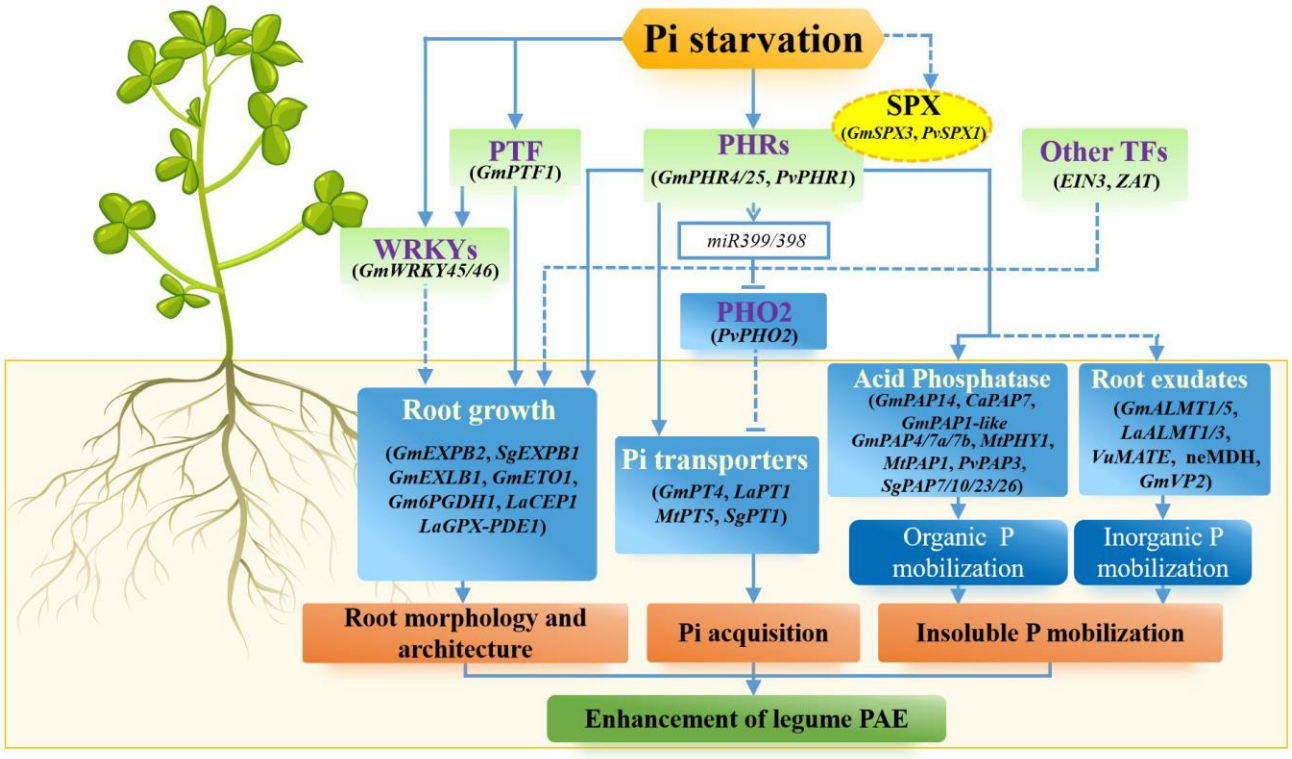

Figure 1 Strategies for improving P acquisition efficiency through gene regulation in legumes. In legumes, P acquisition efficiency can be achieved by remodeling of root morphology and architecture, inducing high-affinity Pi transporters, increasing root exudates to facilitating P mobilization, and activating Pi signaling network. A variety of Pi starvation-induced (PSI) genes have been implicated in improving P acquisition efficiency in legumes. These are related to root growth, Pi uptake, insoluble P mobilization and Pi signaling network (Adopted from Chen et al., 2023)

3.2 Genetic regulation of root cell fate and differentiation

Multiple genetic systems consisting of transcription factors and microRNAs and hormonal signaling pathways work together to control the development of legume roots. The SHORTROOT–SCARECROW (SHR–SCR) module functions as a key regulator that determines cortical cell fate while being vital for nodule organogenesis (Dong et al., 2020). The NODULE INCEPTION (NIN) transcription factor controls both nodule and lateral root development through its regulation of downstream targets that include ASL18/LBD16a and NF-Y subunits (Soyano et al. 2019; Suzaki, 2023). The microRNAs miR166 and miR2111 control transcription factor expression and systemic signaling pathways which affect both root development and nodule formation (Gautrat et al. 2020; Boualem et al., 2008). Plant hormones including auxin and cytokinin and abscisic acid and strigolactones function as either opposing or cooperative factors that regulate the development of lateral roots and nodules (Bensmihen, 2015; Gauthier-Coles et al., 2019; Goto et al., 2022).

3.3 Symbiotic nodulation and integration with root development

The symbiotic nodulation process in legumes happens through root nodule development which serves as a habitat for nitrogen-fixing rhizobia. The process of nodule formation starts when Nod factors from rhizobia initiate cell division in cortical tissue and organ formation which work alongside root development mechanisms (Luo et al., 2023; Ferguson et al., 2010; Suzaki, 2023). The development of nodules in legumes followed lateral root developmental pathways through common genetic elements and signaling mechanisms which included NIN and hormonal interactions (Soyano et al., 2019). The system regulates nodule development through autoregulation of nodulation (AON) and small peptide and microRNA feedback systems to determine proper nodule numbers and resource distribution (Djordjevic et al., 2015; Gautrat et al., 2020).

3.4 Comparative aspects with non-legume model plants

While the basic organization of root developmental zones is conserved between legumes and non-legumes like Arabidopsis thaliana, legumes possess unique features. The SHR–SCR module in legumes enables cortical cytokinin response to initiate cell division in cortical cells which results in nodule organ formation that Arabidopsis lacks (Gauthier-Coles et al., 2019; Dong et al., 2020). The process of root and nodule development follows different hormonal control patterns because legumes developed unique signaling pathways to link symbiotic and environmental signals (Bensmihen, 2015; Gauthier-Coles et al., 2019; Lin et al., 2021). The distinct characteristics between these two species reveal the evolutionary changes which led to their unique root development.

4 Application of scRNA-Seq in legume root development studies

4.1 The research establishes multiple cell types which develop through distinct pathways within Legume root systems

The single-cell RNA sequencing technique enables scientists to develop complete root cell atlases which help them identify every root cell type and developmental phase in legume roots. Scientists use scRNA-seq to analyze thousands of individual cells which helps them construct developmental pathways and predict cell fate transitions from stem cells to differentiated root tissues (Ryu et al., 2019; Serrano-Ron et al., 2021; Coll et al., 2024). The analysis of pseudotime enables researchers to track cell progression from meristematic zones through elongation and differentiation stages (Denyer et al., 2019; Bawa et al., 2022).

4.2 Gene expression heterogeneity and discovery of rare cell populations

The scRNA-seq technique provides exceptional capabilities to detect gene expression diversity in root tissues which leads to the discovery of infrequent cell types that were unknown before. Standard bulk analysis methods lack the ability to detect two cell populations which the technology has identified as quiescent center cells and specialized epidermal cells (Ryu et al., 2019; Shaw et al., 2020). Scientists can understand root functions and developmental flexibility through the identification of these rare cell types (Shaw et al., 2020; Liao and Wang, 2023).

4.3 Insights into root meristem activity and cell cycle regulation

The research delivers information about root meristem cell operations and their cell cycle management systems. High-throughput scRNA-seq has revealed root meristem molecular operations by identifying the factors which control cell cycle progression and stem cell maintenance. The analysis of cell cycle continuums and differentiation trajectories through scRNA-seq has revealed how meristematic activity relates to root growth and regeneration (Ryu et al., 2019; Liao and Wang, 2023). Scientists can use this method to observe the cell reprogramming process and root cell commitment during regeneration.

4.4 Mapping root cell-specific responses to environmental signals

scRNA-seq allows for the mapping of cell-type-specific responses to environmental cues such as nutrient deficiency and abiotic stress. The analysis of gene expression at a single-cell level by scientists reveals particular root cell type reactions to environmental changes which helps them understand stress adaptation mechanisms and signaling pathways (Shaw et al., 2020; Bawa et al., 2022). The research provides essential information about how legumes change their behavior when soil nutrient availability and environmental conditions change.

4.5 Integration of scRNA-seq with other omics tools

The combination of scRNA-seq with ATAC-seq and spatial transcriptomics and proteomics allows scientists to study root development at multiple levels of detail. The combination of different methods in multimodal approaches allows researchers to build gene regulatory networks and find gene expression locations and link transcriptomic data to protein functions and chromatin states (Serrano-Ron et al., 2021; Zheng et al., 2021). The combination of these approaches enables researchers to study root biology as a whole system which helps them understand how cells decide their fate and how they respond to their environment.

5 Case Study

5.1 scRNA-seq in *Medicago truncatula* root nodulation

Scientists use single-nucleus RNA sequencing (sNucRNA-seq) technology to analyze cell-type-specific responses that occur during the initial nodulation process in Medicago truncatula. The researchers established a complete gene expression map through profiling of nuclei from roots that received mock inoculation and rhizobia inoculation which resulted in 25 distinct cell clusters that they identified using Medicago and Arabidopsis marker genes. The analysis showed that root hair cells together with cortex cells and endodermis cells and pericycle cells demonstrated the most significant changes in gene expression following rhizobial infection. The sNucRNA-seq method revealed existing genes and newly discovered signaling pathways which control symbiotic communication start-up and delivered detailed knowledge about nodule development and root cell transformation (Figure 2) (Cervantes-Pérez et al. 2022).

5.2 Application in *Lotus japonicus* Root Development

While the provided search did not yield direct scRNA-seq studies in Lotus japonicus, the methodologies and insights from Medicago truncatula are highly relevant. The model legume Lotus japonicus exhibits identical root developmental patterns and symbiotic processes to Medicago and researchers can directly use established protocols for nuclei extraction and cell-type identification through cross-species marker gene analysis. The research of Lotus japonicus through single-cell methods will enhance our understanding of root and nodule development because it allows cell-type-specific gene expression analysis that extends the findings of Medicago research (Cervantes-Pérez et al., 2022).

5.3 Lessons learned and methodological improvements

The research studies demonstrate multiple essential findings together with new methodological techniques.

The technique sNucRNA-seq enables researchers to detect both frequent and infrequent cell types and their particular reactions to symbiotic signals. The research by Cervantes-Pérez et al. (2022) shows that Arabidopsis marker genes can effectively identify cell types in legumes even when there are limited cell-type-specific markers available. Discovery of Novel Pathways: Single-cell approaches uncover previously unrecognized genes and regulatory networks involved in nodulation and root development. Technical adaptations: the use of nuclei rather than whole cells overcomes challenges posed by plant cell walls, improving the feasibility and quality of single-cell transcriptomic studies in legumes.

6 Challenges and Future Directions in scRNA-Seq for Legume Root Development

6.1 Technical limitations: protoplasting, cell viability, and biases in cell capture

The main technical hurdle in plant scRNA-seq analysis requires protoplasting to remove cell walls but this process triggers stress reactions that damage cells and produces false transcriptional data (Shaw et al., 2020; Sun et al., 2024). The dissociation process fails to properly break down cells with thick or lignified walls which results in uneven cell collection and incomplete cellular mapping (Shaw et al., 2020; Sun et al., 2024). The analysis of single-cell RNA sequencing data faces two major challenges which scientists need to solve by designing experiments correctly and implementing data normalization methods to remove technical artifacts and batch effects (Shaw et al., 2020; Sun et al., 2024).

6.2 Need for cross-species root cell atlas development in legumes

The root atlases of Arabidopsis and several crop species exist in detail yet most legume species do not have complete cross-species cell-type maps. The creation of these atlases serves multiple purposes because it enables researchers to conduct comparative studies and transfer marker genes and identify both shared and distinct features of root biology in legumes. The study of single cells across different legume species will help scientists identify common regulatory systems and unique control mechanisms for each species (Shaw et al., 2020; Bawa et al., 2022; Sun et al., 2024).

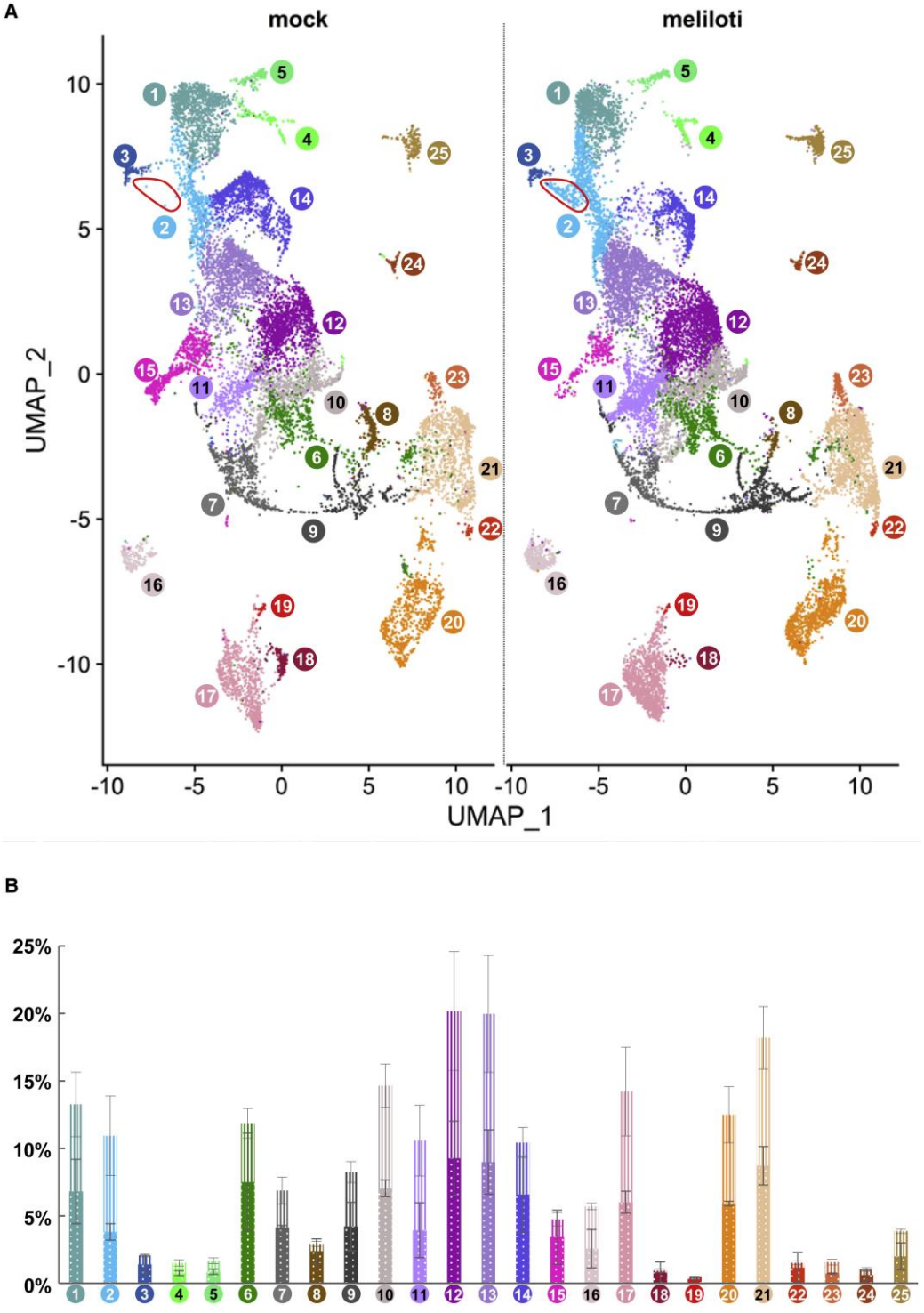

Figure 2 Single-nuclei RNA-seq of the *M. truncatula* roots reveals 25 different root clusters (Adopted from Cervantes-Pérez et al. 2022)

6.3 Integration of scRNA-seq with spatial and temporal resolution approaches

The spatial information gets lost when using scRNA-seq as a standalone method which makes it hard to determine where specific genes are expressed in the tissue. The integration of scRNA-seq with spatial transcriptomics and lineage tracing and time-course experiments enables scientists to construct developmental pathways while observing cell-cell interactions in their natural tissue environment. The integration of scRNA-seq with ATAC-seq and proteomics enables researchers to better understand gene regulatory networks and epigenetic landscapes (Denyer et al., 2019; Shaw et al., 2020; Zheng et al., 2021).

6.4 Prospects for translating findings into crop improvement and breeding strategies

The large dataset from scRNA-seq allows researchers to discover essential regulators of root development and stress adaptation and symbiotic interactions which scientists can use to develop genetic engineering and breeding targets. Single-cell methods will establish themselves as essential tools for improving legume crops through improved protocols and reduced costs which will allow scientists to create new crop varieties with superior nutrient absorption and stress resistance and symbiotic relationships (Shaw et al., 2020; Bawa et al., 2022; Zheng et al.,2021).

7 Challenges and Future Directions in scRNA-Seq for Legume Root Development

7.1 Technical limitations: protoplasting, cell viability, and biases in cell capture

The main technical hurdle in plant scRNA-seq analysis requires protoplasting to remove cell walls but this process triggers cellular stress reactions and cell death and generates artificial gene expression modifications. Cell dissociation methods fail to effectively break down cells with thick or lignified walls which leads to unbalanced cell collection and incomplete cellular mapping. The study of single-cell RNA sequencing data faces two major challenges which include technical noise and batch effects that need proper experimental design and data normalization methods (Shaw et al., 2020; Sun et al., 2024).

7.2 Need for cross-species root cell atlas development in legumes

The root atlases of Arabidopsis and several crop species exist in detail but most legumes do not have complete cross-species cell-type maps. The creation of these atlases serves three main purposes which include comparative research and marker gene transfer and identification of shared and distinct root biology features in legumes. Scientists can discover common regulatory patterns and unique control systems of each plant species through studying single cells across different legume species (Shaw et al., 2020; Bawa et al., 2022).

7.3 Integration of scRNA-seq with spatial and temporal resolution approaches

The main challenge of scRNA-seq data analysis arises from its inability to maintain spatial data which prevents researchers from identifying specific tissue locations for gene expression. Researchers can create developmental pathways and observe cell-cell interactions in their native tissue locations through the combination of scRNA-seq with spatial transcriptomics and lineage tracing and time-course experiments. The integration of scRNA-seq with ATAC-seq and proteomics enables researchers to study gene regulatory networks and epigenetic landscapes better (Denyer et al., 2019; Shaw et al., 2020; Zheng et al., 2021).

7.4 Prospects for translating findings into crop improvement and breeding strategies

Scientists can identify essential regulators of root development and stress responses and symbiotic relationships through scRNA-seq data which serves as a basis for genetic modification and plant breeding programs. Single-cell methods will drive forward legume crop development through improved methods and reduced costs to produce new varieties that show better nutrient absorption and stress resistance and symbiotic relationships (Shaw et al., 2020; Zheng et al., 2021; Bawa et al., 2022).

8 Conclusion

Single-cell RNA sequencing (scRNA-seq) has transformed the study of legume root development because it allows scientists to analyze cell diversity and detect infrequent cell populations while building comprehensive models of developmental progression. The technology has delivered complete information about cell fate determination and gene regulatory mechanisms and root and nodule development processes in legumes. The

analysis of thousands of individual cells through scRNA-seq has enabled researchers to study how different cell types react to environmental signals and identify essential factors that control root development and cell differentiation and symbiotic processes.

The application of scRNA-seq technology produces results which extend past legume research to create a new approach for studying plant development. The development of methods to detect cell states and transitions in complex tissues has enabled scientists to study plant stem cell maintenance and regeneration and environmental stress adaptation. Scientists employ integrated single-cell analysis techniques that combine spatial transcriptomics with epigenomics to examine plant development by analyzing both spatial and temporal patterns.

ScRNA-seq technology shows promising applications for enhancing agricultural practices in legume crops. The technology enables researchers to discover cell-type-specific gene expression patterns and regulatory networks which leads to new targets for breeding and genetic engineering to boost nutrient uptake and stress resistance and symbiotic performance. Single-cell methods and technical solution advancements will establish scRNA-seq as a core analytical method for studying legume biology and crop research which will propel sustainable agricultural development.

ScRNA-seq has revolutionized legume root development research through its discoveries which now benefit both plant science and agricultural practices. Future research on legume crops will gain from continuous method development and combined research approaches which will open up fresh opportunities for their advancement.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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