Development of a single-cell atlas for woodland strawberry (*Fragaria vesca*) leaves during early *Botrytis cinerea* infection using single cell RNA-seq

Yibo Bai, Hui Liu, Haimeng Lyu, Liyao Su, Jinsong Xiong and Zong-Ming (Max) Cheng

Correspondence: Zong-Ming (Max) Cheng (zmc@njau.edu.cn)

1 College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

Full list author information is available at the end of the article

Running title: Woodland strawberry leaves single-cell atlas upon *B. cinerea* infection

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Abstract

Pathogen invasion leads to fast, local-to-systemic signal transduction that initiates plant defense responses. Despite tremendous progress in past decades, aspects of this process remain unknown, such as which cell types respond first and how signals are transferred among cell types. Here, we used single-cell RNA-seq of more than 50,000 single cells to document the gene expression landscape in leaves of woodland strawberry during infection by Botrytis cinerea and identify major cell types. We constructed a single-cell atlas and characterized the distinct gene expression patterns of hydathode, epidermal, and mesophyll cells during the incubation period of B. cinerea infection. Pseudotime trajectory analysis revealed signals of the transition from normal functioning to defense response in epidermal and mesophyll cells upon B. cinerea infection. Genes related to disease resistance showed different expression patterns among cell types: disease resistance-related genes and gene encoding transcription factors were highly expressed in individual cell types and interacted to trigger plant systemic immunity to B. cinerea. This is the first report to document the of single-cell transcriptional landscape of the plant pathogenic invasion process, it provides new insights into the wholistic dynamics of host-pathogen interactions and can guide the identification of genes and the formulation of strategies for resistant cultivar development.

Introduction

Plant pathogens cause billions of dollars in economics losses by reducing or eliminating crop yield or quality. Plants respond to external infections and defend themselves against pathogen invasion by initiating two branches of the innate immune system. The first branch identifies and responds to microbial molecules, including those from non-pathogens, by means of pattern recognition receptors (PRRs) located on the surface of cell membranes. PRRs recognize pathogen-associated molecular patterns (PMAPs) and induce PAMP-triggered immunity (PTI) in plants. The second branch of the innate immune system responds to pathogen factors. When pathogenic bacteria escape the PTI response through secreted protein factors, plant receptor R proteins recognize the secreted effector proteins and induces effector-triggered immunity (ETI) by interacting with them directly or indirectly.
This process has been studied in *Botrytis cinerea*, a necrotrophic fungus of global importance that causes grey mold disease and infects more than 1000 species worldwide, including over 200 crop species, such as grapes and strawberry. In hot, humid environments, conidia of *B. cinerea* invade through stomata, hydathodes, or wounds, and early disease diagnosis may protect plants from damage. In recent years, mechanisms of strawberry defense against the early stage of grey mold disease have been studied by various omics approaches and conventional omics have been used to characterize the response to *B. cinerea* at the whole-organ level. However, such experiments can obscure the characteristics of different cell populations, and cell heterogeneity in the biotic stress response may affect disease assessment and targeted treatment. For this reason, single-cell sequencing technology has potential utility for studying the heterogeneity of cell responses during the pathogen incubation period of plants.

Single-cell technology has recently been applied to plant biology because of its ability to map the transcriptional landscape with a high degree of spatial resolution. With this technology, it is possible to explore the heterogeneity among different tissue and cells types and to identify unknown cell types. This enables predictions of developmental trajectory based on different states of the cells. The limited research to date has established protocols for distinguishing among various heterogeneous cell types, and these protocols have been applied to *Arabidopsis* root system development, stomatal development in *Arabidopsis* leaves, and construction of a single-cell atlas for maize ears. Single-cell sequencing technology has also been applied to studies of plant stress. For example, the root cells of *Arabidopsis* exhibit heterogeneous responses to heat stress. Under low-phosphate conditions, *Arabidopsis* roots increase the density of vascular cells and root hairs to maintain normal physiological activities. Under high salinity, low nitrogen, and iron deficiency, the roots and above-ground parts of rice seedlings show heterogeneity in stress response among cell type. However, there has been no single-cell research on plants under disease stress. In this study, we used scRNA-seq to construct a single-cell transcriptome atlas of woodland strawberry leaves at three stages of *B. cinerea* infection: 0 h post inoculation (hpi, Mock), 6 hpi (S6), and 12 hpi (S12). We identified the features of cells and molecules in woodland strawberry leaves at different disease stages and characterized cells from primary infection sites, such as hydathode, upper epidermal and mesophyll cells, that respond first to *B. cinerea* infection. In addition, we analyzed the
pseudotime trajectories of upper epidermal and mesophyll cells across different lesions. Finally, we documented the expression profiles of disease-related genes and genes that encode transcription factors. Our atlas provides new insights into the cellular heterogeneity during plant-pathogen interactions.

**Results**

**A single-cell atlas of woodland strawberry leaves**

To characterize the single-cell profiles of woodland strawberry, we performed scRNA-seq on the first true leaves of ‘Hawaii 4’ seedlings (Fig. 1a). Large-scale single-cell isolation yielded at least 20,000 cells that were combined with gel beads carrying cell tag sequences and wrapped in droplets (Fig. 1a). After removing double cells and low-quality cells, we obtained a single-cell transcriptome of 15,039 cells from the mock sample; it contained 861,223,068 reads, 65.50% of which mapped to the *F. vesca* v4.01 genome. The median number of unique molecular identifiers (UMIs) of per cell was 15,233, and an average of 3,344 genes were expressed per cell. Expression of 21,392 genes was detected in the mock sample (Table S1).

When the bulk RNA-seq and scRNA-seq data were compared, gene expression in the bulk-sequenced and single-cell-sequenced true leaves showed a significant correlation (*R* = 0.86, *P* < 2.2e-16). In general, the scRNA-seq data obtained in this experiment were of high quality (Fig. 1c, S2a, b).

After filtering and removal of mitochondria and chloroplast organelles, 15,018 cells were used for dimensionality reduction and divided into 12 cell clusters based on their heterogeneity using the t-SNE tool (Fig. 1b). Because there are no previous reports on cell heterogeneity in strawberry, the *Arabidopsis* orthologs of cluster-specific genes were used to annotate these clusters (Table S3). Ep3 and WRKY29 are highly expressed and specific to hydathode cells. In epidermal cells, we identified lipid-transfer proteins (LTP1, LTP3), and 3-ketoacyl-CoA synthase 6 (CER6) as marker genes. The cell cluster enriched in waxy layer-related genes was identified as the upper epidermal, and the clusters enriched in FMA was identified as the lower epidermal. Ribulose bisphosphate carboxylase small chain 1A (RBCS1A), chlorophyll a-b binding protein of the LHCII type (CAB1), chlorophyll a-b binding protein (LHCA2), and light harvesting complex photosystem II subunit 6 (LHCB6) are known marker
genes for mesophyll cells\textsuperscript{14,27-29}. Calmodulin-like protein 1 and phloem protein 2-A10 (PP2-A10) served as markers for phloem cells\textsuperscript{20,25}. Cyclin-dependent kinase B2;1 (CDKB2;1), CYCLIN B2;4 (CYCB2;4), and G2/mitotic-specific cyclin (CYCB1;2) are highly expressed in meristems and were used as meristem cell markers genes\textsuperscript{30}. Chitinase-like protein 2 (CTL2) and xylem cysteine peptidase 1 (XCP1) were used as representative xylem marker genes\textsuperscript{21,25}. Bundle sheath cells were enriched in EXORDIUM like 2 (EXL2) and NAD(P)-binding Rossmann-fold superfamily protein (VEP1)\textsuperscript{31}. MYB domain protein 59 and sulfate transporter 91 (SULTR3;3) were used as marker genes for xylem parenchymal cells\textsuperscript{31}. Based on these markers, we identified 12 cell types: hydathode cells (cluster 0), mesophyll_1 (cluster 1), mesophyll_2 (cluster 2), mesophyll_4 (cluster 4), bundle sheath cells (cluster 3), upper epidermal cells (cluster 5), phloem cells (cluster 6), lower epidermal cells (cluster 10), xylem parenchymal cells (cluster 8), meristem cells (cluster 9), and xylem cells (cluster 11). Heat shock protein was enriched in cluster 7, but it was not possible to determine the cell type of this cluster using the current marker genes (Fig. 1d).

GO enrichment was used to further classify each cell type (Fig. S3c), different cell types were enriched in distinct sets of biological processes (padj \leq 0.05). For example, mesophyll cells were rich in biological processes related to photosynthesis, and epidermal cells were mainly enriched in biological processes related to fatty acid synthesis\textsuperscript{24}. As a conductive tissue, the vasculature was enriched in genes that participate mainly in the conduction of various plant signal molecules and in the transport of metal ions\textsuperscript{32}.
Fig. 1 Cellular heterogeneity cluster analysis of the *F. vesca* leaf single-cell transcriptome. **a** Details of single-cell library preparation. Protoplasts were obtained by enzymatic hydrolysis. The mRNA released from the rupture of the single cell suspension was combined with gel bead and emulsion to form GEMs. The mRNA of the cell was independently reverse transcribed in each GEM, tagged cDNA will be mixed and amplified to library construction. **b** t-SNE visualization divides 15,018 *F. vesca* leaf cells into 12 clusters. **c** Correlation analysis of scRNA-seq and bulk RNA-seq gene expression. **d** Violin plots of representative cluster-specific marker genes in different cell types.

Identification of different cell types

To assess the conservation of cell type gene expression between woodland strawberry and *Arabidopsis*, we compared the woodland strawberry scRNA-seq data to the published *Arabidopsis* leaf dataset\(^1\) to identify single-copy orthologues using Orthofinder (Table S4). We integrated the single cell data from *Arabidopsis* and woodland strawberry, then grouped the
cells into eight clusters through dimensionality reduction (Fig. 2a, b). T-SNE visualization and Pearson’s correlation coefficients showed that there was high homology between cell types of *Arabidopsis* and woodland strawberry (Fig. 2c, d). Furthermore, shared marker genes between the two species further confirmed the conservation of homologous cell types (Fig. S4a). For instance, glycosylphosphatidylinositol-anchor lipid transfer protein 1 (LTPG1) was shown to be highly expressed in the epidermal and to participate in wax monomer transport.

To confirm the expression of different tissue-specific genes, woodland strawberry leaves were divided into upper epidermal, lower epidermal, mesophyll, and vascular tissues by laser microdissection (Fig. 2e-h). Using RT-qPCR, we found that *FvH4_4g01980* (KCS2) and *FvH4_6g09980* (LTP3) were highly expressed in the upper epidermal, and *FvH4_1g23290* and *FvH4_4g15850* which promoted differentiation of stomatal guard cells were specifically expressed in the lower epidermal. *FvH4_3g41620* (PSAG) and *FvH4_6g40970* (LHCB2.1) were highly expressed in mesophyll cells, and *FvH4_3g12160* (PP2-A10) and *FvH4_2g16940* (XCP1) were highly expressed in vascular cells (Fig. 2e-h). These results further verify the accuracy of the strawberry leaf cell type classification.

![Fig. 2](https://academic.oup.com/hr/advance-article/doi/10.1093/hr/uhab055/6511240)
strawberry (dark green) and *Arabidopsis* (orange) cell types. e-h Expression of known tissue-specific marker genes for vascular (e), upper epidermal (f), mesophyll (g), and lower epidermal tissue (h).

**Single-cell analysis of F. vesca responses to B. cinerea infection**

A major challenge in studying the plant responses to biotic stress is the inconsistent degree of reactions among cell types. To determine how each strawberry leaf cell type responds to *B. cinerea* and how the systemic signals are generated and propagated from the infection site, we explored differences in leaf cell responses to the pathogen. At 6 hpi (S6), when the hyphae of *B. cinerea* had gathered on the surface of the leaves (Fig. S1e), we captured 18,223 cells with a median expression of 3,144 genes per cell and a total of expressed 21,629 genes; 64.5% of the sequenced reads could be mapped to the *F. vesca* v4.01 genome (Table S1). At 12 hpi (S12), when the *B. cinerea* hyphae had penetrated the leaf epidermal (Fig. S1f), we captured 17,065 cells with a median expression of 3,199 genes per cell and a total of 21,555 expressed genes, 65.4% of the sequenced reads could be mapped to the genome (Table S1). To study cellular heterogeneity during pathogen infection, we integrated the three samples and corrected batch effects using CCA+ (Fig. 3a, b). Nine cell types were identified based on cluster-specific marker genes (Fig. 3c). The proportions of these cell types changed when leaves were infected with *B. cinerea*. For instance, the proportion of hydathode cells in the transcriptome was 16.6% and 16.3% at 6 and 12 hpi, an increase of 5% compared with the mock sample (Fig. 3d). As the treatment duration increased, the transcriptome proportion of mesophyll_1 cells decreased from 13% to 9% at 6 hpi and 3.5% at 12 hpi. These results were suggestive of cellular and physiological differences at different periods of *B. cinerea* infection.

To provide insights into the gene expression pattern in each cell type during the *B. cinerea* infection process, we identified differentially expressed genes (DEGs) between infection time points for specific cell types and performed GO analysis of the DEG sets (Fig. 3e, f, Fig. S5). The number of DEGs in each cell type increased over the course of the *B. cinerea* infection process. The GO terms ‘response to biotic stimulus’ and ‘defense response’ were enriched in DEGs of all cell types at 6 hpi and 12 hpi relative to 0 hpi. Some biological process GO terms were enriched in DEGs from specific cell types, for example, ‘defense response to fungus’ was enriched in DEGs from epidermal and hydathode cells at 6 hpi. ‘Response to oxidative stress’
was enriched in DEGs from hydathode cells at 6 hpi, and this enrichment intensified as time elapsed (Fig. S5). At 12 hpi, ‘response to oxidative stress’ was enriched in DEGs from multiple cell types: meristem, hydathode, mesophyll_2, and bundle sheath. Furthermore, the GO terms ‘response to stress’ and ‘sodium ion transport’ were also enriched in specific cell types at 12 hpi.

Fig. 3 Single-cell transcriptome analysis of infected and control samples. a t-SNE plot of 50,327 woodland strawberry leaf cells from different infection stages (Mock: 15,039 cells; S6, 18,223 cells; S12, 17,065 cells) and cell types (right). S6 represents the sample taken at 6 hpi by *B. cinerea*, and S12 represents the sample taken at 12 hpi. b t-SNE plot of separated Mock, S6, and S12 single cells. c Expression patterns of cell-type marker genes for each cell cluster. Dot diameter indicates the percentage of cluster cells that expressed the gene. Color indicates the average expression across cells in that cluster. d Bar plot showing the relative proportion of each cell type in Mock, S6, and S12 samples. e Upset plot of upregulated DEGs at 6 hpi by *B. cinerea*. The bar plot on the left shows the number of upregulated DEGs for each cell type. The upper bar plots show the number of upregulated DEGs. (|logFC| ≥ 0.8, FDR ≤ 0.01). f GO terms enriched in upregulated DEGs at 6 hpi in multiple cell types. (FDR ≤ 0.05).

Characterization of the single-cell expression profiles for the cell lineages first infected by *B. cinerea*

To identify the cell types that were first infected by *B. cinerea*, we analyzed the percentage of cells and UMI values across multiple lesions. In general, we observed that the proportion and UMI values of hydathode, upper epidermal, and mesophyll cells increased significantly during lesion progression and the hydathode, mesophyll_2, and upper epidermal cell types...
appeared to respond first to the *B. cinerea* stimulus (Fig. 3d, 4a). Surprisingly, the hydathodes, mesophyll, and upper epidermal showed distinct expression patterns during the *B. cinerea* infection process (Fig. 4c), genes involved in transferase activity and polysaccharide catabolic process were expressed mainly in hydathode cells, whereas genes involved in signaling pathways of translation and binding were expressed mainly in the mesophyll_2 cells, and genes involved in channel activity and response to stress signaling pathways were expressed mainly in the upper epidermal (Table S7). Expression of genes in the pathways related to disease symptom development increased in the upper epidermal and mesophyll as the infection progressed (Fig. 4, Fig. S6).

![Figure 4](https://academic.oup.com/hr/advance-article-doi/10.1093/hr/uhab055/6511240)

**Fig. 4** The scRNA profiles for cell types that respond first to the infection stimulus across different infection stages. a Distribution of total UMI for individual cell types in integrated single cell data. b, c t-SNE plot showing the distribution of hydathode, upper epidermal, and mesophyll cells for integrated sample. d Heatmap showing the expression of selected functionally relevant genes that were differentially expressed between the three cluster types. e The most enriched GO terms in upregulated genes of mesophyll cells in S6 (6 hpi) and S12 (12 hpi) samples (FDR ≤ 0.05).

**Cell trajectory analysis of epidermal and mesophyll cells during the different stages of *B. cinerea* infection**

To evaluate the differences in expression profiles at different stages, pseudotime analysis was performed on mesophyll and epidermal cells. For mesophyll cells, the three samples were projected to three ends of the pseudotime trajectory, including five trajectory states, and
gathered mainly at one of the large ends (Fig. 5a). We separated the cell trajectories of the three stages and found that the cells shifted gradually from state 4 to the state 1 as the processing time increased (Fig. 5b), which summarized the developmental process of mesophyll cells stimulated by B. cinerea. The gene expression patterns of cells in different states were calculated in pseudotime order using Monocle 2. Differentially expressed genes were divided into four clusters, reflecting changes in differential gene expression from the beginning to the end of the pseudotime. GO terms related to photosynthesis and translation which function in mesophyll cells were enriched at the beginning of the pseudotime. Genes related to biotic stress response such as FvH4_1g06520 and FvH4_1g06570 were enriched in the middle stage of pseudotime (Fig. 5e), whereas genes related to protein glycosylation and chitin catabolic processes such as FvH4_1g10650 and FvH4_1g21000 were particularly prominent at the later stage (Fig. 5f).

Similarly, the upper epidermal cells from different time points had two distinct trajectories (Fig. S7a, b). The differentially expressed genes across branch points were divided into three clusters (Fig. S7c, d). Cluster 3-1 contained mainly genes involved in translation and fatty acid biosynthetic processes, such as FvH4_1g18550 and FvH4_4g06700, which were indicative of the function of epidermal cells (Fig. S7c). Cluster 1 and cluster 3-2 were enriched in defense response and metabolic genes, consistent with the transition in epidermal cell state during the infection response (Fig. S7c, d).
Fig. 5 Pseudotime analysis of mesophyll cells in Mock, S6 and S12. a Pseudotime trajectory of mesophyll cells. Each dot represents a single cell. Color represents the pseudotime score (left). Color represents different states (right). b The mesophyll cells distribution on the trajectory for mock, S6 and S12. c Pseudotime heatmap of GO analysis of differentially expressed genes (FDR ≤ 0.05). Color bar indicates the relative gene expression level. d-f Gene expression kinetics of representative genes along a pseudotime progression in the beginning (d), middle (e) and later (f) stage.

Changes in the expression of the defense-related genes during lesion development

We observed significant upregulation of genes encoding receptor-like proteins (FvH4_1g01370) in the lower epidermal during the incubation period of B. cinerea infection (Fig. 6a, b). Leucine-rich repeat (LRR) family proteins were expressed in mesophyll_2 cells. Subsequently, we identified other genes related to recognition and signaling. For example, FvH4_6g09300, related to calmodulin, was expressed mainly in hydathode cells at 6 hpi. However, at 12 hpi, FvH4_6g09300 (CML42) was expressed in almost cell types, demonstrating that genes expressed in response to B. cinerea may be upregulated in one or several cell types during the incubation period of the infection (Fig. S8a).
To further investigate the expression patterns of defense-related genes, we measured their expression levels in different cell types. The disease-related protein PR4 (*FvH4_3g05950*) was expressed in hydathode, mesophyll_2, and upper epidermal cells at 6 hpi, and its expression in other cell types increased as the infection progressed at 12 hpi (Fig. 6c, d). Peroxidase PA2 (*FvH4_3g44360*) was first expressed in hydathode cells at 6 hpi (Fig. 6e, f). With increasing infection duration, its expression level gradually increased in other cell types. These results were consistent with Fig. 4a, implying that hydathode, upper epidermal and mesophyll cells have the highest levels of disease response-related transcriptional variation during early infection.
Transcriptional regulatory network during the incubation period of strawberry infection by *B. cinerea*

Transcription factors (TFs) were also expressed in different cell types during the infection process. At 6 hpi, members of the NAC, HSP, WRKY, VQ and TIFY TF families were highly expressed in each cell type at 6 hpi. At 12 hpi, they were even more highly expressed. The expression distribution of these genes was analyzed using FeaturePlot and RT-qPCR. Student’s t-test was performed to determine the significance of the differences, with *P<0.05 and **P<0.01. The expression of *FvH4_3g05950* (PR4) and *FvH4_3g44360* (PA2) were shown to be significantly different in different processing stages.
expressed in woodland strawberry (Fig. 7a). Interestingly, HSP90 (FvH4_2g38300) was expressed mainly in hydathode and bundle sheath cells, WRKY75 (FvH4_4g23480) was expressed mainly in the upper epidermal, and ZAT11 (FvH4_6g14410) was highly expressed in the phloem. Protein-protein interaction network analysis demonstrated the interaction of TFs expressed in different cell types at 6 hpi, these TFs presumably work together to regulate the strawberry immune response to defend against *B. cinerea* (Fig. 7b). Subsequently, we examined a clustered heatmap of TF expression at 12hpi and found that the WRKY family gene, WRKY75 (FvH4_4g23480) exhibited high expression in each cell type (Fig. 7c). Likewise, FvH4_6g53770 (WRKY DNA-binding protein), FvH4_1g16030 (HSF4), FvH4_6g14410 (ZAT11) and FvH4_7g21880 (ZAT12) had high levels of expression in most cell types. FvH4_3g11860 (NAC042) and FvH4_3g35050 (zinc finger protein) were specifically expressed in the hydathode and lower epidermal cells, respectively. To identify the regulatory relationships among upregulated TFs, we constructed a co-expression network of TFs upregulated expressed in response to *B. cinerea* at 12 hpi (Fig. 7d). The 30 TFs were connected to each other through 69 edges, and transcription factors that were highly expressed in multiple cell types, such as FvH4_4g23480 (WRKY75), had a large number of edges.
Fig. 7 Analysis of core transcription factors (TFs) in different stages of infection. a Heatmap showing the expression of TFs in each cell type at 6 hpi. b The protein-protein interaction (PPI) network of TFs upregulated expressed in response to *B. cinerea* at 6 hpi. c Heatmap showing the expression of TFs in each cell type at 12 hpi. d The co-expression network of TFs upregulated expressed in response to *B. cinerea* at 12 hpi.

**Discussion**

Gray mold caused by the necrotrophic fungus *B. cinerea* is one of the most devastating diseases in crop plants such as strawberry\(^{33}\). Infection development is a continuous process of host/pathogen interaction, and our understanding of how the plant invokes and transduces relevant signals from the local to the systematic level and our attempts to design the best strategy for resistant cultivars development have been hampered by the heterogeneous nature of plant tissues\(^{34}\). The recent advent of single cell transcriptomics in combination with laser-capture microscopy makes it possible to dissect the plant response at the level of individual cell types, identify cell-type-specific genes involved in signal transduction, and select promising candidate genes for gene editing and engineering. In pursuit of these goals, we have developed
a single-cell transcriptome pipeline for studying the interaction between woodland strawberry
(F. vesca) and B. cinerea.

Construction of a single-cell atlas of strawberry leaves

Fragaria vesca ‘Hawaii4’, as an ideal model plant, serves for cultivated strawberry and
the Rosaceae family. In this study, we first captured the major cell types of woodland strawberry
leaves to construct a high-resolution transcriptome atlas (Fig. 1b). Because there was no exact
marker gene for each strawberry leaf cell types, we used multiple known orthologous marker
genes from Arabidopsis to annotate strawberry leaf cell types. Based on the
conservation of gene expression patterns in strawberry and Arabidopsis and the verification of
specific marker genes by laser microdissection, strawberry leaves could be separated into 12
cell types. Cells from the hydathode, upper epidermal, bundle sheath, xylem, phloem, lower
epidermal, xylem parenchyma, and meristem could be clearly identified based on the marker
genes of distinct cell clusters (Table S4). Because there were not enough marker genes to
determine its identity, we could not determine the cell type of cluster 7 (Fig. 1b). Interestingly,
we detected the presence of meristem cells in the leaves, and we speculated that they may occur
in vascular tissues.

Distinct cell type responses to B. cinerea infection

Based on a single-cell expression atlas from the three time points during B. cinerea
infection, we identified the hydathode, upper epidermal and mesophyll cells may mount the
greatest initial response to infection. These results are similar to those of abiotic stress studies.
When Arabidopsis roots experience heat stress, the hairs, nonhair epidermal cells, and cortex
cells respond more quickly. In rice seedlings, the mesophyll, parenchyma, and epidermal cells
respond strongly to salt stress and nitrogen deficiency. We observed that hydathode cells were
enriched in the same pathways (e.g., response to biotic stimulus and defense response) at both
6 and 12 hpi, indicating that hydathode cells have already entered a defensive state at 6 hpi (Fig.
S6a). The upper epidermal cells and mesophyll cells actively respond to B. cinerea infection
with distinct patterns of gene expression in each cell type. The normal function of mesophyll
cells is primarily photosynthesis and light harvesting, however, upon pathogen invasion, their
activities shifted to respond to infection, as suggested by enrichment in chitin catabolic process
and defense response pathways (Fig. 4e, 5c). The upper epidermal cells responded to pathogen
invasion by activating the fatty acid biosynthetic process. Interestingly, the cluster 3 including genes related to lipid biosynthesis process and xyloglucan metabolism, was expressed in the initial and later stages of the pseudotime trajectory (Fig. S7c). The cuticle is an important part of the epidermal and may participate in the plant immune response, as shown in previous research\textsuperscript{39}. Our results demonstrated that single-cell technology provides a high-resolution method for studies of heterogeneity in cell response and permits accurate detection of early pathogen responses.

**Gene expression patterns in *B. cinerea*-infected strawberry leaves**

During plant-pathogen co-evolution, plants have evolved a complex immune defense system, including pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), function synergistically to optimize the plant systemic immune response\textsuperscript{40,41}. In this study, we found many genes related to recognition and signaling in the early stage of *B. cinerea* infection, such as Leucine-rich repeat (LRR) family protein, cysteine-rich receptor-like protein (Table S9). Genes responding to stresses and involved in the secondary metabolism were identified upon *B. cinerea* infection, including peroxidase and pathogenesis-related protein (Table S9).

However, we found that not all cell types in the leaf tissue expressed the same disease resistance genes (Fig. 6, S8). At 6 hpi, genes related to calmodulin, CML42\textsuperscript{42} were highly expressed in the hyathodes (Fig. 6a). By contrast, WRKY75 was highly expressed in upper epidermal cells (Fig. 7a, Table S9), which has been shown to participate in JA signaling pathways to regulate plant defense\textsuperscript{43}. Interestingly, WRKY75 has also been classified as a specific marker gene in the root epidermal of *Arabidopsis*, where it participated in root system development\textsuperscript{44}. ZAT11, a dual-function transcriptional regulator\textsuperscript{45}, was highly expressed in phloem (Fig. 7a, Table S9).

Previous studies have revealed that ZAT11 is involved in the formation of vascular tissue in a process mediated by the PXY transcriptional regulatory network\textsuperscript{46}. These results implied that these differentially expressed genes not only played an important role in resistance to external pathogen stimuli to ensure plant growth, but functioned in plant growth and development. Even at 12 hpi, we also detected small but important differences in the expression of upregulated genes (Fig. 6b, 7b), for instance, most of the differentially expressed genes were enriched in the mesophyll cells, suggesting that these cells may serve as the main site of plant defense responses.
To identify the role of TFs in plant disease response, we analyzed the expression of transcription factors in distinct cell types. HSP90.7, BIP2, JAZ2, ANAC002, WRKY75, and ZAT11 may act as key transcription factors to maintain communication between cell types at 6 hpi (Fig. 7b, Table S9). WRKY, HSF, NAC, TIFY, ERF, bHLH, C2H2, and MYB family genes were identified as differentially expressed in cell types at 12 hpi (Fig. 7c, Table S9). Co-expression network analysis suggested that these TFs do not act alone but may form a powerful immune network. Taken together, these results indicated that each cell type actively participates in the transcriptional regulation process through distinct expression patterns, and different cell types may communicate with each other to build a complex transcriptional regulatory network to resist B. cinerea.

In conclusion, we established markers related to different cell types of strawberry leaves and constructed a high-resolution single-cell gene expression atlas for the early process of strawberry response to B. cinerea infection. This is the first report of a single-cell transcriptomes in woodland strawberry leaves, and the cell type markers developed here can be used to separate the heterogeneous tissues into more specific cell types, not limited to Arabidopsis and field crops. These findings lay a foundation for further investigation of the dynamic process of B. cinerea infection and the functional characterization and manipulation of candidate genes to develop resistant cultivars.

Materials and Methods

Plant material, growth conditions, and fungal treatment

Woodland strawberry (Fragaria vesca, ‘Hawaii4’) was used for the scRNA-seq experiment. Seeds were sown on Murashige and Skoog (MS) medium containing 1.0% sucrose under a light intensity of 100 μmol m$^{-2}$ s$^{-1}$ and long-day conditions (16 h light / 8 h dark) at 25°C. For the pathogen infection, Botrytis cinerea strain (Bc05.10) was cultured on CM agar plates in the dark at 25°C as previously described. Fifteen-day-old seedlings were sprayed with 10⁶ spores/mL conidial suspensions in SMB buffer (10g/L Mycological peptone, 40g/L Maltose). The first true leaf tissues were harvested at 0, 6, and 12 hpi for immediate protoplast isolation, each sample contains 50 pieces of true leaf tissues.

Scanning electron microscopy
Leaf samples collected at 0, 6, and 12 hpi were fixed separately in 2.5% glutaraldehyde. After 8 h, the samples were washed three times with 0.1 M phosphate buffer, dehydrated in an ethanol gradient (50%, 70%, 80%, and 90%) that was replaced three times with tert-butanol, and dried in a freeze dryer. The samples were covered with a 10-nm gold film using an ion sputtering instrument (MC1000, HITACHI, Japan) and observed under a scanning electron microscope (SU8010, HITACHI, Japan).

**Protoplast isolation and scRNA-seq library construction**

Leaf tissues were cut into pieces and placed in RNase-free enzyme solution (2% cellulose R10, 0.6% macerozyme R10, 0.8 M mannitol, 40 mM KCl, 20 mM CaCl₂, 40 mM MES, 0.05% β-mercaptoethanol and 0.1% bovine serum albumin). The tissues were enzymolyzed at 100 rpm for 4 h at 26°C in the dark, and the protoplasts were purified in W5 solution (0.08 M MES [pH 5.5], 0.1 M KCl, 0.02 M MgCl₂, 0.4 M mannitol, and 0.1% bovine serum albumin), strained twice through a 38.5-μm filter, centrifuged at 200 g for 6 min, and washed two times with 8% mannitol to obtain pure protoplasts. The density of the protoplasts was determined with a hemocytometer and adjusted to 700-1200 cells/μL. The activity of single-cell suspensions was detected by fluorescein diacetate (FDA) staining and trypan blue staining, protoplasts with greater than 90% activity were selected for future analysis.

The scRNA-seq libraries were processed on the 10 x Chromium 3’ Single Cell platform (10 x Genomics, Pleasanton, CA). Briefly, through a microfluidic chip, single cell suspension and beads contained cell barcode were enclosed in droplets to form a single-cell GEMs structure. mRNA of the cell undergoes reverse transcription reaction in the droplet to form cDNA and construct the cDNA library. Single-cell FASTQ sequencing reads were mapped to the woodland strawberry ‘Hawaii 4’ reference genome (Fragaria vesca v4.0.a1)⁹⁴, the genome annotation has a total of 28,588 gene models. The read mapping data were converted to digital gene expression matrices using the Cell Ranger single cell software suite (v3.1.0) provided by 10 x genomics website.

**ScRNA-seq data dimensionality normalization and clustering**

The raw count matrices were analyzed using the Seurat package (v3.2.0) in the R (v4.0.2).

Before analyzing the scRNA-seq data, standard pre-processing steps were performed to remove dead and bimodal cells and to filter out cells with >0.05% mitochondrial and ribosomal...
sequences. The number of raw gene counts from each cell was normalized relative to the total number of counts, and we identified the top 2000 highly variable genes for use in cluster analysis. Principal component analysis (PCA) was used for dimensionality reduction. The ‘RunTSNE’ function was used to visualize cell clusters, ‘FindAllMarkers’ was used to identify cluster-enriched genes (marker genes), and ‘FindIntegrateData’ was used to integrate the data from the three samples. CCA+ anchors (Seurat v3) were used to remove batch effects between different samples.\(^{50}\)

The ‘Find Markers’ function in Seurat was used to identify differentially expressed genes (DEGs) between samples based on a dual threshold of \(|\log_{2}\text{FC}| \geq 0.8\) and \(\text{FDR} \leq 0.01\).\(^{51}\) The intersections of differentially expressed genes between samples were visualized using the UpSetR function in R.\(^{52}\)

**Comparison of interspecies scRNA-seq data**

To identify different cell types, we used the known marker genes from *Arabidopsis* to annotate strawberry cell types. Orthofinder was used to cluster single-copy orthologous protein sequences between woodland strawberry and *Arabidopsis*. The protein sequences were downloaded from the GDR (https://www.rosaceae.org) and TAIR website (https://www.arabidopsis.org).

To integrate the single cell data from *Arabidopsis* and woodland strawberry, we downloaded the published *Arabidopsis* leaf scRNA-seq data from the NCBI (GSE161482). SCTransform was used to normalize and standardize the differences between the *Arabidopsis* and strawberry scRNA-seq data according to a published method.\(^{53}\) After t-distributed stochastic neighbor embedded (t-SNE) dimensionality reduction, eight cell clusters were obtained. The ‘AverageExpression’ function was then used to calculate the average gene expression level of each cluster. The cluster relationship between woodland strawberry and *Arabidopsis* were represented by Pearson’s correlation coefficients.

**Gene Ontology term analysis**

Marker genes and differentially expressed genes in specific samples were annotated using biological process of Gene Ontology (GO terms). GO terms and functional annotations were assigned based on the *Fragaria_vesca_v4.0a1_go* file of the *Fragaria vesca* v4.0.a1 genomes.
The clusterProfiler R package was then used to perform GO enrichment analyses with default parameters\textsuperscript{54}.

**Bulk RNA-seq**

The first true leaves of 3-day-old woodland strawberry uninfected seedlings were harvested for RNA extraction. Total RNA was extracted from unprotoplasted and protoplasted leaf tissues using the Plant Total RNA Isolation Kit Plus (Foregene, Chengdu, China). Each sample contains three replicates. One microgram of RNA was used to construct mRNA libraries using chain-specific method by NEBNext \textsuperscript{®} Ultra\textsuperscript{™} Directional RNA Library Prep Kit (NEB, USA) following manufacturer's recommendations. The sequencing of cDNA library using Illumina novaseq platform to generated 150bp/150bp paired-end reads. Raw reads of fastq format were firstly processed through perl scripts. After pre-processing and quality control, the clean reads were mapped to the *Fragaria vesca* v4.0.a1 genome using HISAT2 with default parameters. Differentially expressed genes were identified using DESeq with a dual threshold of $|\log_2 \text{FC}| \geq 1$ and FDR $\leq 0.01$). The log$_2$ (mean RPM+1) and Pearson correlation coefficients of bulk and single-cell RNA sequence data were calculated in R.

**Laser capture microdissection and RT-qPCR analysis**

The first true leaves were embedded on a cryostat (Leica, Germany) using optimal cutting temperature compound, then immediately frozen. The embedded blocks were trimmed, sliced (18 μm thickness), transferred to a Leica PET-membrane 1,4 μm microscopy slide, and dehydrated with 100% absolute ethanol. Laser microdissection was performed with the Leica Microsystems CMS GmbH system. The first true leaves of strawberry were divided into four cell types: upper epidermal, lower epidermal, mesophyll, and vascular tissue. Approximately 300 pieces of each cell type were pooled for immediate RNA extraction.

Total RNA was extracted from different cell types using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, USA), there were three replicates of each cell type and 0, 6, and 12 hpi inoculation treatments. The relative expression of tissue-specific genes and differentially expressed genes in response to disease was measured by quantitative real-time PCR (RT-qPCR), these genes had been identified in the scRNA-seq experiment. cDNA was synthesized using the Prime Script RT reagent kit with gDNA Eraser (Takara, Dalian, China).

The RT-qPCR primers were listed in Table S8. RT-qPCR was performed using SYBR Premix...
Ex Taq II (TaKaRa) on a Light Cycler480 II instrument (Roche). Relative gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method.

**Pseudotime analysis**

The single-cell data were converted to a Cell Data Set object with the ‘as.CellDataSet’ function in Seurat. Monocle 2 was used to reconstruct the pseudotime developmental trajectory in R\textsuperscript{55}. The ‘estimateSizeFactorsand’ and ‘estimateDispersions’ functions to standardize the differences between cells. ‘differentialGeneTest’ was used to select genes that define cell processes. ‘setOrderingFilter’ function to mark the ordered genes and used the “DDRTree” method to perform dimensionality reduction processing. The cells were sorted twice with the ‘orderCells’ function, the root_state parameter was set when the second execution of ‘orderCells’ function. Cell pseudotime development trajectories were visualized using the ‘plot_cell_trajectory’ function.

**Co-expression network of differentially expressed transcription factors**

Differentially expressed transcription factors were analyzed at specific lesion time points ($|\log_{2}FC| \geq 0.8$ and FDR ≤ 0.01). First, we connected highly expressed transcription factors of each cell types at 6hpi in a protein-protein (PPI) interaction network using the STRING database. Then, we calculated the Pearson correlation coefficients (PCCs) between differentially expressed transcription factors at 12 hpi and constructed a co-expression network using Cytoscape_v3.7.2 (PCCs ≥ 0.6, P < 0.05).

**Data availability**

All high-throughput sequencing data have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation, Chinese Academy of Sciences, under accession number CRA004848 (https://ngdc.cncb.ac.cn/gsa)

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**Author contributions**
Y.B. and Z.-M.C. designed this research. Y.B. performed the experiments. Y.B., H.L., H.L., L.S., J.X. participated in single cell transcriptome analyses. Y.B. and Z.-M.C. wrote the manuscript. All the authors approved the final manuscript.

Conflict of interest

The authors declare no competing interests.

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