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Spotlight

Yet uninfected? Resolving cell states of plants under pathogen attack

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Although we have made significant strides in unraveling plant responses to pathogen attacks at the tissue or major cell type scale, a comprehensive understanding of individual cell responses still needs to be achieved. Addressing this gap, Zhu et al. employed single-cell transcriptome analysis to unveil the heterogeneous responses of plant cells when confronted with bacterial pathogens.

The plant biology community has embraced single-cell transcriptome (single-cell RNA sequencing [scRNA-seq]) technologies, allowing researchers to view plant tissue with the compound lens of genomics and cell biology.[1] In addition to enabling granular and comprehensive analyses of known cell types, scRNA-seq can reveal different states of each cell type that are influenced by cell-intrinsic and cell-extrinsic factors. The cell cycle represents a cell-intrinsic factor, whereas environmental stimuli contribute as cell-extrinsic factors. A particularly heterogeneous environmental stimulus is pathogen infection because their distribution is non-uniform and individual cells of pathogens respond to plants independently using genetic programs co-evolved with the hosts. For instance, distinct fungal pathogen *Magnaporthe oryzae* infection stages were observed in a single rice leaf.[2] Plant cell responses can also be highly variable depending on their spatial relationships with pathogen cells and activity. Moreover, different plant cell types possess the distinct potential to respond to microbial signals, as previously shown by transcriptome analyses of sorted cell populations.[3]

Key questions in plant-pathogen interactions that can be addressed through scRNA-seq analyses include: (1) how does the spatial heterogeneity of infection contribute to molecular heterogeneity in individual cells? (2) How do known immune and susceptible pathways function in individual cells? (3) Can single-cell analyses identify specific cell states in pathogen-infected plants and reveal genes and pathways that operate in distinct cell states, which may have been overlooked in previous bulk tissue analyses?

Zhu et al.[4] tackled these questions by employing a model plant pathosystem, where *Arabidopsis thaliana* leaves are challenged by the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000). *Pst* DC3000 uses effector molecules and toxins to subvert the plant immune system, proliferating in the leaf intercellular space (apoplast) and causing disease symptoms in plants.[5] Observation of fluorescently labeled *Pst* DC3000 revealed a heterogeneous distribution of the pathogen cells during infection in *A. thaliana*. For scRNA-seq analysis, the authors isolated cells (protoplasts) by digesting the cell wall of leaves 24 h after infection (syringe infiltration). Because *Pst* DC3000 mainly proliferates in the apoplast surrounded by mesophyll cells, the authors employed a protoplast method known as the "Tape-Arabidopsis Sandwich method" that enriches this cell type. The authors also performed bulk RNA-seq with and without the protoplasting process and identified 7,548 genes induced by protoplast treatment; these genes were removed from scRNA-seq analyses.

As expected, most cells (~94.7% of 11,895 cells) profiled by scRNA-seq were annotated as mesophyll cells, but these cells showed diverse transcriptome patterns in pathogen-infected leaves. The authors identified five mesophyll subpopulations (clusters) affected by pathogen infection. Gene Ontology analysis identified distinct clusters enriched with genes responsive to different plant hormone pathways, salicylic acid (SA) and jasmonic acid (JA), as well as an intermediate cluster. Based on known genes involved in immunity and susceptibility against the bacterial pathogen, the authors confirmed that the clusters with SA and JA responses were enriched with immunity and susceptibility genes, respectively. Together, the scRNA-seq data identified distinct cell states within a cell type spanning from immunity to susceptibility states influenced by pathogen infection.

The authors then sought to understand how cells transition between identified cell states during pathogen infection. They employed pseudotime analysis, a method commonly applied for inferring developmental trajectories by aligning cells as trajectories based on their gene expression patterns. Pseudotime analysis applied to mesophyll cells revealed a mostly linear trajectory that progressed through non-pathogen-responsive clusters, followed by immune clusters, an intermediate cluster, and ended in the susceptible clusters. Furthermore, the authors identified genes strongly expressed at distinct phases of the disease progression trajectory, providing valuable information for further characterizing each cell state.

The authors validated gene expression dynamics predicted by pseudotime analysis using transgenic reporter lines that visualize promoter activity associated with selected genes. Reporter plants were

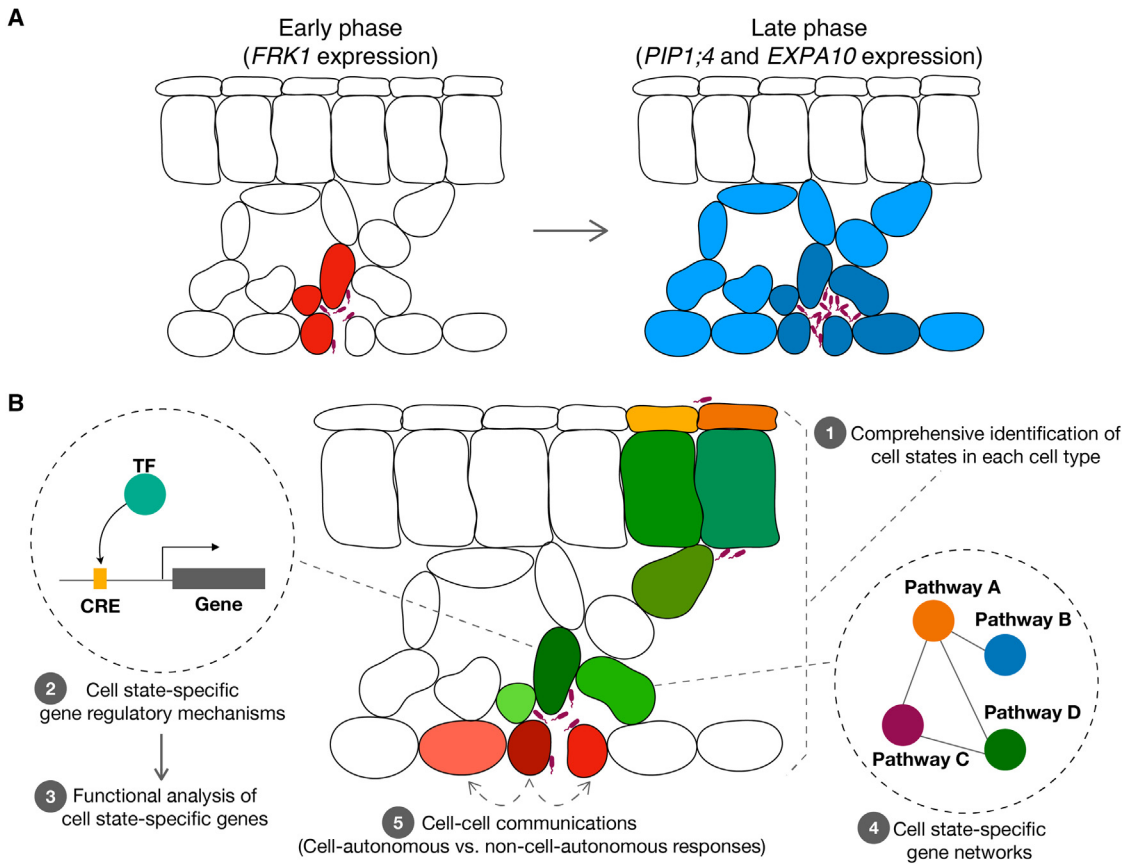

Figure 1. Resolving plant-pathogen interactions at the single-cell and spatial resolution

(A) Schematic diagrams of the spatial expression patterns of immune and susceptible markers in a plant leaf at different stages of infection by a bacterial pathogen. The immune marker *FRK1* (red) is induced locally at an early stage of infection, whereas the susceptible markers *PIP1;4* and *EXPA10* (blue) are induced broadly at a later stage of infection.

(B) Single-cell and spatial omics technologies can address important future topics in plant-pathogen interactions. TF, transcription factor; CRE, *cis*-regulatory element.

surface-inoculated with the pathogen to mimic natural infection. Three marker genes of immune clusters—*FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*), *LipoP1* (*LIPOPROTEIN 1*), and *CBP60g* (*CALMODULIN BINDING PROTEIN 60g*)—were locally induced surrounding bacterial colonies at an early stage of infection (Figure 1A), consistent with the pseudotime prediction. In contrast, three marker genes of susceptibility clusters—*EXPA10* (*EXPANSIN A10*), *PIP1;4* (*PLASMA MEMBRANE INTRINSIC PROTEIN 1;4*), and *ILL5* (*IAA-leu-resistant-like5*)—were induced broadly at later infection stages, also in line with the scRNA-seq data (Figure 1A).

Apart from observing common trends among selected immune or susceptible marker genes, the authors also observed gene-specific spatial expression patterns. When surface-inoculated, expression of *FRK1*—a well-known early marker gene of pathogen-triggered immune responses—was frequently observed in cells surrounding substomatal cavities colonized by bacteria at an earlier time point. In contrast, *LipoP1* and *CPB60g* exhibited more diverse spatiotemporal expression patterns that appeared to be associated with bacterial population size. Diverse expression patterns were also observed among the susceptible marker genes. Together, simultaneous imaging of an immune marker gene and bacterial colonization provides insights into where different cell states are localized.

The pioneering work by Zhu et al.[4] demonstrated the potential of integrating scRNA-seq and imaging techniques to unveil spatiotemporal dynamics of cell states during pathogen infection in plants. The diverse spatial expression patterns of immune and susceptible marker genes highlight the need to simultaneously analyze multiple genes in the same tissue to capture fine-grained cell-state information. However, transgenic reporter analysis is time-consuming and limited in the number of genes that can be analyzed simultaneously. Emerging spatial transcriptomics technologies enable high-throughput spatial mapping of hundreds or thousands of genes at single-cell resolution.[6] Expanding the use of single-cell and spatial omics technologies provides an opportunity to tackle essential unanswered questions in plant-pathogen interactions (Figure 1B): (1) what cell states exist beyond mesophyll cells, and when and where do they emerge? Single-nucleus RNA-seq (snRNA-seq) can facilitate

this investigation as nuclei isolation is more accessible to different cell/tissue types with minimal artifact during sample preparation. Studying other plant-pathogen models is also valuable in broadening our knowledge of potential cell states that a plant can take; a recent study identified cell-type-specific immune responses against the fungal pathogen *Colletotrichum higginsianum* using scRNA-seq.[7] In addition, integrating sc/snRNA-seq with spatial transcriptomics can comprehensively map identified cell states in the tissue context.[8] (2) How are genes regulated in individual cell states? Single-cell epigenome (or multiome) analysis holds promise for identifying gene regulatory modules (e.g., transcription factors, *cis*-regulatory elements, and target genes) at single-cell resolution.[8] (3) What are the functions of individual cell states and their marker genes? For instance, the function of the newly identified immune marker *LipoP1* is still elusive. Cell-type/state-specific genetic manipulation is crucial in addressing this question.[9] (4) How can known interactions between pathways be explained at the single-cell level? Plant hormone pathways (e.g., SA, JA, and ethylene pathways) are known to tightly interact to form the immune network.[10] It is still unknown whether such interactions between hormone pathways occur within the same cell or if multiple different cells collectively explain these pathway interactions. To address this question, single-cell omics analysis of mutants lacking one or more pathways would provide a powerful approach. (5) How do plant cells communicate with each other? Understanding spatial relationships between cells with molecular information is critical for understanding their potential interactions. Particularly, studying the responses of cells that initially recognize pathogens is key to understanding immune signal propagation and distinguishing between cell-autonomous and non-cell-autonomous responses.[11] As cell-cell interactions occur in a three-dimensional context, a new method enabling multiplexed spatial gene expression analysis in whole-mount tissues holds great promise.[12] By harnessing the power of cutting-edge single-cell and spatial omics techniques, we gain an extraordinary glimpse into the intricate world of plant-microbe interactions.

DECLARATION OF INTERESTS

J.R.E. serves on the scientific advisory board of Zymo Research, Inc and Cibus, Inc.

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